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In Vivo Effects of Intercalating and Nonintercalating Drugs on the Tertiary Structure of Kinetoplast Deoxyribonucleic Acid†

Jean Bénard* and Guy F. Riou

ABSTRACT: The kinetoplast DNA (kDNA) of cultured *Trypanosoma cruzi* consists mostly in a large network of numerous minicircular molecules (~25 000), with a very low degree of superhelicity. When the intercalating drugs ethidium bromide and 9-hydroxyellipticine were added to the growth medium in concentrations producing trypanocidal effects, the superhelicity of the kDNA was significantly increased. In contrast, the nonintercalating drug berenil had no effect on the superhelicity of kDNA. In the kDNA extracted from trypanosomes resistant to ethidium bromide or 9-hydroxyellipticine, those drugs induce similar effects, although to a lesser extent than in the wild strain.

In unicellular flagellates of the order Kinetoplastida, a modified region of the mitochondrial apparatus, the kinetoplast, contains DNA (kDNA) at high concentration [see reviews by Simpson (1972) and Englund (1979)]. As shown by electron microscopy, the kDNA of *Trypanosoma cruzi* consists in a complex network of more than 25 000 covalently closed minicircles of 1.4 kilobase pairs and longer molecules in small proportion (Riou & Delain, 1969a). These molecules are apparently held together by topological interlocking. In other species of trypanosomes, the longer molecules have been identified as "maxicircles" and can be removed by some restriction endonucleases (Kleisen et al., 1976; Riou & Saucier, 1979).

A number of studies have established that the kDNA of trypanosomes may be considered as a target for DNA-binding drugs [for a review, see Steinert (1971)]. Ethidium bromide, a representative intercalating dye (Crawford & Waring, 1967; Le Pecq & Paoletti, 1967) has trypanocidal activity (Watkins & Woolfe, 1956). This drug binds to kDNA, inhibits its replication, and induces its progressive and complete loss (Riou 1968, 1970; Riou et al., 1980). The intercalating drug 9-hydroxyellipticine has high affinity for DNA (Festy et al., 1971; Le Pecq et al., 1974), decreases the rate of growth of trypanosomes (Bénard & Riou, 1976), and affects the in vitro kDNA transcription process (Bénard & Riou, 1977). Ethidium bromide and 9-hydroxyellipticine have no base composition specificity. Berenil, a nonintercalating drug which preferentially binds to AT-rich DNA (Festy et al., 1970a), inhibits kDNA replication and is also a trypanocidal drug (Brack et al., 1972; Newton, 1972).

Recently we have determined by sedimentation velocity–ethidium bromide titration (Wang, 1969) the degree of superhelicity of closed minicircles in the network of *T. cruzi*: the normal superhelix density of kDNA is very low and varies with the physiological state of the trypanosomes in different phases of growth (Bénard et al., 1979).

Direct measurements of the in vivo binding of intercalating drug to kDNA cannot be done because it is not possible to obtain intact kinetoplasts from *T. cruzi* and because the intercalation process is reversible. An approach consists in measuring the changes in conformation of the minicircles after treatment of the trypanosomes with drugs. Since intercalating drugs unwind the DNA helix (Wang, 1974) and assuming the presence of in vivo enzymatic nicking–closing activities, an increase in the number of superhelical turns per DNA minicircle would be an index of in vivo intercalation. This indirect procedure has been successfully used by Smith et al. (1971)

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with mitochondrial DNA (mtDNA) of HeLa cells.

In the present work we estimate the amount of intercalating drug bound in vivo to the kDNA of *T. cruzi*. We show that the degree of superhelicity of the kDNA increases significantly upon treatment of the trypanosomes by ethidium bromide and 9-hydroxyellipticine. This effect increases with drug concentration; in contrast, the nonintercalating drug berenil has no effect on the tertiary structure of kDNA. Moreover, in trypanosomes resistant to each of these two drugs, the degree of superhelicity of kDNA is found to be lower than that of sensitive cells treated under the same conditions. These data suggest differences in drug permeability in resistant and nonresistant cells.

Materials and Methods

The chemicals were obtained from the following sources: ethidium bromide (EthBr) was from Boots Pure Drug Co., Ltd., Nottingham; berenil, 4,4′-(diazoamino)dibenzamidine diaceturate, was from Hoechst AG, Germany; 9-hydroxyellipticine (9-OH-El) was a generous gift of Dr. Dat-Xuong, Gif sur Yvette, France.

