Noncovalent Drug–DNA Binding Interactions That Inhibit and Stimulate (A)BC Excinuclease†

Christopher P. Selby* and Aziz Sancar
Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

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ABSTRACT: (A)BC excinuclease from *Escherichia coli* catalyzes the initial step of nucleotide excision repair. It recognizes and binds to many types of covalent modifications in DNA and incises the damaged strand on both sides of the lesion. We employed a variety of noncovalent DNA binding drugs to examine in vitro the mechanisms and the nature of the DNA–drug interactions responsible for two phenomena: inhibition of excision repair by caffeine and other noncovalent DNA binding compounds; incision of undamaged DNA produced by (A)BC excinuclease in the presence of the bisintercalating drug ditercalinium. All of the chemicals examined (e.g., actinomycin D, caffeine, ethidium bromide, and Hoechst 33258) inhibited incision of a covalent adduct by (A)BC excinuclease, and direct evidence is given for a common mechanism in which UvrA is depleted by binding to drug-undamaged DNA complexes. In the absence of significant amounts of undamaged DNA, another mechanism of inhibition was observed, in which enzyme bound to noncovalent drug–DNA complexes in the vicinity of the lesion prevents formation of preincision complexes at the lesion. Ditercalinium and unexpectedly all of the other drugs examined promoted the incision of undamaged DNA when the enzyme was present at high concentration. Thus, this activity contrary to previous assumptions is not unique to bisintercalators. Another unexpected finding was stimulation of incision at certain sites of photodamage in DNA produced by low concentrations of noncovalent DNA binding chemicals. Thus, enzyme inhibition, the stimulation of incision at certain damaged sites, and the production of enzymatic incisions at undamaged sites in DNA are effects that many noncovalent DNA binding chemicals have on (A)BC excinuclease, including intercalating drugs and minor groove binders with high and low sequence specificity.

(A)BC excinuclease initiates nucleotide excision repair in *Escherichia coli* by producing incisions in the damaged DNA strand on both sides of the lesion (Sancar & Rupp, 1983). The incision process is initiated when the $(UvrA)_2(UvrB)_1$ complex, guided by UvrA's affinity for damaged sites, binds to a lesion. UvrB, which has no affinity for DNA, is "loaded" onto the lesion by UvrA. UvrA then dissociates from the UvrB-damaged DNA complex, and incisions are made upon binding of UvrC to this complex (Orren & Sancar, 1989, 1990). The incision process is followed by displacement of the oligomer containing the damage, synthesis of a repair patch by *Pol*I, and ligation by DNA ligase (Sancar & Rupp, 1983). This repair pathway enables cells to survive damage produced in their DNA by a wide range of genotoxicants because (A)BC excinuclease recognizes an enormous range of DNA damage as substrate (Selby & Sancar, 1990a).

A deficiency in nucleotide excision repair, brought about by enzyme inhibitors or genetic manipulations, greatly enhances cellular sensitivity to mutation and killing by DNA-damaging treatments. Caffeine and a number of other intercalating and nonintercalating DNA binding chemicals have been shown to sensitize *E. coli* cells to mutation and/or killing (Lieb, 1961; Witkin, 1961; Kihlman, 1977). In vivo/in vitro studies have shown that caffeine inhibits the incision step of repair in *E. coli* (Shimada & Tagaki, 1967; Seeberg & Strike, 1976). Snyder (1987) found that all six of the strong intercalating drugs examined inhibited the incision step in cultured human fibroblasts. We found that in vitro, caffeine inhibits incision by (A)BC excinuclease and promotes the binding of UvrA to areas of DNA with no known covalent damage. We suggested that caffeine inhibits incision by depleting the amount of UvrA subunit available to catalyze incision. Ethidium bromide and chloroquine also promoted the binding of UvrA to undamaged DNA (Selby & Sancar, 1990b,c). Thus, it is possible that UvrA trapping, leading to inhibition of nucleotide excision and cellular sensitization, may be brought about by many intercalators and possibly other noncovalent DNA binding drugs. However, the properties of the noncovalent drug–DNA complexes that evoke UvrA trapping and consequently the scope of compounds that may inhibit the enzyme by this mechanism are unknown.

The bisintercalating drug ditercalinium is quite toxic to *E. coli* even in the absence of DNA-damaging treatments. Interestingly, an in vivo study associated this toxicity with the presence of a functional UvrA protein (Lambert et al., 1988). An in vitro study with purified (A)BC excinuclease showed that, similar to caffeine, ditercalinium promoted the binding of enzyme to apparently undamaged DNA. However, ditercalinium also promoted the enzyme-mediated incision of undamaged DNA (Lambert et al., 1989), an activity that was not observed with caffeine (Selby & Sancar, 1990b) and has not to our knowledge been demonstrated with any other drug. It was proposed that the mechanism of ditercalinium cytotoxicity in *E. coli* arose from (1) formation of stable undamaged DNA–repair enzyme complexes and/or (2) repeated enzymatic incision of DNA, both of which could interfere with DNA metabolism and possibly deplete the repair enzyme (Lambert et al., 1988, 1989).

The purpose of our investigation into the effects of noncovalent DNA ligands on (A)BC exinuclease activity was to more closely examine (1) the UvrA trapping mechanism

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* Address correspondence to this author.

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proposed for enzyme inhibition, (2) the structural features of compounds that inhibit by this mechanism, and (3) why ditercalinium promotes incision in undamaged DNA and other drugs (e.g., caffeine) apparently do not. Our data provide direct evidence of a UvrA trapping model of enzyme inhibition by showing that inhibition of incision is associated with binding of UvrA to undamaged DNA and failure of UvrA to form preincision complexes, and the fact that inhibition can be reversed by supplementing incision reactions with additional UvrA. Inhibition by UvrA trapping was brought about by many noncovalent DNA ligands which include intercalators and minor groove binders with different sequence specificities. Unexpectedly, all of the drugs studied were also found to promote incision of undamaged DNA when high concentrations of enzyme were used, although incision in the presence of caffeine was quite low even under these conditions. Interestingly, we also found that all of the drugs studied also enhanced incision at certain sites of UV photodamage.

Materials and Methods

Materials. The DNAs used in our experiments included a 138 bp oligomer with a uniquely located psoralen adduct (HMT-DNA, Van Houten et al., 1987), a 137-mer with a thymine–thymine cyclobutane dimer (T<>T-DNA; Selby & Sancar, 1990d), and a 48-mer with a thymine dimer (Lin & Sancar, 1990). Each was synthesized and either end-labeled (137- and 138-mers) or internally labeled (48-mer) with ^{32}P as described in the references given. In some experiments, we used control oligomers that lacked the modification or control oligomers or plasmids that were irradiated with germicidal UV light. Supercoiled pBR322 and pDR3274 (6.24 kbp; Thomas et al., 1985) were purified by cesium chloride/ethidium bromide density gradient centrifugation, and in some cases, [^{3}H]dT-labeled pBR322 was used [preparation described in Selby and Sancar (1988)]. The text and figures give amounts of plasmid DNA in terms of nanomoles of plasmid molecules, not nanomoles of base pairs.

