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Selective inhibition by harmane of the apurinic/apyrimidinic endonuclease activity of phage T4-induced UV endonuclease

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ABSTRACT

1-Methyl-9H-pyrido-[3,4-b]indole (harmane) inhibits the apurinic/apyrimidinic (AP) endonuclease activity of the UV endonuclease induced by phage T4, whereas it stimulates the pyrimidine dimer-DNA glycosylase activity of that enzyme. E. coli endonuclease IV, E. coli endonuclease VI (the AP endonuclease activity associated with E. coli exonuclease III), and E. coli uracil-DNA glycosylase were not inhibited by harmane. Human fibroblast AP endonucleases I and II also were only slightly inhibited. Therefore, harmane is neither a general inhibitor of AP endonucleases, nor a general inhibitor of Class I AP endonucleases which incise DNA on the 3'-side of AP sites. However, E. coli endonuclease III and its associated dihydroxythymine-DNA glycosylase activity were both inhibited by harmane. This observation suggests that harmane may inhibit only AP endonucleases which have associated glycosylase activities.

INTRODUCTION

Castellani and Setlow (1) recently reported that 1-methyl-9H-pyrido-[3,4-b]-indole (harmane) inhibited excision of pyrimidine dimers from DNA in human cells. The mechanism of the inhibition could not be determined because their results did not distinguish between inhibition at the incision step and actual excision of the photoproducts. The mechanism of incision of irradiated DNA has recently been elucidated for the phage T4-induced and Micrococcus luteus UV endonucleases (2-9). In both of these systems incision occurs in two steps; hydrolysis of the glycosylic bond of the 5' nucleotide of the dimer to produce an apyrimidinic site (AP site), is followed by phosphodiester cleavage of the DNA on the 3'-side of this AP site (Fig. 1). Although it is not known whether repair of pyrimidine dimers occurs by a similar mechanism in human cells, Kuhnlein et al. (10) have demonstrated that fibroblasts cultured from persons with xeroderma pigmentosum complementation group D, which are DNA repair deficient, lack an AP endonuclease activity. This enzyme, termed AP endonuclease I, incises depurinated DNA on the 3'-side of the AP site to produce 3'-deoxyribose and 5'-phosphomonoester termini, as does the T4 UV endonuclease acting on this substrate (11). This similarity

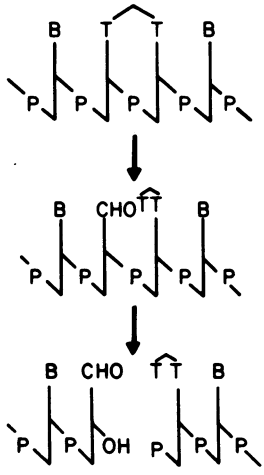

Fig. 1. Mechanism of incision of UV-irradiated DNA by T4 UV endonuclease.

has suggested that AP endonuclease I might play a role in DNA repair in human cells similar to that of the AP endonuclease activity of T4 UV endonuclease in infected E. coli. However, the AP endonuclease I is not totally analogous to the T4 UV endonuclease because it does not cleave UV-irradiated DNA nor has it been shown to have any associated DNA glycosylase activity.

Behmoaras et al. (12) have reported that lysyltryptophanyl lysine binds to DNA by stacking of the indole ring with the nucleic acid bases. This stacking is greater in single-stranded DNA than in double-stranded DNA, but is particularly high in DNA containing AP sites. However, the binding of the tripeptide to depurinated DNA is greatly reduced following incision of the DNA at the AP sites. The peptide apparently binds to locally destabilized regions of DNA following base damage. Furthermore, both this peptide and lysyltyrosinyl lysine catalyse incision of the DNA at AP sites (12, J. Laval, personal communication), although the nature of the termini produced by such incisions have yet to be determined. Hayashi et al. (13) have shown that harmane, which is a tryptophan analog, also intercalates and unwinds the DNA. These observations suggest that harmane may inhibit pyrimidine dimer excision by binding to AP sites in DNA, thereby preventing incision by AP endonuclease activities when such incision precedes dimer excision. The T4 UV endonuclease activity provides a convenient system to test this possibility. Also, numerous other AP endonucleases from E. coli and human cells are available for comparison.