Culture of Trypanosomes. The present experiments were performed with the culture form of a *T. cruzi*, whose origin was the Tehuantepec strain of the Institut Pasteur. The trypanosomes were grown in a biphasic medium, as previously described (Riou & Yot, 1977).

EthBr- or 9-OH-El-Resistant Strains. Strains of trypanosomes resistant to intercalating drugs (EthBr or 9-OH-El) were obtained by subculturing 7-day cultures in progressively higher concentrations of the drugs. Under these conditions, a strain resistant to 4.4×10^{-6} M EthBr (eb^R) was obtained by Riou (1976). By use of the same procedure, cells resistant to 3.4×10^{-5} M 9-OH-El were similarly obtained (el^R). The trypanosomes were considered as resistant when the growth curves became similar during several subcultures in the presence of drug. When stabilized at 3.4×10^{-5} M 9-OH-El, el^R trypanosomes exhibit, after 7 days of culture, a growth inhibition of 30%, when compared to control cells.

Purification of kDNA Networks in Their Two Conformations. Cells were harvested and lysed with 1% sarkosyl in 0.15 M NaCl–0.015 M sodium citrate, pH 7.2 (SSC), for 15 min at 37 °C. The lysate was submitted to Pronase treatment (1 mg/mL) for 2 h at 37 °C and deproteinized with a chloroform–isoamylic alcohol mixture (24:1). After dialysis against SSC, the kDNA networks were pelleted by centrifugation at 9000*g* for 30 min, at 4 °C, resuspended in SSC, and centrifuged again in the same conditions. This procedure was repeated 3 times. The kDNA networks were submitted to a CsCl–EthBr gradient as previously described (Riou & Delain, 1969a) and separated according to their conformation. The lower band contained kDNA network form I, in which most of the minicircles are in their superhelical conformation. The upper band was composed mainly of kDNA network form II, in which all the minicircles have at least one single-strand break. In some experiments, nuclear DNA (<2%) was also detected in the latter band.

Determination of the Degree of Superhelicity of the kDNA Networks. The sedimentation coefficients of kDNA networks were measured by band sedimentation analysis. Centrifugation was performed at 3600 rpm by using an ANJ rotor in a Spinco Model E ultracentrifuge. Aliquots of 0.5–1 μg of purified kDNA, previously dialyzed against 2 M NaCl–10^{-2} M Na_3-EDTA (pH 8.0) (Wang, 1969), were sedimented in 3 M CsCl, at 20 °C. Under these conditions, only kDNA networks in their two forms are capable of forming a band, the nuclear DNA remaining unsedimented. The sedimentation coefficients were expressed without correction for the solvent. For each kDNA sample, the degree of superhelicity of the kDNA network was evaluated by sedimentation velocity–EthBr titration and expressed in ν^0_c values; ν^0_c is the number of bound ethidium molecules per DNA nucleotide, calculated according to Wang (1969) from the free EthBr concentrations corresponding to the minimum of the curve $s = f(\text{EthBr})$. EthBr concentrations were measured by using $\epsilon_M = 5.45 \times 10^3$ (Saucier et al., 1971).

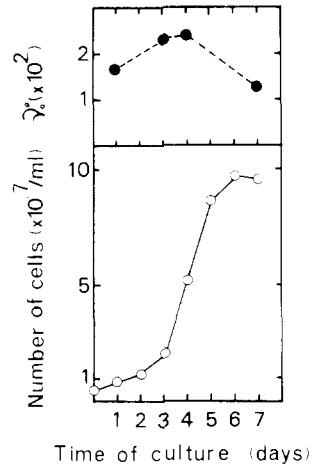

FIGURE 1: Degree of superhelicity of kDNA form I as a function of the growth phase of *T. cruzi*. Trypanosomes were harvested 1, 3, 4, and 7 days after inoculation in the growth medium. Their kDNAs were purified and analyzed by sedimentation velocity–EthBr titration. The degree of superhelicity is expressed in ν^0_c, calculated from the critical free EthBr concentration according to Wang (1969).

