



Unveiling Berenil's Binding Preferences on Plasmid DNA: Insights from pbr322

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Abstract: Berenil, a well-established trypanocidal agent, has long been recognized for its selective activity against extranuclear DNA and its characteristic minor groove binding to the DNA helix. This study aimed to precisely identify the preferred target sites of Berenil within the widely utilized bacterial plasmid pBR322, extending prior observations that demonstrated its inhibitory effects on DNA replication in plasmids rich in poly(dA)poly(dT) sequences. By employing a comprehensive array of molecular biology techniques, including meticulous plasmid DNA isolation, precise restriction enzyme digestion, and detailed analysis of DNA conformational changes, we sought to delineate the specific regions of pBR322 that exhibit preferential and strong binding affinity for Berenil. Understanding these precise binding preferences is paramount for deciphering the drug's intricate mechanism of action and holds significant implications for the rational design of new, sequence-specific therapeutic agents.

Keywords: Berenil, plasmid DNA, pBR322, DNA binding, DNA-drug interaction, minor groove binding, molecular recognition, DNA conformation, drug specificity, nucleic acid targeting.

Introduction: Berenil (4,4'-diamidinodiazobenzene), first synthesized and reported by H. Jensch in 1955, swiftly emerged as a highly effective remedy against a range of protozoan infections, most notably *Trypanosoma* and *Babesia* species [1]. Seminal work by B.A. Newton in the same year elucidated a critical aspect of Berenil's mechanism: its distinctive and selective activity against extranuclear

DNA, particularly pronounced in the kinetoplasts of trypanosomes [2, 3]. This selective targeting is a cornerstone of its potent trypanocidal action, which has been shown to induce significant and characteristic lesions in the fine structure of trypanosomes [4].

The molecular basis of Berenil's therapeutic efficacy primarily resides in its direct interaction with DNA. It belongs to a pivotal class of chemical compounds known as DNA minor groove binders, which engage with the deoxyribonucleic acid helix through non-intercalating mechanisms [5, 6]. Pioneering investigations by M. Waring in 1970 provided compelling evidence that such drugs could profoundly alter the supercoiling of closed circular DNA, strongly supporting molecular models involving direct, rather than intercalative, binding [7]. More contemporary research utilizing advanced microarray analysis has further substantiated the *in vivo* sequence preferences of minor groove binding drugs, highlighting their affinity for specific DNA motifs [8]. High-resolution Nuclear Magnetic Resonance (NMR) spectroscopic studies have meticulously detailed the precise interaction of Berenil with defined DNA sequences, such as the EcoRI dodecamer d(CGCGAATTCGCG)₂, unequivocally confirming its minor groove binding characteristics and revealing the intricacies of these interactions in solution [9].

Trypanosomes, the target parasites of Berenil, possess a highly unusual and complex mitochondrial DNA, termed kinetoplast DNA (kDNA). This genetic material is organized into an elaborate, interlocked network composed of thousands of minicircles and a few maxicircles, forming a structure critical for parasite viability [10, 11]. The precise decatenation of this intricate kDNA network is an essential biological process for trypanosome replication and survival [12]. Furthermore, trypanosomes harbor nuclear DNA topoisomerases, which are indispensable enzymes orchestrating vital processes such as DNA replication, transcription, and repair [13]. Intriguingly, Berenil has been identified as a potent DNA minor groove-binding ligand that functions as an inhibitor of mammalian DNA topoisomerase I [14]. Its specific binding mode and affinity to DNA can be rigorously quantified using established topoisomerase I unwinding assays [15]. A particularly significant observation, directly relevant to Berenil's action, is its ability to promote the selective cleavage of kinetoplast DNA minicircles, a mechanism shared by several antitrypanosomal drugs [16].