MATERIALS AND METHODS

Materials. Unlabelled PM2 DNA and PM2 DNA labelled with [^{3}H]thymidine (2100 or 5400 cpm/nmol nucleotide) were prepared as described (9,10). 1-Methyl-9H-pyrido-[3,4-b]indole (harmane) was purchased from Sigma Chemical Co. Stock solutions of harmane were prepared in ethanol. E. coli endonuclease VI (the AP endonuclease associated with exonuclease III) was purchased from Bethesda Research Labs, Inc. or was prepared as previously described (14). E. coli endonuclease IV was a gift from T. Lindahl. T4 UV endonuclease was purified through the phosphocellulose chromatography step as described (9). Uracil-DNA glycosylase was purified about 300X from E. coli by fractionation with ammonium sulfate (15) and chromatography on phosphocellulose. E. coli endonuclease III and human fibroblast AP endonucleases I and II were purified as described (11,16). E. coli endonuclease III has both AP endonuclease and dihydroxythymine-DNA glycosylase activities (2,16).

Methods. DNA containing pyrimidine dimers was prepared by UV-irradiation of PM2 DNA in 0.01 M Tris chloride, pH 7.5 containing 0.02 M NaCl. The DNA was irradiated for 30 seconds at a distance of 20 cm from 2 General Electric 15 watt germicidal lamps to produce 2 to 3 pyrimidine dimers per duplex circle of DNA. Partially depurinated DNA was prepared by incubation of [^{3}H]-DNA at 70°C for 15 or 20 minutes in 10 mM sodium citrate, pH 5.0, containing 100 mM NaCl (17). The resulting DNA contained 2 to 3 AP sites per duplex circle of DNA.

The fluorescence procedure described by Putcher and Morgan (18) was adapted for assaying pyrimidine dimer-DNA glycosylase activity on UV-irradiated PM2 DNA (9). The assay mixture contained 17 mM NaCl, 10 mM EDTA, 65 µg bovine serum albumin per ml, 2 mM TrisCl, and 100 mM potassium N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate (HEPES) buffer, pH 7.4 and irradiated DNA, as indicated in the figure legends. This assay will determine not only the number of nicked PM2 DNA molecules produced during enzyme incubation, but also the number of molecules containing unnicked AP sites because AP sites lead to strand breakage during the 95°C treatment in alkali.

The AP endonuclease activities were assayed using partially depurinated PM2 [^{3}H]-DNA as substrate (10,17). The assay conditions used for each AP endonuclease were: endonuclease III, 50 mM HEPES, pH 7.8, 2 mM EDTA, 25 mM KCl, 2 mM dithiothreitol, 20 mM potassium phosphate, pH 7.5; endonucleases IV and VI, 25 mM TrisCl, pH 7.5, 10 mM $MgCl_2$; human AP endonucleases I and II, 50 mM TrisCl, pH 7.5, 10 mM $MgCl_2$; and T4 UV endonuclease, 25 mM TrisCl, pH 7.5; 2.5 mM EDTA for the experiment in Fig. 2 or 25 mM potassium phosphate, pH 7.5, 170 mM NaCl, 80 µg bovine serum albumin per ml for the experiment in Fig. 5.

Following incubation at 37°C the number of nicked circles was determined as described (10,17).

When both nicks and AP sites were to be determined, the UV-irradiated PM2 DNA was incubated as described above for the pyrimidine dimer-DNA glycosylase assay, and portions were removed at various times for measurement of nicked circles by the glycosylase assay (measures nicks plus unnicked AP sites) and AP endonuclease assay (measures nicks only). For both of the above assays the absolute number of AP sites and/or nicks introduced into the DNA population was calculated using the first term of the Poisson distribution (17).

