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COVALENT BINDING OF ETHIDIUM AZIDE ANALOGS TO SALMONELLA DNA IN VIVO: COMPETITION BY ETHIDIUM BROMIDE

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SUMMARY: The photoreactive analogs of ethidium bromide (ethidium mono- and diazide) have been developed as drug probes to determine the actual molecular details of ethidium bromide interactions with DNA. In an effort to demonstrate that the analogs in fact mimic the parent ethidium, competition experiments were designed using ^{3}H thymidine-labeled DNA in intact Salmonella TA1538, which is reverted by the azide analogs. ^{14}C-labeled ethidium azide analogs were used in combination with the non-labeled ethidium bromide. The results presented here demonstrate that the parent ethidium competes with the azide analogs as a DNA intercalating drug using CsCl density gradient ultracentrifugation.

INTRODUCTION:

Ethidium bromide (3,8-diamino, 5 ethyl-6-phenylphanthridinium bromide) has been used extensively as a probe of the structural and functional properties of nucleic acids (1-5). From previous studies, the in vitro interactions of EBr-nucleic acid interactions have been characterized (6-10); however, these studies represent only an indirect approach for determining the actual binding mechanisms of EBr in biological systems.

This laboratory has successfully utilized and refined the technique of photoaffinity labeling in an effort to analyze the in vivo consequences of EBr interactions with the development and characterization of the 8-azido-3-amino analog (EA_1) of ethidium bromide (11-15). The 3,8-diazido analog (EA_2) developed by Bastos (16) was compared with EA_1 in its ability to mimic the parent EBr in nucleic acid interaction. Interactions of these analogs shown in Figure 1 with EBr binding sites in the dark are expected to mimic those of the parent EBr; however, upon photolysis with visible light, the azide

Abbreviations: Ethidium Bromide - EBr; Ethidium Monoazide - EA_1; Ethidium Diazide EA_2; Ethidium Azide analog - EA.

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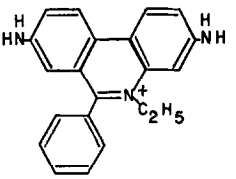
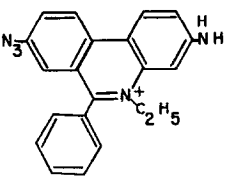
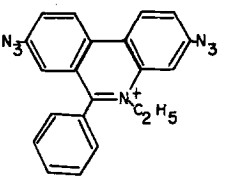


ethidium



ethidium monoazide



ethidium diazide

Figure 1. Chemical structure of ethidium bromide, ethidium monoazide and ethidium diazide.

substituent is activated, generating the reactive nitrene which forms a covalent bond at the binding site, rendering the complex irreversible. The drug-receptor site association may then be retrieved from any system and consequently may be analyzed directly so that the in vivo as well as in vitro targets can be identified, characterized and correlated with biological effects.

The studies presented here confirm covalent binding of these photoreactive drugs to the Salmonella genome in vivo and subsequent retrieval of this drug-nucleic acid complex, using cesium chloride density gradient ultracentrifugation. The ability of EA_1 and EA_2 to mimic the parent EBr is demonstrated through competition by EBr for EA_1 and EA_2 binding sites.

MATERIALS AND METHODS:

a. Ethidium Compounds

EBr, purchased from Calbiochem Laboratories, was recrystallized from a 1:3 methanol:dibutylether mixture. The improved synthesis and purification procedures developed recently (11) have provided azide products with higher specific activity as measured by mutagenicity. Essentially no contaminating by-products or unreacted ethidium bromide were detected by IR spectroscopy or thin layer chromatography.

b. Synthesis of Radiolabeled EA_1 and EA_2

Radiolabeled analogs of EBr were synthesized in this laboratory by using 6[^{14}C]EBr, 17.7 Ci/M (Modichem Developments, Ltd., Manchester, England). Synthesis followed the procedure reported previously (Graves, et al., 1977) with modifications for microquantities. Because both the EA_1 and EA_2 were needed as products, a 1:1:2 stoichiometric ratio of 6[^{14}C]EBr:$NaNO_2$:NaN_3 was used. EBr (11 mg/ 28 mmoles) was dissolved in

1 ml H_2O adjusted to pH 1.6 and cooled to 5°C. $NaNO_2$ (3.85 mg/ 28 mmoles), dissolved in 1 ml H_2O was added slowly with constant stirring and allowed to react 10 minutes prior to the addition of NaN_3 (7.24 mg/ 56 mmoles). EA_1 and EA_2 were then coprecipitated by the addition of 1-2 drops of 10 N NaOH, and allowed to stand at 5°C for 10 minutes prior to filtration. The filtrate was dissolved in H_2O, adjusted to pH 3 with HCl, and eluted through a (20 x 150 cm) cellex carboxymethyl cellulose column (BioRad) with H_2O adjusted to pH 2.8 with HCl. EA_1 appeared first as a yellow fraction and provided about a 30% yield. The EA_2 fraction followed as an orange band and gave a 50% yield. Purity of products was established by UV-Vis spectroscopy and thin layer chromatography.