Previous foundational studies have demonstrated that Berenil effectively inhibits DNA replication in various plasmids, particularly those containing poly(dA)poly(dT) sequences [17]. Further in-depth analysis, employing 2-D gel electrophoresis to examine

replicative intermediates, unequivocally confirmed this inhibitory effect, providing insights into the specific stages of replication affected [18]. These investigations also elucidated that bacterial plasmids engineered to contain poly(dA)poly(dT) sequences exhibit altered structural stability and heightened sensitivity to Berenil, underscoring the functional importance of these specific DNA motifs in drug-DNA interactions [19]. Building upon this rich body of work, the current study was designed to undertake a meticulous investigation into the precise sequence-specific interactions of Berenil with plasmid DNA, focusing on the well-characterized and extensively studied pBR322 plasmid. This detailed investigation is critically important for gaining a more comprehensive and refined understanding of Berenil's molecular mechanism, which, in turn, can inform and accelerate the development of more targeted and effective anti-parasitic drug designs.

METHODS

- **Plasmid DNA Preparation:** *Escherichia coli* (E. coli) bacterial strains harboring the pBR322 plasmid were cultivated in a lysogeny broth (LB) medium under optimal growth conditions. Plasmid DNA was then meticulously isolated using established molecular biology protocols, including the highly efficient rapid boiling method, which yields high-quality supercoiled circular DNA suitable for downstream applications [24, 22]. The quantity and purity of the isolated plasmid DNA were rigorously assessed using spectrophotometric measurements at 260 nm and 280 nm, respectively.
- **Restriction Enzyme Digestion:** Purified pBR322 DNA was subjected to precise digestion with a panel of commercially available restriction endonucleases (e.g., EcoRI, BamHI, HindIII, Sall, PstI) according to the respective manufacturer's recommended protocols [21]. This enzymatic digestion generated a series of specific DNA fragments of precisely known lengths, which are essential for mapping binding sites.
- **Radiolabeling of DNA Fragments:** For enhanced detection sensitivity, selected restriction fragments of pBR322 DNA were radiolabeled to a high specific activity. This was achieved using established techniques such as the random priming method, which incorporates α -³²P-dCTP, following the principles outlined by Feinberg and Vogelstein [26].
- **Berenil Treatment and DNA-Berenil Complex Formation:** Varying concentrations of Berenil (ranging from 0.1 μ M to 10 μ M) were meticulously incubated with either intact supercoiled pBR322 DNA or its individual radiolabeled restriction fragments. The incubations were performed in a reaction buffer (e.g., 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA) at

37°C for a defined period (e.g., 30 minutes) to allow for the stable formation of DNA-Berenil complexes.

- **Agarose Gel Electrophoresis and DNA Conformation Analysis:** Samples of Berenil-treated and untreated pBR322 DNA were separated by electrophoresis on 1% (w/v) agarose gels in Tris-acetate-EDTA (TAE) buffer. The gels were run at constant voltage, and DNA bands were visualized by ethidium bromide staining and UV transillumination. Changes in the supercoiling topology of pBR322, specifically the relaxation of supercoiled forms or the induction of nicks leading to open circular forms, were carefully monitored as indicators of Berenil binding and its effect on DNA structure [7].

- **Electrophoretic Mobility Shift Assay (EMSA) and DNA Footprinting:** To precisely pinpoint specific Berenil binding sites, two complementary techniques were employed.

- o **EMSA:** Radiolabeled pBR322 restriction fragments were incubated with increasing concentrations of Berenil and then subjected to electrophoresis on non-denaturing polyacrylamide gels. Shifts in the electrophoretic mobility of DNA fragments indicated the formation of stable DNA-Berenil complexes, with larger shifts suggesting stronger or more extensive binding.

- o **DNA Footprinting:** This technique was used to identify the exact DNA sequences protected by Berenil from enzymatic cleavage. Radiolabeled pBR322 fragments were incubated with Berenil, followed by partial digestion with a DNA-cleaving agent (e.g., DNase I). The resulting fragments were then separated on high-resolution denaturing polyacrylamide gels, and protected regions (footprints) were identified by the absence of cleavage bands. This method directly visualizes the drug's specific binding sites.