Uracil-DNA glycosylase activity was assayed using phage T5 DNA labelled with [6-^{3}H]uridine (1.3×10^{4} cpm in dUMP per nmol dUMP) as substrate (19,20). Incubations were for 5 minutes at 37°C. The dihydroxythymine-DNA glycosylase activity of E. coli endonuclease III was assayed in 300 µl using 5 nmol T7 DNA (84000 cpm [^{3}H]thymine per nmol nucleotide) treated with 0.4% osmium tetroxide (16). Following incubation at 37°C for 60 minutes, the DNA was precipitated by addition of 30 µg calf thymus DNA and 900 µl 100% ethanol. Then 30 µl of 20 mM 5,6-dihydroxythymine was added as an internal standard, and after 30 min at -70°C the samples were centrifuged at 7,000xg for 10 min in a Sorvall SE12 rotor. The supernatant solution (1 ml) was evaporated to dryness at 32°C using an Evapo-Mix (Buchler Instruments), 300 µl of equilibration buffer was added, and 200 µl was analysed in a Waters model ALC/GPC 244 liquid chromatograph equipped with a Supelcosil LC-18 column (25 cm x 0.46 cm) equilibrated with 2.5 mM potassium phosphate, pH 3, containing 2.5% methanol. Isocratic elution was carried out at a flow rate of 2.0 ml per min at about 2500 psi, and 0.2 ml fractions were collected. This system resolves thymine and dihydroxythymine, and the dihydroxythymine-containing fractions were counted to determine the amount released from the DNA.

RESULTS

The effect of harmane on the AP endonuclease activity of the T4 UV endonuclease was assayed using partially depurinated PM2 DNA as the substrate (Fig. 2). Harmane inhibited this activity *in vitro* in the same concentration range which inhibited pyrimidine dimer excision *in vivo* in human cells (1). Therefore, harmane does inhibit the AP endonuclease activity associated with T4 UV endonuclease.

The effect of harmane on the incision of UV-irradiated DNA by the T4 UV endonuclease was then tested (Fig. 3). The two distinct activities of the

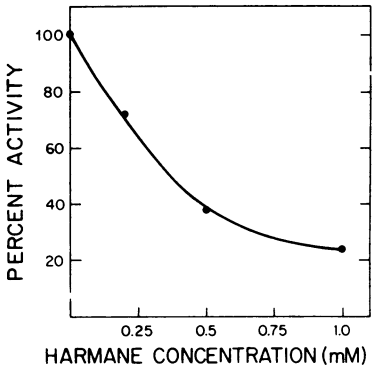

Fig. 2. Effect of harmane on the AP endonuclease activity of T4 UV endonuclease. Partially depurinated PM2 [^{3}H]-DNA (50 fmol DNA circles per 50 µl assay mix) was incubated with T4 UV endonuclease at 37°C in the presence of various amounts of harmane, and after 4 min the samples were assayed for the number of nicks introduced. In the absence of harmane, 0.66 nicks were introduced per DNA circle during the incubation.

UV endonuclease can be measured simultaneously. The assay of Futcher and Morgan (18) measures the total number of nicked plus unnicked AP sites produced during the reaction, whereas the nicked circle assay (10,17) measures only the number of DNA molecules actually nicked. The difference between these two assays is a measure of the number of AP sites produced but not nicked during the enzyme incubation. Because the T4 UV endonuclease generates AP sites at pyrimidine dimers as an intermediate in the overall nicking reaction, the total production of nicked and unnicked AP sites equals the pyrimidine dimer-DNA glycosylase activity. Addition of 0.5 mM harmane inhibited the nicking of AP sites produced by the pyrimidine dimer-DNA glycosylase acting on irradiated DNA. Under the assay conditions used, and in the absence of harmane, the rate of production of AP sites by the pyrimidine dimer-DNA glycosylase was 4.3 fmol per min, whereas the rate of nicking of the AP sites produced was 3.1 fmol per min. In contrast, in the presence of 0.5 mM harmane the rate of production of AP sites by the glycosylase was 8.0 fmol per min and the rate of nicking of AP sites was only 1.5 fmol per min. Therefore, harmane inhibits the second step of the reaction, but stimulates the initial step, and inhibits the AP endonuclease whether acting on depurinated DNA (Fig. 2) or on AP sites produced as intermediates in the overall nicking reaction of T4 UV endonuclease on UV-irradiated DNA (Fig. 3).