Chemicals were from Aldrich (acriflavine, crystal violet, methylene blue, quinacrine, and toluidine blue), Fluka (acridine orange), Molecular Probes (Hoechst 33258), and Sigma (actinomycin D, caffeine, chloroquine, and ethidium bromide), except ditercalinium which was graciously provided by Drs. B. Lambert and J.-B. LePecq. All drugs were dissolved in water except chloroquine, which was brought to pH 7.1–7.5 in 1 M Tris. The ditercalinium concentration was determined by using a molar extinction coefficient of 65 000 at 262 nm, and other drug concentrations were determined by weight, using a molecular weight of 232 for acriflavine. The Uvr proteins were purified as described in Thomas et al. (1985).

Methods. Incision by (A)BC excinuclease was examined by digesting substrate oligomers with enzyme in ABC reaction buffer (50 mM Tris, 50 mM KCl, 10 mM $MgCl_2$, 2 mM ATP, 5 mM dithiothreitol, and 50 μg/mL BSA, pH 7.4) and analyzing the products on sequencing gels, or by digesting supercoiled substrate plasmids and quantitating the conversion to a nicked form as detected by mobility on agarose gels (Selby & Sancar, 1988). The superhelical and nicked forms were quantitated either by scanning densitometry of photographs of the gels or by excising the ^{3}H-labeled DNAs and scintillation counting.

Binding of UvrA and/or UvrB to the uniquely modified HMT-DNA substrate or to control, undamaged substrate was examined by DNase I footprinting (Selby & Sancar, 1990b). In some cases, the effect of drug on binding of UvrA to undamaged oligomers was measured by the gel retardation technique (Garner & Rezvin, 1981). In this approach, the

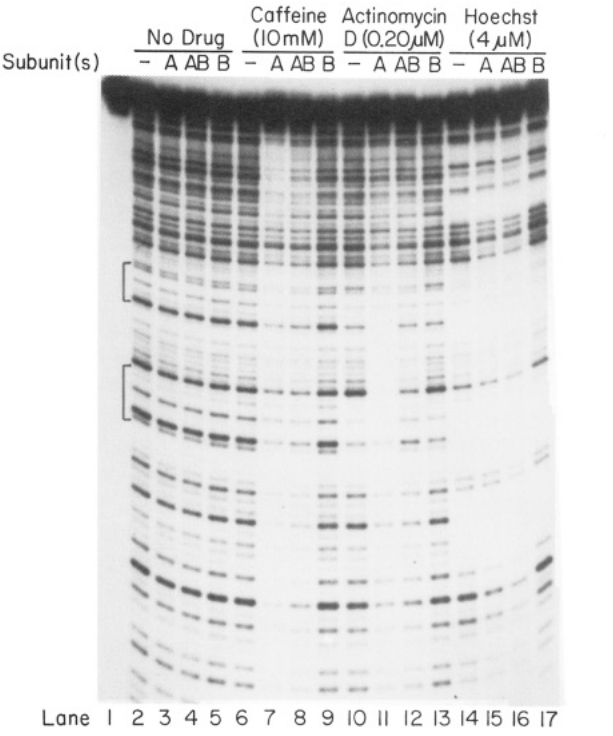

FIGURE 1: Protection of unmodified DNA from DNase I by noncovalent DNA binding drugs plus either UvrA or UvrA plus UvrB. The control ^{32}P-labeled 137-mer that lacks covalent modification was incubated with the drugs shown at the indicated concentration for 30 min. UvrA (4.2 nM) and/or UvrB (60 nM) was then added, and after 30-min incubation, the DNA was digested with DNase I. Products were analyzed on an 8% sequencing gel followed by autoradiography. The brackets to the left locate two partially occupied actinomycin D binding sites (compare lanes 2 and 10). In lane 1, the DNA was incubated without drug, protein, or DNase I. (A, UvrA; B, UvrB.)

^{32}P-labeled undamaged DNA substrate, drug, and protein were incubated for 20–30 min at room temperature and then loaded onto a 4.5% polyacrylamide gel (running and gel buffers, 50 mM Tris–borate, pH 8.2, and 1 mM EDTA) running at 50–100 V, and electrophoresis was for 2–4 h. Gels were autoradiographed to visualize the amount of free and protein-bound DNA. Experiments with drugs were conducted under dim yellow illumination.

Results

Inhibition of Incision and Nonspecific Binding of UvrA. It has been proposed that caffeine inhibits incision by (A)BC excinuclease by "trapping" UvrA in complexes with undamaged DNA and depleting the amount of UvrA available for reaction. To examine this model of enzyme inhibition and the scope of compounds that inhibit in this manner, we first surveyed a number of noncovalent DNA binding drugs for their capacity (1) to promote binding of UvrA to undamaged DNA and (2) to inhibit incision by (A)BC excinuclease.

Figure 1 gives examples of drug-induced binding of UvrA to undamaged DNA using the DNaseI footprinting technique. Lane 1 shows the unmodified 137-mer, and lane 2 shows the DNase I digestion pattern of the 137-mer in the absence of drug or protein. Lanes 3 and 4 show that in the absence of drug, there is minimal binding of UvrA or UvrA and UvrB to the DNA at the protein concentrations used. Lane 5 shows that UvrB does not protect the DNA from DNaseI, which is expected because it only binds to DNA when "loaded" onto damaged sites by UvrA (Orren & Sancar, 1989, 1990). Lanes 6, 10, and 14 show the effects of caffeine, actinomycin D, and

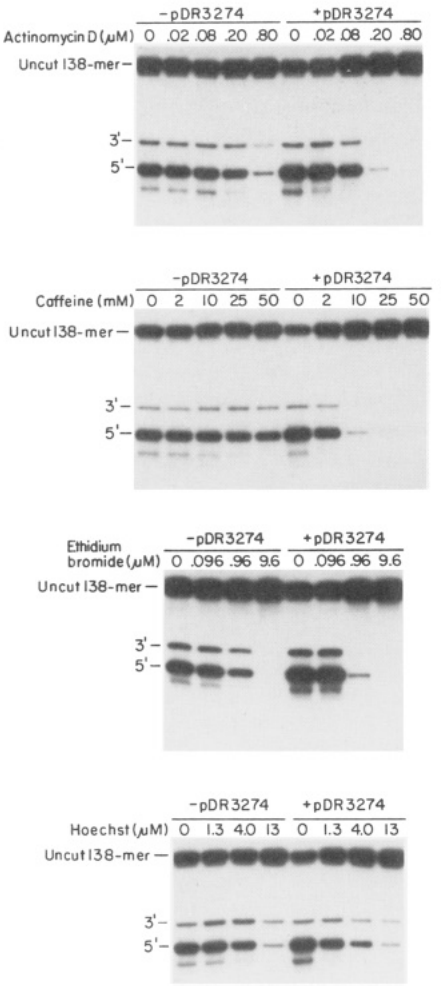

FIGURE 2: Inhibition of incision by noncovalent DNA ligands. The ^{32}P-labeled 138-mer HMT-DNA, either alone or mixed with unlabeled pDR3274 (0.6 nM), was incubated with actinomycin D, caffeine, ethidium bromide, or Hoechst 33258 at the indicated concentrations for 30 min. Then UvrA (4.2 nM) and UvrB (60 nM) were added, and after 30 min, UvrC (140 nM) was added and digestion proceeded for 15 min at 37 °C. Reactions were then stopped, and the products were analyzed on 8% sequencing gels followed by autoradiography. The location of the uncut substrate 138-mer is indicated. The source of the major digestion product (indicated by 5′), and a minor product (indicated by 3′), is described in the text. We note that the additional faint incision band 5′ to the psoralen adduct is probably due to damage to a guanine residue 5′ to the adduct produced by phosphotriester synthesis (Van Houten et al., 1987). The four panels show autoradiograms of four separate gels.