c. Salmonella Tester Strain

Salmonella typhimurium tester strain TA1538 (17-20) provided by Dr. Bruce Ames, University of California at Berkeley, was utilized in these experiments. This strain is auxotrophic for histidine as a result of a frameshift mutation in the histidine operon. It was used because EA_1 effectively reverts this frameshift strain without enzymatic activation by the S-9 fraction (rat liver microsomal preparation), when it is activated by a short photolysis period, presumably allowing the drug to attach covalently at its site of interaction (15). This strain lacks excision repair which maintains higher revertant counts, and it is also an rfa^- mutant which renders the cell wall extremely permeable.

d. Competitive Binding between EBr and EA_1 or EA_2

Salmonella typhimurium (500 ml) were grown to late log phase in 100 µCi [^{3}H]-thymidine treated growth medium as described previously. Cells were harvested, washed and resuspended in 25 ml 0.15 M saline-0.1 M EDTA buffer, pH 8.0. From this, 3 ml aliquots (1 x 10^{10} cells/ml) were then diluted with 7.0 ml sterile growth medium in order to reproduce the conditions of the mutagenesis experiments. [^{14}C]EA_1 was introduced into each sample at a final concentration of 1.5 x 10^{-5} M, and EBr added simultaneously to obtain a final concentration range of 5 x 10^{-6} to 1 x 10^{-4}M. Resulting concentration ratios of EBr:EA_1 were 1:3, 1:1, 2:1, 4:1 and 7:1. The [^{14}C]EA_2 binding competition studies were done analogously to those of the [^{14}C]EA_1; however, the lower relative binding affinity of EA_2 for nucleic acids as compared to EA_1 necessitated using the higher [^{14}C] labeling. EBr and EA_2 were added simultaneously with concentration ratios of EBr:EA_2 equal to 1:1, 5:1 and 10:1. After establishing drug concentrations, the samples were incubated 30 minutes in the dark and then irradiated for 15 minutes with a GE 30W fluorescent lamp at 2 x 10^4 erg/cm^2/sec. Cells were again collected by centrifugation and washed four times in saline-EDTA buffer. Cells were lysed by the addition of 2 ml of 3% sarcosyl (K and K Laboratories) to the cell pellet. To the lysed suspension, 0.05 ml Pancreatic RNase (1 mg/ml in saline-citrate) and 0.02 ml T_1 RNase (2500 units, Sigma) were introduced, and digestion allowed to proceed for 30 minutes at 37°C. Cellular proteins were degraded with 0.02 ml Pronase (10 mg/ml saline-citrate, Sigma), allowing 45 minutes at 37°C. T_1 RNase was preincubated for 10 minutes at 90°C and Pronase for 2 hours at 37°C before they were added to the cells, in order to destroy contaminating DNase activity. After enzymatic digestion, 1.65 ml of the cell lysate was placed in a nitrocellulose tube (5/8 x 3") containing 6.15 ml CsCl (Gallard-Schlessinger), and 0.2 ml Micrococcus lysodeikticus DNA (Sigma), which provided a density reference. The samples having a final volume of 8.0 ml in saline-citrate and a density of 1.690 g/ml, were centrifuged in a Beckman L2-65B preparative ultracentrifuge, using a type 65 fixed angle rotor at 35,000 rpm for 65 hours at 25°C. 65 fractions per sample were collected from the bottom of the tubes, and the buoyant density was determined by weighing 100 µl of every 5th fraction and then adjusting the samples, using the density of M. lysodeikticus DNA (1.731 g/ml) as a reference. DNA was then coprecipitated with BSA (0.1

ml of 1 mg/ml, Sigma) by the addition of 1.0 ml cold TCA (10%) to each fraction, followed by a 24 hour incubation at 5°C. DNA was collected on glass fiber filters (Whatman GF/C) and samples were counted in Aquasol (New England Nuclear) using an Isocap 300 LSC (Searle). Spillover of counts between [^{3}H] and [^{14}C] was corrected by standard curves.

e. Method of Analysis of Binding

Based on the final drug concentrations of the EA_1 and EA_2 analogs per sample, the number of drug molecules were calculated to be 8.03×10^{16} and 1.5×10^{17} molecules respectively. The number of base pairs per sample was determined on the assumption of 9×10^6 base pairs (21) per bacterium and 1.14×10^{-14} grams (22) DNA per genome, presuming 3×10^{10} bacteria per sample, which was determined experimentally through plating of serial dilutions.