Results

Variation in the Degree of Superhelicity of the kDNA during the Growth of the Trypanosomes. The sedimentation velocity–EthBr titration in neutral 3 M CsCl at 20 °C was used to evaluate the degree of superhelicity of the kDNA networks. This dynamic procedure has the advantage that it takes into account only the supercoiled minicircles of the network and that it follows their relaxation by EthBr during the titration. Moreover, the validity of this method has been previously established by using kDNA networks from I nicked by DNase I and resealed with T4 DNA ligase in the presence or absence of EthBr. Under these conditions, the superhelix density values, expressed as ν^0_c, are reproducible within ±10% limits (Bénard et al., 1979).

When analyzed at different stages of culture, the kDNA networks form I exhibit significant variations in their tertiary structures. Under our culture conditions, *T. cruzi* grows at first slowly (preexponential phase) and then goes through an exponential phase before reaching a stationary phase.

The ν^0_c values, obtained for kDNA networks extracted during these three phases, are significantly different. The results are plotted in Figure 1: the ν^0_c value of kDNA is higher during the exponential growth phase than during the preexponential ($\nu^0_c = 1.7 \times 10^{-2}$) and the stationary phases ($\nu^0_c = 1.3 \times 10^{-2}$). This probably reflects the interactions of unwinding proteins during replication and transcription of kDNA. The degree of superhelicity found in kDNA is very low when compared to that of other circular DNA molecules: for SV40 DNA, ν^0_c was evaluated to be 5.8×10^{-2} (Gray et al., 1971) and for mtDNA from HeLa cells, $\nu^0_c = 4.2 \times 10^{-2}$ (Smith et al., 1971).

The Degree of Superhelicity of the kDNA Changes after Treatment with DNA Intercalating Drugs. Since the number of tertiary turns in the kDNA closed minicircles varies during

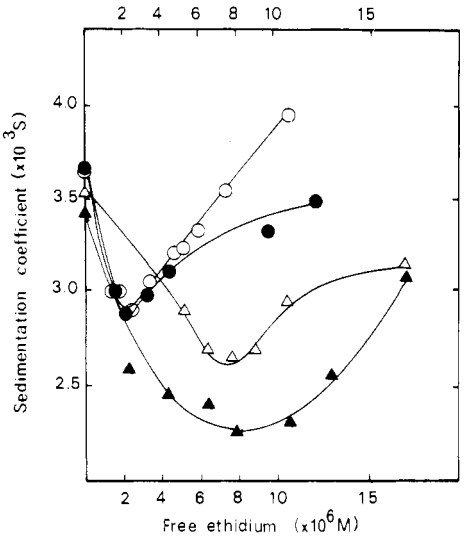

FIGURE 2: Sedimentation coefficients in 3 M CsCl of kDNA form I as a function of EthBr concentration. The kDNA was extracted from trypanosomes in the stationary growth phase. The control kDNA was from 8-day cells. The drug was added after 7 days of culture, the cells were collected 24 h later, and the kDNA was extracted. (○) Control kDNA; (△) kDNA from 1.1×10^{-5} M EthBr-treated trypanosomes; (▲) kDNA from 3.4×10^{-5} M 9-OH-El-treated trypanosomes; (●) kDNA from 3.8×10^{-5} M berenil-treated trypanosomes.

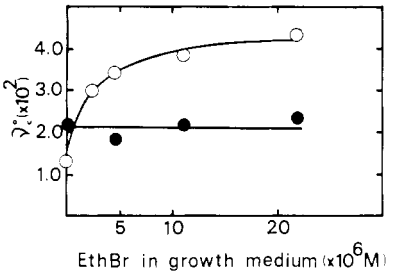

FIGURE 3: ν^0_c values of kDNA form I as a function of EthBr concentration in the growth medium. kDNA form I was analyzed by sedimentation velocity–EthBr titration and ν^0_c was calculated as described under Materials and Methods. (○) Trypanosomes sensitive to 4.4×10^{-6} M EthBr; (●) trypanosomes resistant to 4.4×10^{-6} M EthBr (eb^R).

the growth of trypanosomes and since intercalating drugs induce the loss of kDNA in dividing cells (Riou, 1968; Simpson, 1972), we have chosen to study the action of these drugs on kDNA extracted from trypanosomes during the stationary phase of growth. Under these conditions, changes in superhelicity could only reflect drug interaction with kDNA in vivo.