Hoechst, respectively, on the DNase I digestion. Actinomycin D and Hoechst bind with high affinity to certain sequences as indicated by striking areas of protection from DNase I. The drugs promote the binding of UvrA (lanes 7, 11, and 15) and possibly also UvrA–UvrB complexes (lanes 8, 12, and 16) to undamaged DNA, as indicated by the enhanced protection against DNase I which cannot be accounted for by a simple summation of drug and protein binding. The drugs did not promote the binding of UvrB alone (lanes 9, 13, and 17).

Figure 2 shows incision of the HMT-DNA by (A)BC excinuclease in the presence of four noncovalent DNA binding drugs. The enzyme makes incisions on both sides of the adduct; however, because the radiolabel is located at the 5′ end of the damaged strand, the major product seen on the sequencing gels is the 5′ incision product (indicated by 5′), and the band marked 3′ indicates a product formed at low yield when the enzyme makes a 3′ incision that is not coupled with a 5′ incision. (Coupling of the two incisions will be addressed in a later section.) Figure 2 clearly demonstrates inhibition of incision by the drugs.

If caffeine and other noncovalent DNA binding drugs inhibit (A)BC excinuclease by making UvrA bind to undamaged DNA, then it follows that inhibition of incision by caffeine and related drugs should be sensitive to the amount of drug, enzyme, and unmodified DNA present in the reaction. To examine this prediction, we included undamaged DNA in the form of unlabeled pDR3274 in the incision reactions shown in Figure 2. In the absence of drug, the addition of plasmid slightly stimulates incision by removing UvrA from the preincision UvrB–DNA complex (Bertrand-Burggraf et al., 1991). More importantly, incision was more strongly inhibited by the drugs in the presence of plasmid than in the absence of plasmid. This observation is consistent with the UvrA trapping model of inhibition, since the undamaged plasmid DNA provides additional sites for nonspecific binding of UvrA which leads to further depletion of UvrA and inhibition of incision.

In our survey of noncovalent DNA binding drugs, we measured incision and UvrA binding using several concentrations of each drug. These experiments showed that the drug concentrations required to inhibit incision also promoted binding of UvrA to undamaged DNA (e.g., compare Figures 1 and 2). This association was observed when the following drugs were examined (data not shown): acridine orange, acriflavine, actinomycin D, caffeine, crystal violet, chloroquine, ethidium bromide, Hoechst 33258, methylene blue, quinacrine, and toluidine blue. Thus, these results show that inhibition of incision by (A)BC excinuclease and the capacity to promote binding of UvrA to undamaged DNA are related properties shared by many noncovalent DNA binding drugs.

Failure To Form UvrA–UvrB Preincision Complexes. In the UvrA trapping model, incision complexes fail to form at the site of covalent damage in DNA because caffeine and related compounds promote enzyme binding to undamaged DNA instead. To test this model, we conducted DNase I footprinting experiments using end-labeled HMT-DNA to visualize preincision complexes formed by UvrA and UvrB at a covalent adduct. For unmodified DNA, we used unlabeled pDR3274. The trapping model predicts that the presence of appropriate amounts of undamaged plasmid and drug would prevent binding of UvrA and UvrA–UvrB to the covalent adduct in the HMT-DNA.

Before describing the effect of drugs on the enzyme footprints, it is necessary to consider the mechanism of damage recognition by UvrA and UvrB (Orren & Sancar, 1989, 1990) and the corresponding footprints that these subunits make at the site of damage (Bertrand-Burggraf et al., 1991). UvrA and UvrB form a $(UvrA)_2(UvrB)_1$ complex in solution which recognizes and binds to covalent damage in DNA (Orren & Sancar, 1989, 1990). The DNA-bound complex, which consists of UvrA and UvrB possibly in a 2:1 ratio, makes a 48 bp footprint around the psoralen adduct in the HMT-DNA and a DNase I hypersensitive site at the 11th phosphodiester bond 5′ to the lesion, as illustrated in Figure 3 (lane 10). The addition of plasmid has no effect on the hypersensitive site but causes the footprint to shrink to about 19 bp (Figure 3, lane 14). The footprint shrinks because added plasmid favors dissocation of UvrA from the nominal $(UvrA)_2(UvrB)_1$ complex. The remaining 19 bp footprint consists primarily of UvrB loaded onto the damaged site (Bertrand-Burggraf et al., 1991); this complex leads to incision upon addition of UvrC (Orren & Sancar, 1990). Adding plasmid stimulates incision reactions (e.g., Figure 2), because the presence of UvrA in the UvrA–

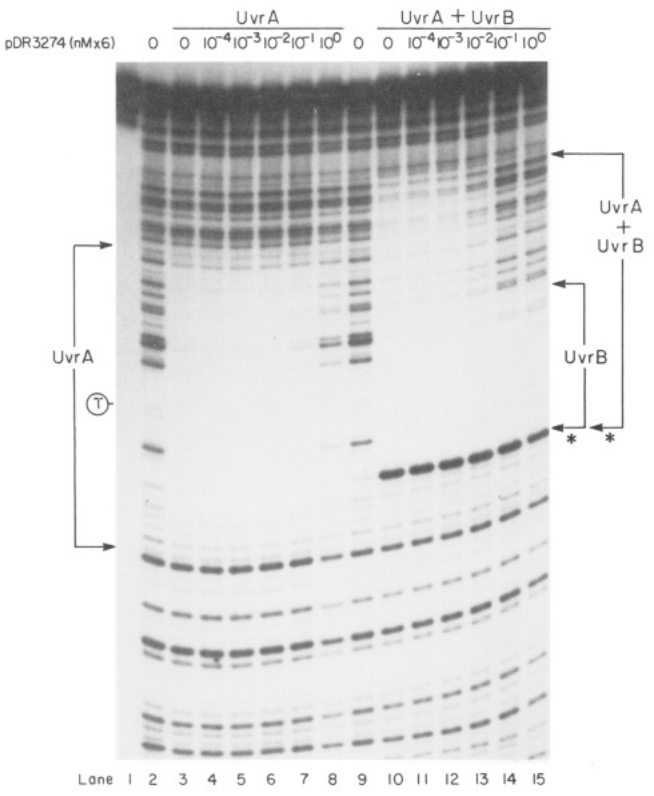

FIGURE 3: Effect of unmodified plasmid on UvrA and (UvrA)UvrB footprints as a function of plasmid concentration. The 5′ end-labeled HMT-DNA was mixed with the indicated concentrations of unlabeled pDR3274. The DNAs were incubated with UvrA (4.2 nM) plus or minus UvrB (60 nM) for 30 min. The DNAs were then digested with DNase I. Reactions were stopped, and products were analyzed on an 8% sequencing gel followed by autoradiography. In lane 1, DNA was incubated without Uvr proteins or DNase I, and in lane 9, DNA was incubated with UvrB (60 nM) only and digested with DNase I. The circled T marks the location of the psoralen adducted thymine, brackets indicate the UvrA, UvrA–UvrB, and UvrB footprints, and the (UvrA)UvrB-hypersensitive site is located by the asterisks.