RESULTS:

Determination of the *in vivo* binding similarities between EBr and the azide analogs was accomplished by examining and comparing, in the presence and absence of varying concentrations of EBr, the extent of *in vivo* binding to *Salmonella* DNA by EA_1 and EA_2.

Covalent attachment of EA to DNA following photolytic activation was demonstrated using CsCl density gradient profiles (Figure 2). The interaction sites of these photoreactive probes could be compared directly to those of the parent ethidium molecule by showing competition for binding to *Salmonella* DNA. When [^{3}H]-thymidine-labeled *Salmonella typhimurium* were exposed to 1.5×10^{-5}M [^{14}C]EA_1 or 2.5×10^{-5}M [^{14}C]EA_2 in the dark, the [^{14}C] counts were negligible, demonstrating that no azide was covalently bound without photolysis, as shown in Figure 2 (A and C). Photolysis caused extensive EA_1 binding (Figure 2 B), but labeling by EA_2 at a concentration 1.7 times higher than that of EA_1 was only 40% as effective (Figure 2 D). These binding studies revealed that 1.34% of the labeled EA_1 which was added to each sample (in the absence of EB) was bound within the cells, and of that, 43% (or 0.58% of the total) was attached to the DNA. Thus, 1 out of approximately 600 base pairs was occupied by EA_1. By comparison, only 0.19% of the total EA_2 (in the absence of EB) was covalently attached to DNA, producing a binding ratio of 1 drug per 900 base pairs.

Competition by the EBr for binding sites was examined using 1.5×10^{-5} M [^{14}C]EA_1 and various EBr concentrations. A decrease in binding of [^{14}C]-

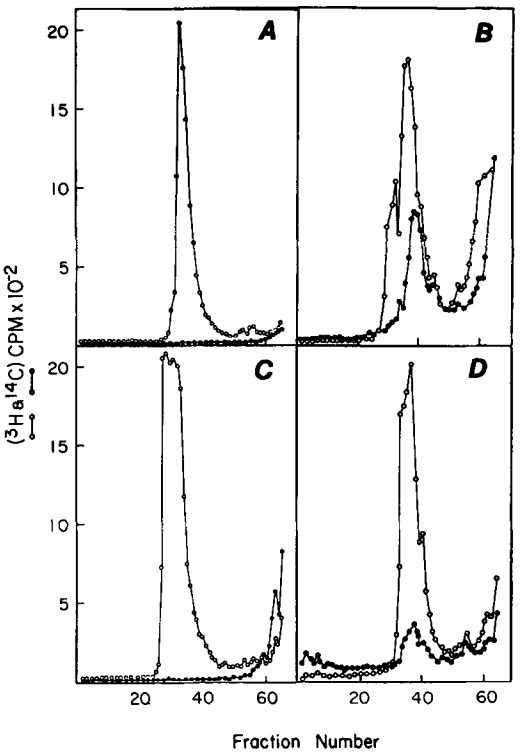


Figure 2. CsCl profile showing the covalent attachment of EA analogs to [^{3}H]-labeled DNA (O-O) of Salmonella: A, 1.5 x 10^{-5}M [^{14}C]EA_1 (●-●) with no photolysis; B, 1.5 x 10^{-5}M [^{14}C]EA_1 (●-●) photolyzed; C, 2.5 x 10^{-5}M [^{14}C]EA_2 (●-●) with no photolysis; D, 2.5 x 10^{-5}M [^{14}C]EA_2 (●-●) photolyzed.

labeled monoazide was seen with increasing EBr concentration (Figure 3). Table I gives the specific decrease in [^{14}C] label that resulted with increasing concentration ratios of EBr:EA_1 as the EBr eliminated azide binding. One EBr molecule does successfully compete with one EA_1 molecule for an ethidium binding site, but at a concentration ratio of 4:1 EBr:EA_1, the parent ethidium became more effective in competing with the monoazide interaction. This enhancement of competition was accompanied by a buoyant density shift of the Salmonella DNA from 1.706 to 1.695 g/ml, indicating an irreversible change in the DNA conformation had occurred due to the covalent binding of EA_1 in combination with the high concentration of EBr. This density shift is consistent with high concentrations of EA_1; conversely, when only high EBr concentrations