EthBr or 9-OH-El was added after 7 days of culture and the cells were harvested 24 h later. The degree of superhelicity of the kDNA was analyzed by sedimentation velocity in the presence of EthBr as titrating dye. The experimental results, obtained with several concentrations of EthBr present in the growth medium, are presented in Table I and in Figure 2. The ν^0_c values, obtained for the kDNA of treated trypanosomes, are significantly higher than those for the kDNA of control trypanosomes. When plotted as a function of drug concentration in the growth medium, the ν^0_c values increase progressively before reaching a plateau at $\sim 1.5 \times 10^{-5}$ M (Figure 3). This indicates a saturation process, either in the uptake of the drug by the kinetoplast or in its binding to kDNA.

The drug 9-OH-El exhibits similar in vivo effects on the degree of superhelicity of the kDNA. For a low concentration of 9-OH-El (3.4×10^{-6} M), the ν^0_c value is the same as that

Table I: ν^0_c Values of kDNA Form I from Control and Drug-Treated Trypanosomes

kDNA from	ν^0_c (x10^2)[a]
untreated trypanosomes (control)	1.3
EthBr-treated trypanosomes	
sensitive strain	
2.2×10^{-6} M	3.0
4.4×10^{-6} M	3.4
1.1×10^{-5} M	3.9
2.2×10^{-5} M	4.2
resistant strain[b]	
no drug	2.1
4.4×10^{-6} M (eb^R)	1.9
1.1×10^{-5} M	2.1
2.2×10^{-5} M	2.4
9-OH-El-treated trypanosomes	
sensitive strain	
3.4×10^{-6} M	1.4
3.4×10^{-5} M	4.1
resistant strain[b]	
3.4×10^{-5} M (el^R)	1.7
berenil-treated trypanosomes	
sensitive strain	
3.8×10^{-5} M	1.3

[a] The ν^0_c values were determined by sedimentation velocity–EthBr titration in 3 M CsCl and calculated from the critical free EthBr concentration according to the relation given by Wang (1969). [b] Resistant strain maintained in the absence of drug during three subculture passages before extraction of kDNA.

of the control kDNA. For a higher concentration of 9-OH-El (3.4×10^{-5} M), a ν^0_c value of 4.1×10^{-2} is observed (Figure 2 and Table I). Moreover, the curve $s = f$(EthBr) has a wide profile, suggesting a heterogeneity in the degree of superhelicity of the kDNA networks. In contrast, the nonintercalating drug berenil does not induce any change in the tertiary structure of the kDNA closed minicircles (Figure 2 and Table I).

These data show that EthBr and 9-OH-El intercalate in vivo in kDNA and that nicking–closing cycles may occur during the stationary growth phase of trypanosomes. In addition, the sedimentation velocity–EthBr titration is a suitable method to measure the molar ratio of drug/nucleotide in the complexes formed in vivo.

Superhelicity of kDNA in Resistant Trypanosomes. Strains of trypanosomes resistant to 4.4×10^{-6} M EthBr (eb^R) or 3.4×10^{-5} M 9-OH-El (el^R) were obtained as described under Materials and Methods. The resistant cells were first grown in the absence of drug for three 7-day passages. The resistant and sensitive trypanosomes were then treated with drug under the same conditions. The drug (EthBr or 9-OH-El) was added at several concentrations during the stationary growth phase and the trypanosomes were harvested 24 h later. The degree of superhelicity of kDNA form I from sensitive and resistant cells was evaluated and compared. Table I shows the ν^0_c values obtained with sensitive and resistant (eb^R) cells when using EthBr in various concentrations. The ν^0_c values obtained with resistant cells remained nearly constant [$(1.9–2.1) \times 10^{-2}$] in the absence of drug or with 4.4×10^{-6}, 1.1×10^{-5}, and 2.2×10^{-5} M EthBr, though higher than the value for the control kDNA (1.3×10^{-2}). In contrast, the ν^0_c values obtained for sensitive trypanosomes increase with the drug concentration, before reaching a constant value significantly higher than the value for resistant trypanosomes. For trypanosomes resistant to 9-OH-El, the ν^0_c value increases slightly (1.7×10^{-2}) when compared to the control value. In contrast, for the corresponding sensitive cells a high ν^0_c value (4.1×10^{-2}) is observed. This result indicates that there is more 9-OH-El binding to the kDNA of sensitive trypanosomes.