UvrB preincision complex actually interferes with incision (Bertrand-Burggraf et al., 1991). The enzyme is called (A)BC excinuclease with A in parentheses because UvrA is not present in the incision complex (Orren & Sancar, 1989). UvrA in isolation does bind to the psoralen adduct, making a 33 bp footprint (Figure 3); however, this binding of UvrA alone probably is not a step that leads to incision (Bertrand-Burggraf et al., 1991).

Footprinting results obtained in the presence of ethidium bromide are shown in Figure 4. In the absence of plasmid (Figure 4A), ethidium bromide does not affect the UvrA or UvrA–UvrB footprints, per se, but promotes enzyme binding all over undamaged regions of the substrate. Ethidium bromide specifically reduces the intensity of the UvrA–UvrB-hypersensitive site at a concentration (0.96 μM) that corresponds to inhibition of incision (Figure 2). For footprinting experiments in the presence of plasmid, we used pDR3274 at 0.6 nM because at this plasmid concentration (1) the UvrA footprint begins to fade (due to the high affinity of UvrA for undamaged DNA; Figure 3, lanes 2–8), (2) the UvrB–DNA complex becomes discernible (lanes 9–15), and (3) DNase I is not depleted (as in lanes 8 and 15). The results obtained with ethidium bromide in the presence of plasmid (Figure 4B) show that the drug *abolishes* both the UvrA and the UvrA–UvrB footprints, indicating that the unmodified plasmid DNA–drug complexes do effectively compete with the covalently damaged site for enzyme binding. Again, the reduced intensity of the UvrA–UvrB-hypersensitive site at 0.96 μM drug parallels the reduced level of incision (Figure 2). Essentially the same observations were made in footprinting experiments conducted with actinomycin D, caffeine, and Hoechst 33258 (data not shown).

Thus, the footprinting results show that the drugs inhibit incision by preventing the formation of UvrA–UvrB preincision complexes (detected by the UvrA–UvrB-hypersensitive site). The results obtained in the presence of plasmid are entirely consistent with the UvrA trapping model in which preincision complexes do not form because UvrA is nonspecifically bound to the undamaged plasmid DNA. However, results obtained in the absence of plasmid were different in that (1) higher concentrations of the drugs were needed to inhibit incision (Figure 2) and inhibit formation of the UvrA–UvrB-hypersensitive site (Figure 4), and (2) failure to form preincision complexes was associated with extensive binding of enzyme all over the HMT-DNA (Figure 4A). These latter results suggest a different mechanism of inhibition, in which nonspecific binding of enzyme adjacent to the lesion prevents preincision complexes from forming at the site of the lesion. We note that the reaction conditions in which plasmid is added are more physiologically relevant because in the absence of plasmid the DNA (138-mer) concentration is quite low (in the picomolar to nonomolar range) and the ratio of undamaged to damaged DNA is very low (137 bp:1 bp).

Inhibition Results from Depletion of UvrA. The data support a UvrA trapping model of inhibition in which under physiologically relevant conditions, the noncovalent DNA binding drugs promote the binding of UvrA to undamaged DNA and effectively reduce the amount of UvrA available to catalyze repair. However, the data presented to this point do not rule out the possibilities that the drugs inhibit by trapping UvrA–UvrB complexes with undamaged DNA or that UvrA loads UvrB onto drug–DNA complexes, which effectively depletes UvrB. These alternatives are plausible if the enzyme reacts with the noncovalent drug–DNA complex in the same way that it reacts with covalent lesions. Also consistent with these alternatives is our observation that the drugs promoted enzyme binding to undamaged DNA when UvrA was added alone or together with UvrB (Figures 1 and 4A).

In order to examine which subunits are effectively depleted during enzyme inhibition, we measured inhibition of incision under conditions in which each subunit individually was used at a concentration so low that it limited the extent of incision in the absence of drug. We reasoned that if the drugs inhibit by trapping UvrA and lowering its effective concentration, then the enzyme would be more sensitive to inhibition when UvrA was limiting and UvrB and UvrC were in excess than when either UvrB was limiting and UvrA and UvrC were in excess or when UvrC was the limiting subunit. If the drugs inhibit by trapping UvrA–UvrB complexes, then the enzyme would be more sensitive under both the UvrA-limiting and the UvrB-limiting conditions than when UvrC was the reaction-limiting subunit. Finally, if the drugs inhibit because UvrB is loaded onto noncovalent drug–DNA complexes, then the enzyme would be more sensitive under the UvrB-limiting condition than under the UvrA-limiting or the UvrC-limiting conditions.

Enzyme inhibition experiments utilized the plasmid nicking assay in which the enzymatic incision of supercoiled, UV-irradiated (125 J/m^2) plasmid pBR322 (at 1.7 nM) is measured by its conversion to the nicked form. Results are given in Table I. Using relatively high subunit concentrations (14 nM UvrA, 120 nM UvrB, and 160 nM UvrC) gave 1.7 nicks per plasmid.