Because of these unexpected dichotomous effects we investigated the acti-

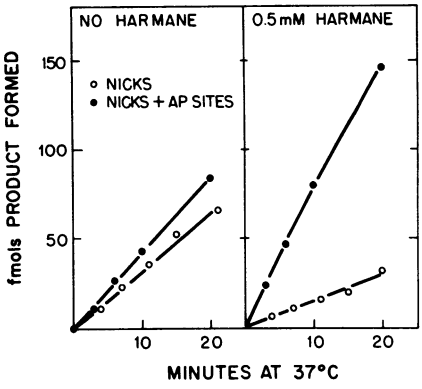

Fig. 3. Effect of 0.5 mM harmane on the pyrimidine dimer-DNA glycosylase and AP endonuclease activities of T4 UV endonuclease. UV-irradiated PM2 [^{3}H]-DNA (50 fmol DNA circles per 10 µl assay mix) was incubated with T4 UV endonuclease at 37°C and at various times 40 µl and 10 µl samples were removed and assayed for nicks plus unnicked AP sites (•) and nicks only (o), respectively. The former represents the total glycosylase activity, and the latter represents the AP endonuclease activity. The results are expressed as fmol nicks introduced per 10 µl sample.

vity of both the glycosylase and AP endonuclease activities as a function of harmane concentration (Fig. 4). The AP endonuclease was inhibited at all concentrations between 0.05 and 1 mM harmane and the glycosylase was similarly stimulated. These results indicate that harmane inhibits the nicking of irradiated DNA by T4 UV endonuclease by selectively inhibiting only the AP endonuclease, while it actually stimulates the pyrimidine dimer-DNA glycosylase activity.

This result is consistent with the hypothesis that harmane inhibits incision of irradiated DNA by T4 UV endonuclease because it inhibits AP endonucleases in general. Therefore we tested the effect of harmane on various AP endonuclease activities, including the AP endonuclease activity associated with E. coli endonuclease VI (also known as exonuclease III) and E. coli endonuclease IV (Fig. 5A). These enzymes were not inhibited by concentrations as high as 1 mM harmane, but were actually stimulated up to 60%. Therefore, harmane is not a general inhibitor of E. coli AP endonucleases.

Mosbaugh and Linn (11) suggested that AP endonucleases could be classified by their mechanism of incision. Class I enzymes are those which cleave on the 3'-side of an AP site to produce 3'-deoxyribose and 5'-phosphomonoester termini, whereas Class II enzymes cleave on the 5'-side of an AP site to pro-