Discussion

EthBr and berenil specifically interact with kDNA and inhibit its replication (Brack et al., 1972; Riou & Delain, 1969b) and its transcription (Simpson & Simpson, 1978). In rapidly dividing trypanosomes, these drugs induce an alteration of the kDNA structure, with a complete loss of kDNA, yielding dyskinetoplastic trypanosomes (Steinert, 1971). A trypanocidal effect is observed: these cells are no longer viable in a drug-free medium. The drug 9-OH-El (Le Pecq et al., 1974) produces the same effect, though with less specificity for kDNA (Bénard & Riou, 1976).

The number of tertiary turns of closed circular DNA molecules may be changed in vitro by nicking the molecules with pancreatic DNase and closing the intermediate relaxed structures with T4 DNA ligase in the presence of controlled amounts of EthBr (Wang, 1969). Since EthBr unwinds the DNA helix (Wang, 1974), the rotation angle between the base pairs increases after removal of the drug and closure and a definite number of superhelical turns appears, the topological number of a closed circular molecule remaining constant. Thus, the number of tertiary turns is proportional to the number of EthBr molecules bound to DNA at the time of closure. A similar nicking–closing process has been found to occur in vivo in prokaryotic and eukaryotic cells [see Champoux (1978)].

We have shown (Figure 1) that, in the absence of drug, the number of tertiary turns of kDNA increases during the exponential growth phase, which suggests the action of unwinding proteins involved in replication or transcription. When the cells are in the stationary phase, the kDNA is less superhelical and the corresponding ν^0_c is very low (Figure 1 and Table I).

The superhelicity of kDNA has been studied by adding intercalating drugs to trypanosomes during the stationary growth phase, to avoid the total loss of kDNA observed in rapidly dividing cells, as well as the variations in kDNA tertiary structure noticed during cell growth. In the present work, we have estimated the number of EthBr and of 9-OH-El molecules intercalated in vivo with kDNA by measuring its degree of superhelicity. Our results show that EthBr and 9-OH-El induce an increase in superhelicity dependent on drug concentration (Table I; Figure 3). The maximal effect was observed with 1 bound molecule of EthBr/30 nucleotides. The difference in ν^0_c between EthBr-treated cells and control cells was 2.9×10^{-2} (Table I; Figure 3). In mtDNA from mammalian cells treated with EthBr, the ratio of drug/nucleotide found was higher (Smith et al., 1971). The lower saturation level of kDNA by EthBr could be explained by topological constraints existing between the minicircles in the network. The increase in tertiary turns after treatment with intercalating drugs indicates that kDNA is subject to nicking–closing cycles during the stationary growth phase. Studies relative to the kinetoplastic nicking–closing enzyme could precise the level of synthesis and activity during the growth phase of trypanosomes. Recently Fairfield et al. (1979) have purified from rat liver mitochondria a DNA nicking–closing enzyme which is sensitive to EthBr and berenil. In our biological system EthBr modifies the superhelicity of the kDNA; in contrast, berenil, which specifically binds to kDNA but does not intercalate (Festy et al., 1970b; Newton, 1972; Brack et al., 1972), has no effect on the superhelicity of kDNA.

The measurement of the in vivo binding of a drug to DNA can be useful for the understanding of the mode of action of the drug and the mechanism of drug resistance. In the absence of drug during three 7-day subcultures, resistant trypanosomes exhibited a kDNA ν^0_c value of 2.9×10^{-2} which was significantly higher than the value of control kDNA (Figure 3). Since no drug interacted in vivo with kDNA after this "wash-out" period, this result indicates that the kDNA of resistant cells is more superhelical than that of control trypanosomes. This suggests a difference between the resistant and the control cells in the level or the activity of the enzymes involved in the regulation of the conformational state of kDNA in vivo, that is, nicking–closing enzymes, DNA gyrase, and RNA polymerase (Champoux, 1978). Moreover, the ν^0_c of resistant cells does not vary with the concentration of EthBr in the growth medium, while a saturation curve is observed with sensitive cells (Figure 3). The latter observation could be explained by drug metabolization or by a decrease in drug permeability. Preliminary experiments support the latter hypothesis (J. Bénard, unpublished results).