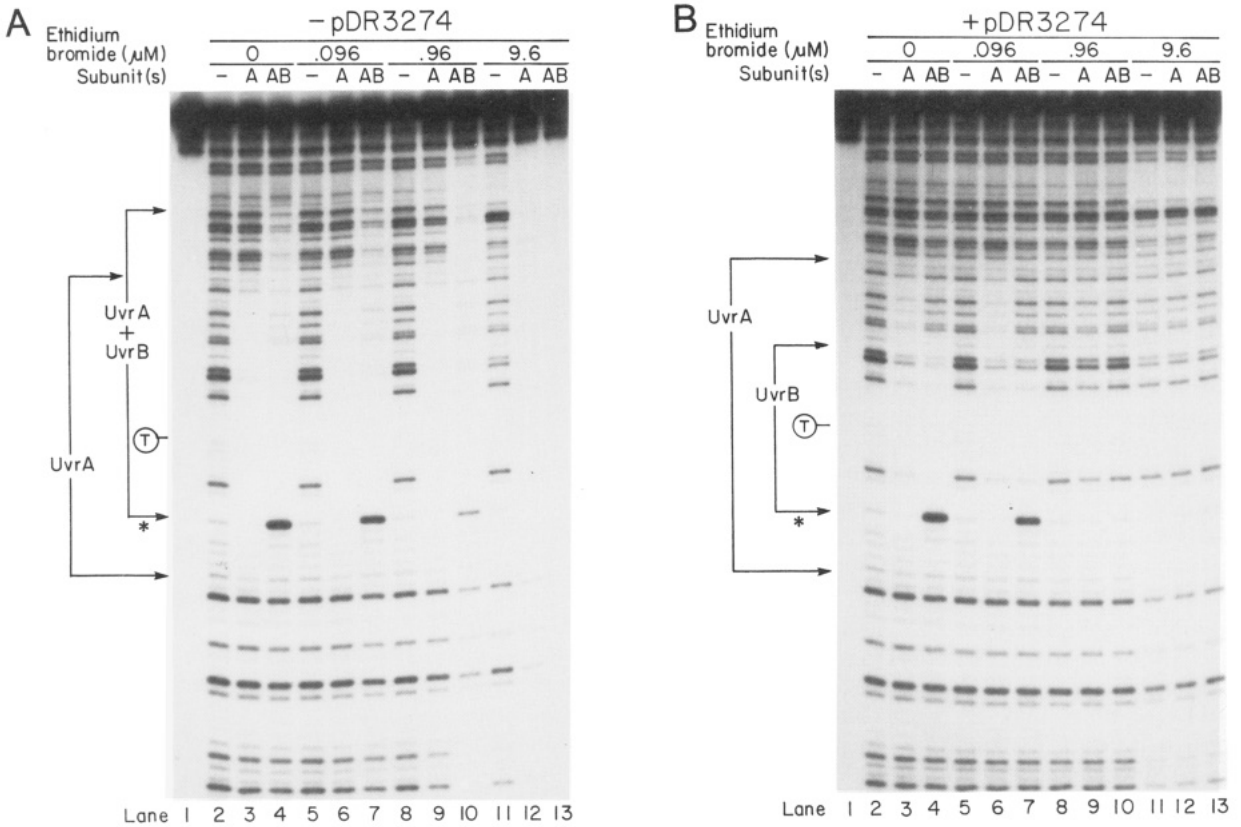

FIGURE 4: Effect of ethidium bromide on the binding of UvrA and (UvrA)UvrB to a psoralen adduct in the absence (A) and presence (B) of 0.6 nM pDR3274. The end-labeled 138-mer HMT-DNA and the unlabeled plasmid were incubated with drug at the indicated concentration for 30 min. Then UvrA (4.2 nM) and UvrB (60 nM) were added as indicated (A, UvrA; B, UvrB) and incubations continued for 30 min before digestion with DNase I. Reactions were stopped and products were analyzed as in the Figure 3 legend. In lanes 1, the DNAs were incubated without Uvr proteins or DNase I. The adducted T residue, footprints, and hypersensitive site are indicated as in Figure 3. Note that at 9.6 μM ethidium bromide gave overall protection from DNase I (compare lanes 2 and 11). Ethidium bromide at 0.96 μM produced only marginal overall protection from DNase I (compare lanes 2 and 8) but drastically reduced the intensity of the UvrA–UvrB-hypersensitive site (compare lanes 4 and 10).

Table I: Inhibition of Incision under Subunit-Limiting Conditions[a]

	subunit concn (nM)			nicks per plasmid			
limiting subunit	UvrA	UvrB	UvrC	no drug	actinomycin D	ethidium bromide	Hoechst
	14	120	160	1.7	1.7	1.5	1.7
UvrA	**1.0**	120	160	0.73	0.40	0.24	0.48
UvrB	14	**12**	160	0.78	0.82	0.70	0.82
UvrC	14	120	**80**	0.50	0.55	0.50	0.44

[a] The pBR322 (1.7 nM) was incubated with no drug, 0.08 μM actinomycin D, 0.76 μM ethidium bromide, or 1.3 μM Hoechst for 30 min, then UvrA and UvrB were added, and incubation was continued for 30 min. Then UvrC was added, incubation was continued at 37 °C for 8 min, and reactions were stopped by adding an SDS–dye loading mixture. Nicked and supercoiled plasmids were separated on 0.8% agarose gels, and the nicks per plasmid were quantitated on the basis of densitometric scans of photographs of the gels. The values given are the difference in values obtained by using UV-irradiated (125 J m^{-2}) and unirradiated plasmids. The concentrations of limiting subunits are in boldface.

When the amount of UvrA was reduced to 1 nM, the extent of reaction was reduced from 1.7 nicks per plasmid to 0.73 nick per plasmid. When UvrB was the limiting subunit (12 nM), there were 0.78 nick per plasmid, and when UvrC was limiting (80 nM), there were 0.50 nick per plasmid. When 0.08 μM actinomycin D, 0.76 μM ethidium bromide, or 1.3 μM Hoescht were included in incision reactions utilizing the various subunit-limiting conditions, inhibition of incision was observed only under the UvrA-limiting condition. A separate experiment (not shown) under identical conditions but with caffeine at 10 mM also showed inhibition of incision only under UvrA-limiting conditions. These results together with our previous findings clearly show that under physiological conditions, the drugs inhibit (A)BC excinuclease by trapping UvrA with undamaged DNA and depleting the amount of UvrA available to catalyze repair. Furthermore, the results in Table I show that the inhibition is reversed by adding more UvrA.

Binding of UvrA to a Drug Binding Site in Undamaged DNA. The simplest model to describe UvrA trapping assumes that UvrA binds to a noncovalent drug–DNA complex much as it binds to a covalent drug–DNA complex. Such a ternary complex would exist until the noncovalently bound drug, UvrA, or both dissociate (Lambert et al., 1989). If such stable complexes exist, then theoretically it should be possible to observe them by footprinting methods. In practice, however, it is not simple to clearly demonstrate these complexes because of numerous complicating factors, for example, the need to locate discrete high-affinity drug binding sites, the demonstrated ability of UvrA bound to DNA to act as a nucleation site for additional UvrA binding, and consequently the requirement that all reactants be present in optimal quantities.

Our best evidence of UvrA binding to undamaged DNA at a noncovalent drug binding site was obtained by using actinomycin D and is shown in Figure 1. The substrate has two high-affinity actinomycin D binding sites that are separated by about 10 bp and are partially occupied in Figure 1 (compare lanes 2 and 10). Lane 11 shows that in the presence of UvrA and actinomycin D, UvrA most strongly inhibits DNase I digestion in the region of the drug binding sites. This enhanced protection from DNase I is most clearly evident in the region between the two drug binding sites, where the two corresponding UvrA footprints would be expected to overlap. Thus, these data demonstrate a drug noncovalently bound to DNA acting as a surrogate lesion for binding by UvrA.

Effect of Chloroquine on Coupled Incisions. In our initial survey of different drugs, we saw unique effects of chloroquine on the apparent coupling of the two incisions that (A)BC excinuclease makes at a covalent lesion. By coupled incisions, we mean that after the digestion reaction is stopped, both the 5′ and 3′ incisions have been made (either simultaneously or sequentially). By uncoupled incisions, we mean that the digestion product has only the 3′ or the 5′ incision.