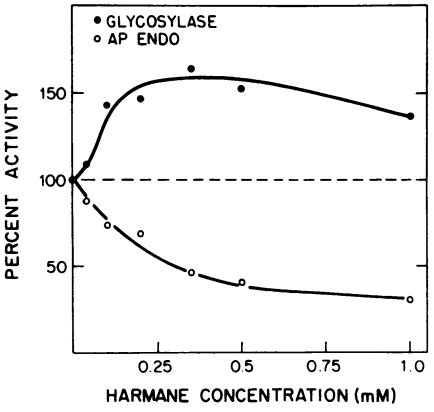

Fig. 4. Effect of harmane concentration on the pyrimidine dimer-DNA glycosylase and AP endonuclease activities of T4 UV endonuclease. UV-irradiated PM2 [^{3}H]-DNA (50 fmol DNA circles per 10 µl assay mix) was incubated with T4 UV endonuclease at 37°C. Samples (40 µl) were removed at 5 minutes and assayed for nicks plus unnicked AP sites (•), and 10 µl samples were removed at 16 minutes and assayed for nicks (o). In the absence of harmane 52 fmol nicks plus unnicked AP sites and 38 fmol nicks were produced per 10 minutes per 10 µl sample.

duce 3'-hydroxyl nucleotide and deoxyribose 5'-phosphate termini. E. coli endonucleases IV and VI are Class II enzymes, whereas T4 UV endonuclease is a Class I AP endonuclease. Thus it seemed possible that harmane might inhibit only Class I AP endonucleases. E. coli endonuclease III is also a Class I enzyme (8) and harmane inhibited the AP endonuclease activity of this enzyme to the same extent as it inhibited that of T4 UV endonuclease (Fig. 5A). Human fibroblasts also contain both a Class I and a Class II AP endonuclease activity (11). Kuhnlein et al. (10) showed that xeroderma pigmentosum complementation group D mutant fibroblasts lack the Class I enzyme, suggesting that it does play some role in DNA repair. However, harmane inhibited these enzymes only slightly, and there was no significant difference between the Class I and Class II enzymes (Fig. 5B), indicating that harmane is not a general inhibitor of Class I AP endonucleases.

We have also tested the effect of harmane on several E. coli DNA glycosylases. E. coli uracil-DNA glycosylase was stimulated slightly by concentrations of harmane between 0.05 and 0.5 mM and inhibited slightly by 1 mM as observed for the human fibroblast AP endonucleases (Fig. 5B and 5C). In contrast the dihydroxythymine-DNA glycosylase activity of E. coli endonuclease III was strongly inhibited at both 0.25 mM and 0.5 mM harmane. Therefore