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Escherichia coli Photoreactivating Enzyme: Purification and Properties†

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ABSTRACT: We have purified large quantities of *Escherichia coli* photoreactivating enzyme (EC 4.1.99.3) to apparent homogeneity and have studied its physical and chemical properties. The enzyme has a molecular weight of 36 800 and a $s^0_{20,w}$ of 3.72 S. Amino acid analysis revealed an apparent absence of tryptophan, a low content of aromatic residues, and the presence of no unusual amino acids. The N terminus is arginine. The purified enzyme contained up to 13% carbohydrate by weight. The carbohydrate was composed of mannose, galactose, glucose, and *N*-acetylglucosamine. The enzyme is also associated with RNA (approximately 10 nucleotides/enzyme molecule) containing uracil, adenine, guanine, and cytosine with no unusual bases detected.

Ultraviolet light (220–300 nm) produces cyclobutylpyrimidine dimers between adjacent pyrimidines on the same DNA strand (Setlow, 1963). These dimers have been shown to be a major cause of death and mutation in procaryotes and in a simple eucaryote and have been implicated in many effects of ultraviolet radiation on higher organisms (Setlow, 1973; Cleaver, 1968; Setlow et al., 1969; Sutherland et al., 1970; Hart et al., 1977). The DNA photoreactivating enzyme (EC 4.1.99.3) repairs UV[1]-irradiated DNA in a two-step reaction. The enzyme binds to the DNA (Rupert, 1962), presumably at the dimer, forming a metastable complex. On absorption of a photon in the range 300–600 nm, the enzyme catalyzes the photolysis of the dimer (Setlow et al., 1965), thus producing two monomer pyrimidines and restoring biological integrity to the DNA.

Although the enzyme is important because of its role in DNA repair and as an enzyme requiring light for catalysis, fundamental characterization of the enzyme has been impeded by several problems. First, there are only 10–20 photoreactivating enzyme molecules per *Escherichia coli* cell (Harm, 1964; Kondo & Kato, 1966). Second, the enzyme is labile, frequently losing activity, especially during ion-exchange chromatography. Third, the enzyme aggregates in solution, making difficult the determination of an accurate molecular weight (Sutherland et al., 1973). Finally, the enzyme has a low affinity for commonly used protein stains, making its localization on gels difficult; furthermore, the low absorbance of the apoprotein at 280 nm hampers its detection by absorbance measurements at that wavelength.

We have used an *E. coli* K-12 strain with greatly increased photoreactivating enzyme levels (Sutherland et al., 1972) as a source of large quantities of enzyme. We have developed a gentle purification procedure which allows the production of large quantities of active enzyme. Using methods designed to minimize aggregation and localizing the enzyme by its associated nucleotide-containing "cofactor", we have determined the molecular weight of the native enzyme to be 36 800 and that of the apoprotein to be 35 200 ± 200. We have also characterized the amino acid and carbohydrate composition of the enzyme. The apparent absence of tryptophan makes unlikely dimer photoreactivation by the enzyme by tryptophan photocatalysis.

Experimental Section

Assay for Photoreactivation. Samples were assayed for dimer photoreactivation by the method of Sutherland & Chamberlin (1973). All assays were carried out by using yellow Sylvania gold lamps as safelights.

Binding Assay. Fractions were tested for their ability to bind to ultraviolet (UV)-irradiated DNA by a filter binding assay, similar to that of Madden et al. (1973). Samples were added to 0.2 mL of an assay mix consisting of 20 mM KPO_4 buffer, pH 7.2, 62.5 mM NaCl, 0.1 mM dithiothreitol (DTT), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 2.5% dimethyl sulfoxide (Me_2SO), 0.25 mM calf thymus DNA, and 30–100 pmol of UV-irradiated ^{32}P-labeled T7 DNA. The mixture was incubated at 37 °C for 10 min, and the reaction was stopped by addition of 1.0 mL of an ice-cold rinse buffer (20 mM KPO_4 buffer, pH 7.2, 62.5 mM NaCl, 0.1 mM DTT, and 1.0 mM EDTA). Samples were then filtered through

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[1] Abbreviations used: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; Me_2SO, dimethyl sulfoxide; $NaDodSO_4$, sodium dodecyl sulfate; i.d., inner diameter; PRE, photoreactivating enzyme; UV, ultraviolet.

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