Analysis of (A)BC excinuclease digestion products of the HMT-DNA on sequencing gels provides some information about the coupling of the two incisions. As noted previously, the HMT-DNA is labeled at the 5′ end of the damaged strand, as indicated in Figure 5C, and the principal digestion product seen on a sequencing gel is the labeled product produced by incision on the 5′ side of the lesion as indicated in Figure 5A, lane 1. This product results when the enzyme makes coupled incisions and if the enzyme makes an uncoupled 5′ incision. The digestion product labeled 3′ (Figure 5A) can only arise from an uncoupled 3′ incision (Van Houten et al., 1987). Approximately 10% of the product made upon digestion of the HMT-DNA with (A)BC excinuclease is the uncoupled 3′ incision product (e.g., Figures 2 and 5A).

It has been found with the HMT-DNA that suboptimal reaction conditions enhance uncoupling (Sancar & Sancar, 1988; Selby & Sancar, 1988). In particular, the 5′ incision is selectively inhibited, but the 3′ incision is relatively unaffected; with the HMT-DNA, this produces a decrease in the 5′ incision band and a concurrent *increase* in the 3′ incision band when the products are separated on a sequencing gel (Selby & Sancar, 1988). A different type of "coupling effect" was observed as the concentration of chloroquine was increased (Figure 5A). Chloroquine produced a reduction in the 5′ band that was *not* associated with an increase in the 3′ band. Thus, chloroquine does not selectively inhibit the 5′ incision but selectively inhibits the formation of both of the coupled incisions. (If chloroquine selectively inhibited the 5′ incision, then since the HMT-DNA is labeled at the 5′ end of the damaged strand, the intensity of the 3′ band would increase.) Furthermore, the different dose–response relationships for inhibition of the coupled (5′ band) versus uncoupled 3′ incisions indicate the existence of two different populations of enzyme complexes: a chloroquine-sensitive population that makes coupled incisions and a chloroquine-resistant population that makes the uncoupled 3′ incision.

Our interpretation of the effect of chloroquine is confirmed by results obtained by using the 48-mer containing a thymine dimer. This substrate, illustrated in Figure 5D, possesses two internal labels; coupled incisions at the dimer by (A)BC excinuclease produce a labeled 13-mer, and uncoupled 3′ incision produces a labeled 30-mer. (All 3′ incisions, coupled and uncoupled, produce a labeled 19-mer.) The effect of chloroquine on incision of the 48-mer is shown in Figure 5B.

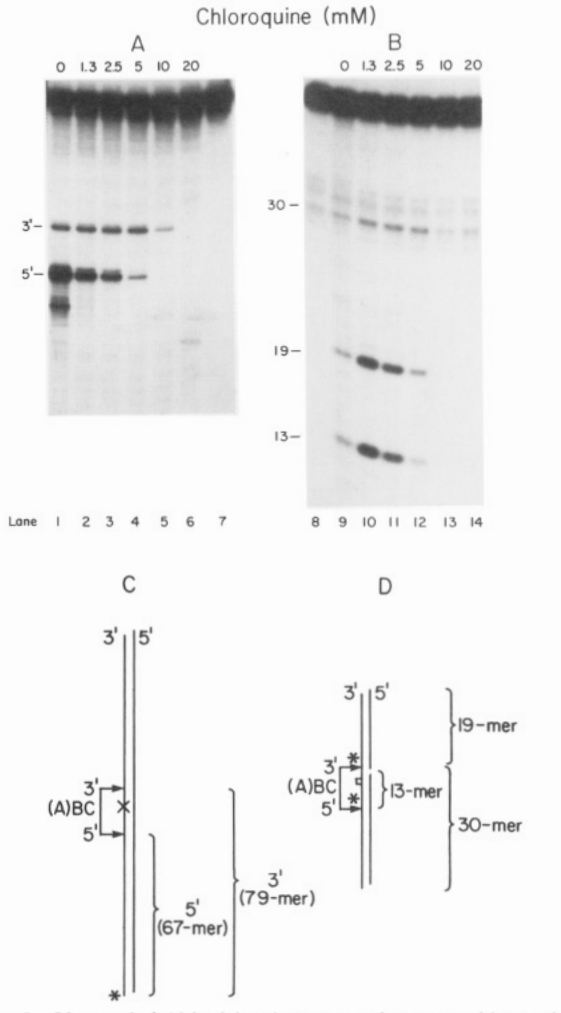

FIGURE 5: Uncoupled 3′ incision is more resistant to chloroquine inhibition than coupled 3′ and 5′ incisions. The 138-mer HMT-DNA (A) and the 48 bp thymine dimer substrate (B) were incubated with chloroquine at the indicated concentrations and digested with 4.2 nM UvrA, 60 nM (A) or 33 nM (B) UvrB, and 140 nM UvrC for 8 min (A) or 10 min (B). Reactions were stopped and products were analyzed as in the Figure 2 legend. Panels C and D show the locations of the radiolabels (indicated by asterisks) for the 138-mer HMT-DNA and the 48-mer thymine dimer substrates, respectively, and show the source of radiolabled products of digestion by (A)BC excinuclease (brackets). The arrows indicate the enzyme incision sites, the psoralen adduct is indicated by an X, and the dimer is indicated by a box. The HMT-DNA (panels A and C) was labeled at the 5′ end of the damaged strand; 3′ indicates the location of the uncoupled 3′ incision product, and 5′ indicates the coupled (and uncoupled) 5′ incision products. The 48-mer in panels B and D had two internal labels (Lin & Sancar, 1990); coupled incisions on the 3′ and 5′ sides of the dimer give a 13-mer, and an uncoupled 3′ incision gives a 30-mer. The 19-mer is produced by incision on the 3′ side, both coupled and uncoupled. An uncoupled 5′ incision would give a labeled 32-mer, which was not observed to any appreciable extent. In lanes 7 and 8, the DNA was not digested with (A)BC excinuclease. The background in (B) (see lane 8) is probably from radiolytic degradation of the internally labeled 48-mer.

Compared to the psoralen adduct, the dimer is weakly incised (Bertrand-Burggraf et al., 1991), and incision of the dimer is stimulated by low amounts of drug (see below). More importantly, (A)BC excinuclease complexes that produce the uncoupled 3′ incision (30-mer) are more resistant to chloroquine than complexes that produce the coupled incisions (13-mer).

We have to this point described two mechanism of inhibition of (A)BC excinuclease, both of which involve drug-induced binding of UvrA to undamaged DNA. First, in the presence of significant amounts of undamaged plasmid DNA, the drugs

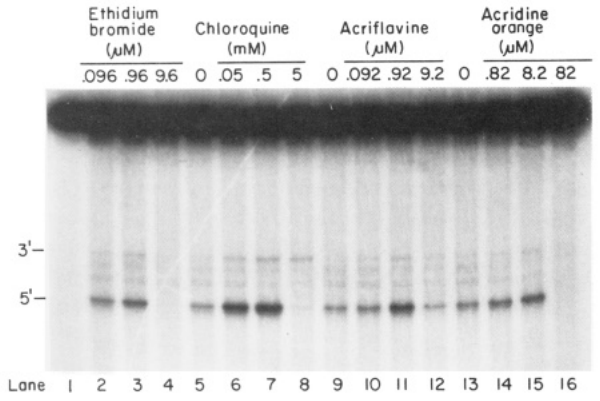

FIGURE 6: Stimulation of incision of the 137-mer T<>T-DNA by various drugs. The DNA was incubated for 30 min with the drugs shown at the indicated concentrations and then was digested with 4.2 nM UvrA, about 100 nM UvrB, and 200 nM UvrC for 15 min at 37 °C. Reactions were stopped and products were analyzed as in the Figure 2 legend. The locations of the 5′ and uncoupled 3′ incision products are indicated. In lanes 1, 5, 9, and 13, no drug was added. In lane 1, the DNA was not digested with (A)BC excinuclease.