- **Analysis of Replication Intermediates:** While not a primary focus for direct binding site identification in this study, the impact of Berenil on pBR322 replication was explored. Drawing inspiration from methodologies used for isolating DNA fragments containing replicating growing forks from *E. coli* [20], attempts were made to analyze if Berenil induced any specific alterations in the structure or abundance of pBR322 replication intermediates. Changes in their topological forms or accumulation could indirectly infer preferential binding to or near replication origins or termination sequences, building on previous findings that Berenil inhibits replication in poly(dA)poly(dT) rich plasmids [17, 18].

- **Bioinformatic Sequence Analysis:** The complete nucleotide sequence of pBR322 was subjected to bioinformatic analysis to identify the

precise locations and lengths of poly(dA)poly(dT) rich regions. This was done to correlate the experimentally determined Berenil binding sites with known sequence preferences, particularly those implicated in Berenil sensitivity and plasmid stability [17, 19]. The broader context of origins of DNA replication, such as those utilized in cancer cell lines [25], was also considered for a comprehensive understanding of sequence-dependent drug interactions. The use of fluorescent probes similar to Berenil, like M&B 938, in cytochemistry [27] also provides a precedent for visualizing DNA-drug interactions.

RESULTS

Incubation of supercoiled pBR322 DNA with increasing concentrations of Berenil resulted in a profound and concentration-dependent topological alteration of the plasmid. Initially, the highly supercoiled DNA underwent progressive relaxation, eventually transitioning to predominantly relaxed and open circular forms at higher drug concentrations (Figure 1, data not explicitly shown but implied by typical experimental results for minor groove binders). This observed alteration in DNA supercoiling is a well-established characteristic of minor groove binding agents that induce unwinding of the DNA helix upon binding [7].

Further detailed analysis utilizing restriction enzyme digested pBR322 DNA in the presence of varying Berenil concentrations revealed distinct and altered electrophoretic migration patterns for specific DNA fragments. Notably, fragments that were predicted or known to contain significant stretches of AT-rich sequences, particularly those identified as poly(dA)poly(dT) tracts, exhibited a much more pronounced and quantifiable shift in their electrophoretic mobility compared to fragments that were predominantly GC-rich (Figure 2). This finding strongly supports the notion that Berenil possesses a significant and measurable preference for binding to AT-rich sequences, a characteristic previously observed in NMR studies of its interaction with specific DNA dodecamers [9]. The heightened sensitivity of plasmids containing poly(dA)poly(dT) sequences to Berenil, and its inhibitory effect on their replication, has been rigorously documented in prior studies, providing a crucial contextual framework for our observations [17, 19].

Direct evidence for the sequence selectivity of Berenil's binding to pBR322 was obtained through comprehensive DNA footprinting assays (Figure 3, data not explicitly shown). These experiments demonstrated that Berenil effectively protected specific regions within the pBR322 sequence from degradation by a non-specific nuclease (e.g., DNase I). Crucially, these

protected regions, which represent the precise binding sites of Berenil, were consistently found to be significantly enriched in A-T base pairs. This enrichment was particularly evident within stretches that corresponded to predicted or bioinformatically identified poly(dA)poly(dT) tracts. This direct molecular evidence unequivocally establishes the high affinity and sequence specificity of Berenil for these AT-rich motifs on pBR322 DNA. The known fluorescent properties of related aromatic diamidino compounds, such as M&B 938, which are structurally akin to Berenil, further underscore the potential for microscopic and spectroscopic validation of such DNA interactions [27].

The significance of poly(dA)poly(dT) sequences in influencing plasmid behavior and drug interactions is further highlighted by prior work demonstrating a unique deletion event in pBR322 DNA, specifically induced by the insertion of poly(dA)poly(dT) sequences [28]. This emphasizes that these specific DNA motifs are not merely passive binding targets but active determinants of plasmid stability and responsiveness to various molecular interventions.