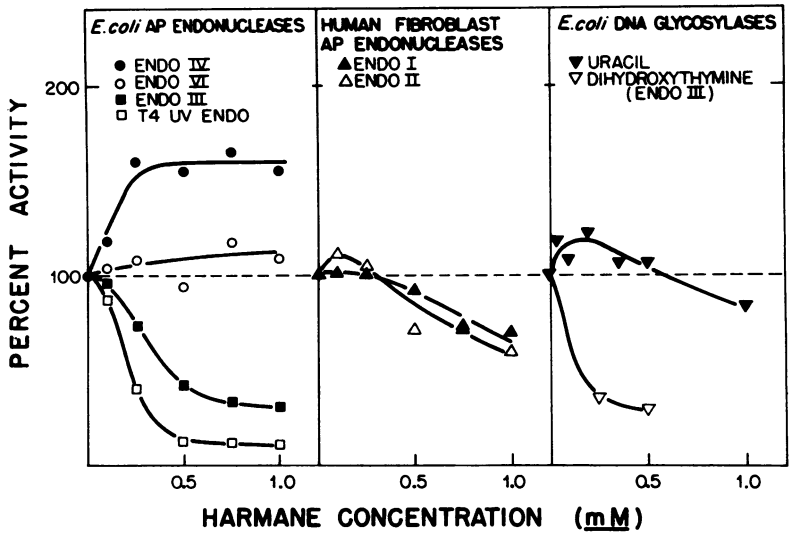

Fig. 5. Effect of harmane on other AP endonuclease and DNA glycosylase activities. The AP endonuclease activities associated with E. coli endonucleases III (■), IV (●), and VI (o), T4 UV endonuclease (□), human fibroblast AP endonucleases I (▲) and II (Δ), and the glycosylase activities associated with E. coli uracil-DNA glycosylase (▼) and endonuclease III (dihydroxythymine-DNA glycosylase) (▽) were assayed in the presence of various concentrations of harmane. The AP endonuclease assays contained 106 fmol DNA circles per 50 µl assay mix and the fmol nicks produced in the absence of harmane after 10 minutes incubation at 37°C were 86, 60, 152, 15, 64 and 105 for E. coli endonucleases III, IV, VI, T4 UV endonuclease and human fibroblast AP endonucleases I and II, respectively. In the absence of harmane, uracil-DNA glycosylase and the dihydroxythymine-DNA glycosylase activity of E. coli endonuclease III released 201 and 10.5 pmol free base, respectively.

harmane affects the three DNA glycosylases tested in three different ways, so no general conclusion about the effect of harmane on DNA glycosylase appears to be valid.

DISCUSSION

Our results indicate that harmane exhibits selective inhibition of the AP endonuclease activity of T4 UV endonuclease, because under the conditions used the pyrimidine dimer-DNA glycosylase activity was not inhibited, but was actually stimulated. This stimulation could result from an enzyme-sparing effect if harmane prevents binding of the enzyme to AP sites in the DNA, thus increasing the amount of enzyme available to act as a glycosylase. We began this work with the idea that harmane might preferentially bind to DNA at AP sites, perhaps preventing the DNA from binding to an essential tryptophan in

the active site of the enzyme. This assumption predicts that harmane might be a general inhibitor of all AP endonucleases. This prediction was not supported by our results with other endonucleases. The AP endonucleases of T4 UV endonuclease and E. coli endonuclease III were the only AP endonuclease strongly inhibited by harmane at the concentrations used in these experiments.

The actual mechanism of the harmane inhibition of incision of UV-irradiated DNA by T4 UV endonuclease has yet to be elucidated, although it is clear that the inhibition occurs at the second step of the reactions shown in Figure 1. It is curious that the only two AP endonucleases observed to be inhibited by harmane in these experiments have associated DNA glycosylase activities. However, in one case the glycosylase activity is stimulated, whereas in the other case it is inhibited. Although this might ultimately provide some clues about the nature of the active sites of these two enzymes, it is important to point out that reaction conditions may affect the interaction between harmane and the activity being measured. For example, when the sodium chloride concentration in the assay mixture is increased from 17 mM to 250 mM, the pyrimidine dimer-DNA glycosylase activity of T4 UV endonuclease is also slightly inhibited by harmane.

Recently, it has been shown that the two activities of T4 UV endonuclease are contained in a single, small polypeptide whose molecular weight is less than 20,000 daltons (4,5,9). Surprisingly, the two activities have quite different rates and the ratio of the two activities is very dependent upon assay conditions. Factors affecting this ratio include assay temperature (7), salt concentration (unpublished data), and pH (5). The results described here indicate that harmane also affects this ratio. These observations suggest but do not prove that the two activities of the enzyme may reside in two completely or partially distinct active sites on the enzyme.

These results do not explain the harmane inhibition of dimer excision in human cells reported by Castellani and Setlow (1). Neither of the human AP endonucleases was strongly inhibited by harmane (Fig. 5B), a fact which suggests that both the mechanisms of the incision of UV-irradiated DNA by the T4 and human UV endonucleases and their inhibition by harmane may be quite different. Whereas it is possible that harmane may bind to DNA and inhibit some AP endonucleases by mimicking a tryptophan- or tyrosine-containing active site, it may also bind to enzymes which recognize and bind to altered thymine residues in DNA. What is clear is that the pyrimidine dimer-DNA glycosylase activity of the T4 UV endonuclease is not inhibited by harmane under conditions which strongly inhibit the AP endonuclease activity of the enzyme. It

is also clear that a variety of indole derivatives may bind to DNA and alter its metabolism. Matsukura et al. (21) have recently shown that 3-amino-harmane is an hepatic carcinogen, and it seems possible that this may be due to some effect on DNA repair.

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