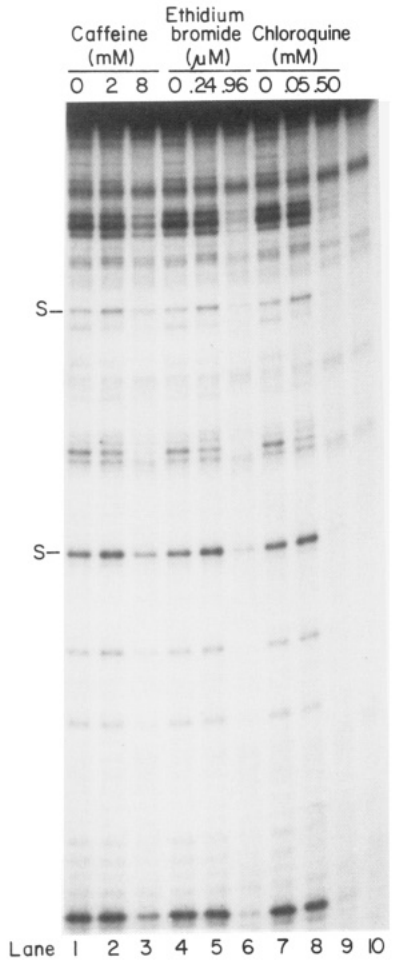

FIGURE 7: Stimulation of incision is limited to certain sites of UV photodamage. The end-labeled 137-mer control DNA that lacks the dimer was irradiated with 10 kJ/m^2 of germicidal UV light, incubated with the drugs shown at the indicated concentrations and with unlabeled pDR3274 (0.6 nM) for 30 min, and then incubated with UvrA (4.2 nM) and UvrB (60 nM) for 30 min. UvrC (140 nM) was added, and digestion reactions were for 8 min at 37 °C. Reactions were stopped and products were analyzed as in the Figure 2 legend. In lane 10, the DNA was not digested with (A)BC excinuclease. Sites where incision is stimulated by the drugs are indicated with an S.

deplete UvrA. Second, in the absence of undamaged plasmid, the drugs promote UvrA binding in the vicinity of the covalent

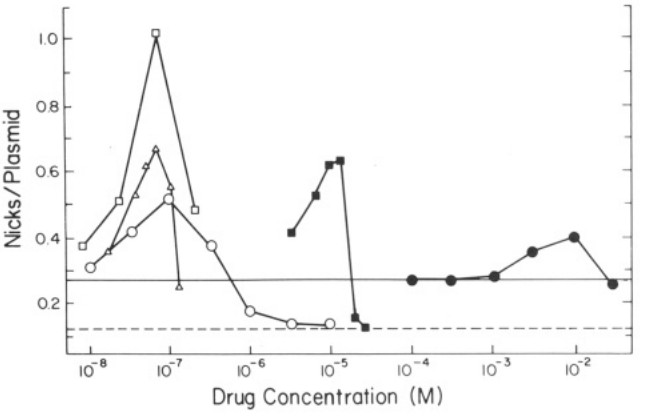

FIGURE 8: Incision of unmodified pBR322 by (A)BC excinuclease in the presence of noncovalent DNA binding drugs. Tritium-labeled plasmid at 1.6 nM was incubated with actinomycin D (triangles), caffeine (closed circles), ditercalinium (open squares), ethidium bromide (open circles), or Hoechst 33258 (closed squares) for 1 h in siliconized Eppendorf tubes and then was digested with UvrA (14 nM), UvrB (130 nM), and UvrC (160 nM) for 10 min at 37 °C. Reactions were stopped by adding an SDS–dye loading mixture, and incision was quantitated by scintillation counting of the supercoiled and nicked reaction products isolated as described under Methods. The dashed horizontal line respresents the level of incision in plasmids incubated without enzyme (0.12 nick per plasmid), and the solid horizontal line is the level of incision in plasmids incubated with enzyme but no drug (0.27 nick per plasmid).

lesion which blocks damage recognition. The experiments in Figure 5 were conducted in the absence of plasmid, and in other experiments when plasmid was added, no "coupling effect" was observed (not shown). Therefore, the second mechanism of inhibition is implicated in the "coupling effect" of chloroquine. As yet, we cannot explain how protein bound nonspecifically in the presence of chloroquine can preferentially inhibit incision complexes that make coupled incisions. It is possible that another unknown mechanism is responsible for the preferential inhibition.

Stimulation of Incision. In our survey of drugs, we noticed that whenever we used the 137-mer T<>T-DNA substrate, subinhibitory concentrations of drug actually stimulated incision, as shown in Figure 6. The T<>T-DNA is constructed around a central 11-mer which contains the dimer. Stimulation was also observed when the modified 11-mer was located in the opposite strand of the 137-mer [constructs described in Selby and Sancar (1990c); data not shown] and was observed when the 11-mer was placed in a 48-mer of entirely different sequence (Figure 5B). Stimulation was also observed by using an oligomer containing photodamage produced by irradiation with far-UV, as shown in Figure 7. In the latter case, stimulation was restricted to a minority of the incision sites, it occurred at near-inhibitory concentrations of drug, and it occurred in the presence of added plasmid. Stimulation of incision of the HMT-DNA was not observed (Figure 2 and data not shown).

Incision of Undamaged DNA. Lambert et al. (1989) reported that ditercalinium promoted incision of undamaged plasmid DNA by (A)BC excinuclease. Incision required all three Uvr subunits and occurred preferentially in supercoiled compared to relaxed plasmids. We found that all of the noncovalent DNA binding drugs tested including ditercalinium promoted incision of unmodified plasmids, when relatively high concentrations of the Uvr proteins were used. The levels of incision observed, shown in Figure 8, were relatively small. Compared to a background of 0.12 nick per plasmid in untreated DNA and 0.27 nick per plasmid produced by adding purified (A)BC excinuclease alone, incisions produced by

enzyme plus drug ranged from 0.4 to 1.0 nick per plasmid. Reducing the amount of Uvr proteins reduced the level of incision (data not shown). For comparison, under the conditions of Figure 8, digestion of UV-irradiated (125 J/m^2) plasmids gave approximately 2 nicks per plasmid. As previously reported for ditercalinium (Lambert et al., 1989), incision produced by enzyme plus the drugs was dependent upon the presence of all three Uvr subunits and was far more extensive in supercoiled than relaxed plasmids (data not shown). In an earlier study of caffeine (Selby & Sancar, 1990b), we reported that a 50-min digestion of unmodified plasmid with (A)BC excinuclease gave 0.31 and 0.16 nicks per plasmid, respectively, in the absence and presence of 10 mM caffeine. The decrease was probably due to inhibition by caffeine of a nuclease contaminant known to be present in the UvrC preparation. Caffeine did not increase the amount of incision in unmodified DNA in our earlier experiments because of the low amount of UvrA (1.7 nM) used in those experiments compared to Figure 8 (14 nM).