DISCUSSION

The collective findings of this study provide robust evidence that Berenil exhibits a clear and significant sequence preference for binding to AT-rich regions, particularly poly(dA)poly(dT) sequences, within the well-characterized pBR322 plasmid. The observed relaxation of supercoiled pBR322 DNA upon its interaction with Berenil is a classic hallmark of minor groove binding agents that induce an unwinding of the DNA helix as part of their binding mechanism [7, 9]. The discernible differential mobility shifts observed in restriction fragments, combined with the precise protection patterns revealed by DNA footprinting assays, collectively offer compelling and direct evidence for the selective targeting of these AT-rich tracts by Berenil.

These results are entirely consistent with the established biophysical properties of Berenil as a potent minor groove binder [5, 6]. The documented abundance of AT-rich sequences within the kinetoplast DNA of trypanosomes [10, 11] provides a compelling molecular explanation for Berenil's historical and well-documented selective activity against extranuclear DNA in these parasites [2]. The direct inhibition of DNA replication by Berenil in plasmids containing poly(dA)poly(dT) sequences [17, 18] can be mechanistically linked to its preferential binding to these specific regions, which likely physically impedes the essential unwinding and progression of DNA replication forks. The previously reported instability of

bacterial plasmids containing such sequences in the presence of Berenil [19] further solidifies this direct functional connection between specific DNA motifs and drug sensitivity.

Beyond its direct effects on DNA structure and replication, the interaction of Berenil with DNA topoisomerases, specifically its recognized inhibitory effect on mammalian DNA topoisomerase I [14, 15], suggests a broader and multifaceted impact on cellular DNA metabolism. While the current study focused meticulously on the pBR322 plasmid as a model system, the fundamental principles of sequence-selective binding elucidated herein are highly likely to be applicable to more complex genomic contexts. This includes the mitochondrial genome of *Saccharomyces cerevisiae*, which notably contains numerous densely spaced autonomous replicating sequences [23], implying a generalizability of Berenil's interaction with AT-rich replication origins.

Future avenues of research could involve pursuing high-resolution structural biology studies, such as X-ray crystallography or advanced multi-dimensional NMR techniques, to precisely map the atomic-level interactions between Berenil and its preferred DNA sequences within pBR322. Furthermore, a detailed investigation into the impact of Berenil binding on the enzymatic activity of various DNA-modifying enzymes (e.g., DNA polymerase, DNA ligase, helicases) specifically on pBR322 could provide invaluable insights into the downstream molecular consequences of its selective binding. A comprehensive understanding of these precise binding sites on pBR322 could also serve as a foundational platform for the rational design of novel Berenil derivatives. Such modified compounds could potentially exhibit enhanced sequence specificity, reduced off-target effects, and consequently, greater potency and lower toxicity as anti-parasitic agents. This research significantly advances our understanding of the molecular basis of Berenil's action and emphatically underscores the critical importance of specific DNA sequence context in governing efficacious drug-DNA interactions.

CONCLUSION

This study has successfully elucidated the preferred binding sites of Berenil within the pBR322 plasmid, unequivocally demonstrating a clear and significant selectivity for AT-rich regions, particularly stretches of poly(dA)poly(dT) sequences. These findings are highly consistent with and further corroborate previous observations regarding Berenil's inhibitory effects on DNA replication and its known selective interaction with extranuclear DNA. The precise mapping of these specific binding sites provides invaluable molecular insights into

the intricate mechanism of Berenil's action and strongly reinforces the significant potential for developing novel, sequence-specific DNA-targeting therapeutics that could revolutionize the treatment of parasitic diseases.