Discussion

Noncovalent DNA binding drugs are a structurally diverse group of chemicals that bind to DNA in different ways. Several drugs were selected for our studies to represent different modes of noncovalent DNA binding in the hope that we could learn more about which features of noncovalently bound drug were important in modulating (A)BC excinuclease activities. Included were the intercalators caffeine, ethidium bromide, and actinomycin D. Actinomycin D exhibits a strong preference for binding to certain GC-rich sequences, a property associated with the bulky peptide groups attached to the intercalating chromophore. Ethidium bromide and caffeine bind with lower sequence specificity. Caffeine, which has a relatively low affinity for DNA, may also bind to the exterior of the helix (Kihlman, 1977; Neidle & Abraham, 1984; Torraletti et al., 1989). We also used Hoechst 33258 which binds in the minor groove with affinity for AT-rich regions (Zimmer & Wahnert, 1986).

The mechanistic studies of repair inhibition provided direct evidence that under the more physiological conditions (plasmid added), actinomycin D, caffeine, ethidium bromide, and Hoechst 33258 inhibit incision by depleting the system of UvrA. UvrA functions to load UvrB onto the damaged site, forming a preinicision complex (Orren & Sancar, 1989, 1990). Repair inhibition was associated with a failure to form preincision complexes at the site of the lesion. In each case, inhibition was reversible by adding more UvrA. The drugs were shown to promote the binding of UvrA to undamaged DNA, and with actinomycin D, this nonspecific binding was shown to occur with higher affinity at drug binding sites. The latter results suggest that UvrA trapping results from the formation of ternary UvrA–drug–DNA complexes. In the absence of plasmid, a different mechanism of inhibition was observed. With only the 138-mer HMT-DNA present, there was insufficient undamaged DNA for the drugs to effectively deplete the reactions of UvrA, and more drug was needed to inhibit incision. Inhibition was associated with extensive binding of enzyme to the entire 138-mer HMT-DNA and fewer (UvrA) UvrB preincision complexes at the lesion, presumably as a result of steric hindrance by nonspecifically bound protein.

The results obtained with the four chemicals discussed above plus the other chemicals surveyed allow one to predict that any chemical that binds noncovalently to DNA with reasonable affinity has the potential to trap UvrA and inhibit (A)BC excinuclease. This would include intercalators and minor groove binders with high and low sequence specificity. This prediction may be relevant to nucleotide excision repair in other organisms as well since (1) Lin and Sancar (1990) have shown that the UvrC protein from *Bacillus subtilis* is isofunctional to the UvrC subunit from *E. coli*, suggesting a common enzyme reaction mechanism, and (2) Snyder (1987) found that intercalators inhibit the incision step of repair in cultured human fibroblasts, suggesting a common mechanism of inhibition. One may make an additional prediction. Knowing that caffeine sensitizes *E. coli* toward killing by DNA-damaging treatments because it inhibits excision repair, one may predict that the other noncovalent DNA binding chemicals that inhibit (A)BC excinuclease in vitro will also be cellular sensitizers. However, we note that in certain cases cellular sensitization may arise from inhibition of other repair enzymes, and cellular sensitization by inhibitors of DNA repair may be difficult or impossible to demonstrate due to other toxic properties of the drugs (for example, caffeine toxicity in human cells; Day, 1975).

When we found that (A)BC excinuclease produced incisions in undamaged DNA in the presence of all drugs tested, we considered the possible model wherein UvrA loads UvrB onto noncovalent drug–DNA complexes, and incisions are produced in combination with UvrC (Lambert et al., 1989). However, the observation that the drugs also stimulate incision at the site of certain covalent lesions in DNA suggests an alternative model. Purified DNA contains low levels of covalent modification, and it is possible that the drugs substantially stimulate incision at these sites which are present even in "unmodified" DNA. One might test the two models by comparing incision sites made in the presence and absence of drug. The model of Lambert et al. (1989) predicts that drug-induced incision sites will occur at drug binding sites, while the latter model predicts that incision sites will be the same in the presence and absence of drug. Our efforts to compare enzyme incision sites have been unsuccessful primarily due to the low signal-to-noise ratio for incision of unmodified plasmid.

The stimulation of incision at certain sites of UV photodamage that we observed with subinhibitory concentrations of practically every drug tested was unexpected and is not easily explained. The failure to stimulate at all incision sites suggests that stimulation does not result from a global effect of the drugs on DNA such as helix stiffening. The drugs used bind with different DNA sequence preferences. Because the sites of stimulation did not vary with the different drugs used, stimulation probably does not result from the known interactions of the drugs with undamaged DNA. It seems more likely that certain lesions possess structural features that favor the binding of many types of noncovalent DNA binding drugs in a way that facilitates damage recognition by (A)BC excinuclease. Relevant is the report by Domon et al. (1970) which demonstrates more extensive binding of caffeine to UV-irradiated DNA versus unirradiated DNA and the report by Ts'o and Lu (1964) which demonstrates more extensive binding of caffeine and other drugs to single-stranded DNA than to double-stranded DNA.

Several aspects of (A)BC excinuclease, as it functions in the absence of drugs, were revealed in this study. An examination of the inhibition of incision by chloroquine identified the source of uncoupled 3′ incisions commonly observed (though at low frequency) in in vitro reactions. This uncoupled incision is the product of a unique population of enzyme complexes that can be referred to as pathological because of their abnormal reaction product. The effects of added plasmid on the DNase I footprints of the enzyme and on incision have

been more extensively examined in a separate investigation (Bertrand-Burggraf et al., 1991).

Conclusions

Intercalating and minor groove binding drugs of diverse structure that bind with different DNA sequence specificities all inhibit (A)BC excinuclease by two distinct mechanisms: by trapping UvrA with undamaged DNA and (in the absence of significant undamaged DNA) by promoting the formation of nonspecific protein–DNA complexes near the covalent lesion which block the enzyme from forming preincision complexes at the lesion. The inhibitory effects on the enzyme are not associated with specific noncovalent DNA binding interactions or chemical constituents, but are produced simply as a consequence of noncovalent binding of drug to DNA. All of the drugs studied also promoted incision of apparently undamaged, supercoiled DNA at high enzyme concentration, and therefore this phenomenon is not produced only by bisintercalation. Certain UV photoproducts (notably thymine–thymine cyclobutane dimers) were incised with higher efficiency in the presence of all drugs studied, but none of the drugs stimulated incision at other covalent adducts (notably a psoralen monoadduct). Finally, uncoupled 3′ incisions were identified as arising from a "pathological" population of (A)BC excinuclease enzyme complexes by their greater resistance to inhibition by chloroquine than coupled 5′ and 3′ incisions.

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