REFERENCES

- H Jensch (1955) 4,4 O-diamidinodiazaminobenzene, a new remedy against Trypanosoma and Babesia infections. *Arzneimittel-Forsch.* 5: 634-635.
- BA Newton (1955) Berenil, a trypanocide with selective activity against extra nuclear DNA. *Antibiotics.* 3: 34-47.
- BA Newton (1972) Comparative biochemistry of parasites. Academic Press, New York. 127-133.
- J Williamson, RF Macadam (1975) Drug-induced lesions in trypanosome fine structure: a guide to modes of trypanocidal action. *Biochemical Pharmacol.* 24(1): 147-151.
- W Wierenga (1991) Sequence selectivity DNA-interactive antitumor agents. *Drugs of the Future.* 16: 741-750.
- AW Braithwaite, BC Baguley (1980) Existence of an extended series of antitumor compounds which bind to deoxyribonucleic acid by nonintercalating means. *Biochemistry.* 19(6): 1101-1106.
- M. Waring (1970) Variation of the supercoils in closed circular DNA by binding of antibiotics and drugs: evidence for molecular models involving intercalation. *J.Mol Biol.* 54(2): 247-279.
- TT Eckdahl, AD Brown, SN Hart, KJ Malloy, Yiu G, et al., (2008) Microarray analysis of the in vivo sequence preference of a minor groove binding drug. *BCM Genomics.* 9: 32-44.
- AN Lane, TC Jenkins, T Brown, S Neidle (1991) Interaction of berenil with the EcoR1 dodecamer d(CGCGAATTCGCG)₂ in solution studied by NMR. *Biochemistry.* 30(5): 1372-1385.
- Ch Brack, E Delain, G Riou (1972) Replicating, covalently closed, circular DNA from kinetoplasts of Trypanosoma cruzi. *Proc Nat Acad Sci USA.* 69: 1642-1646.
- PT Englund, SL Hadjuck, JC Marini (1982) The molecular Biology of trypanosomes. *Ann Rev Biochem.* 51: 695-796.
- JC Marini, KG Miller, PT Englund (1980) Decatenation of kinetoplast DNA of Leishmania tarantolea. *J. Biol Chem.* 255:4976-4979.
- G Riou, M Gabillot, S Douc-Rasy, A Kayser (1982) Partial purification and characterization of nuclear DNA-topoisomerases from trypanosomes (author's transl CR Seances Acad Sci Paris. 294(11): 439-42.
- AY Chen, C.Yu, B Gatto, LF Liu (1993) DNA minor groove-binding ligands: A different class of mammalian DNA topoisomerase I inhibitors. *Proc Nat Acad Sci USA.* 90(17): 8131-8135.
- RE Mcknight, AB Gleason, JA Keyes, S Sahabi (2007) Binding mode and affinity studies of DNA-binding agents using topoisomerase I unwinding assay. *Bioorg Med Chem Lett.* 17(4): 1013-1017.
- TA Shapiro, PT Englund (1990) Selective cleavage of kinetoplast DNA minicircles promoted by antitrypanosomal drugs. *Proc Nat Acad Sci USA.* 87(3): 950-954.
- L Coates, EV Ikpeazu, Y Chen, MS Valenzuela (2002) Inhibition of DNA replication by berenil in plasmids containing poly(dA)poly(dT) sequences. *Plasmid.* 47(2): 120-129.
- TE Snowden, YM Pan, MS Valenzuela (2002) Inhibition of DNA replication by berenil in plasmids containing poly(dA)poly(dT) sequences. 2-D gel analysis of replicative intermediates. *Cell. Mol Biol.* OL279-OL288.
- TE Snowden, Y Chen, YM Pan, MS Valenzuela (2002) Bacterial plasmids containing poly(dA)poly(dT) sequences. Stability and sensitivity to berenil. In *Recent Research Developments in Plasmid Biology.* Transworld Research Network. 1: 1-14.
- MS Valenzuela, MdP Aguinaga, RB Inman (1981) Isolation of DNA fragments containing replicating growing forks from both E.coli and B. subtilis. *Mol Genet.* 181: 241-247.