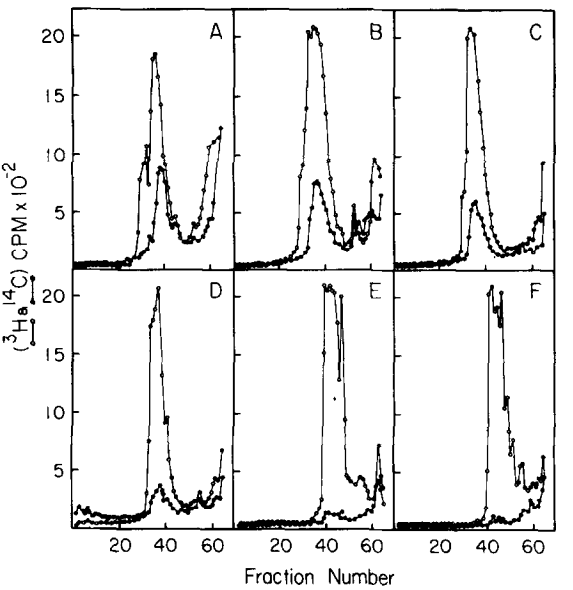


Figure 3. CsCl profile of [^{3}H]-labeled DNA (O-O) from Salmonella showing photolytic binding of 1 x 10^{-5}M [^{14}C]EA$_1$ (●-●) and competition by EBr. Experiment A contained only EA$_1$, while B, C, D, E, and F had EBr:EA$_1$ ratios of 1:3, 1:1, 2:1, 4:1, and 7:1, respectively. Experiments were carried out as outlined in the text.

Table I

Effect of Varying Ethidium Bromide Concentrations on

Ethidium Azide Analog Binding to Salmonella DNA

EBr:EA analog Ratio	EA:Base Pairs (x 10^{-3})	% Competition
EA$_1$ (dark) (1.5 x 10^{-5}M)	0	0
EA$_1$ (photolyzed)	1:0.571	0
1:3	1:0.730	23.0%
1:1	1:1.13	49.6%
2:1	1:1.56	63.5%
4:1	1:4.90	88.4%
7:1	1:8.94	93.6%
EA$_2$ (dark) (2.5 x 10^{-5}M)	0	0
EA$_2$ (photolyzed)	1:0.905	0
1:1	1:2.22	59.1%
5:1	1:21.3	95.7%
10:1	1:48.9	98.1%

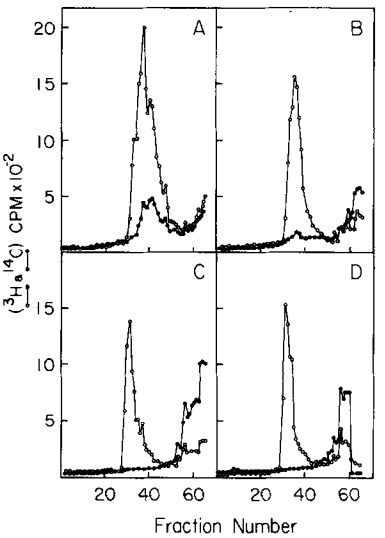


Figure 4. CsCl profile of [^{3}H]-labeled DNA (O-O) from Salmonella showing photolytic binding of 2.5 x 10^{-5}M [^{14}C]EA (●-●) and competition by EBr. Experiment A contained only EA_2, while B, C, and D had EBr:EA_2 ratios of 1:1, 5:1 and 10:1, respectively. Experiments were carried out as outlined in the text.

are added to the system, no buoyant density shift is observed. Such a shift was not seen with EA_2 in combination with EBr. These data suggested that the EBr competed effectively for most binding sites on Salmonella DNA for EA_1, until the monoazide was covalently attached by photolytic activation. Competition for the EA_2 binding sites was examined analogously, as shown in Figure 4, by varying EBr concentrations and maintaining 2.5 x 10^{-5}M EA_2. The results presented in the Table show that the parent ethidium at equimolar concentration with the azide derivatives displaced the diazide more readily than it did the monoazide, since the [^{14}C] EA_2 label was decreased by 60% while the [^{14}C] monoazide label was only decreased by 50% by equimolar concentrations of EBr. These results imply that the noncovalent binding of the diazide is somewhat weaker than that of the monoazide. At higher ratios of EBr:EA_2 (5:1 and 10:1) the diazide showed only minimal binding of 1 molecule per 2.1 x 10^4 base pairs and 4.9 x 10^4 base pairs, respectively.

DISCUSSION AND CONCLUSION:

We have demonstrated that EBr effectively competes with EA_1 and EA_2 for most of the ethidium binding sites in Salmonella DNA. Using CsCl density gradient ultracentrifugation with [^{14}C] labeled azide analogs and [^{3}H] labeled Salmonella DNA, low concentration ratios of EBr:EA decreased the [^{14}C] azide label on a 1:1 basis.

It is interesting to note that where ratios of 4:1 EBr:EA_1 are reached, the EBr not only becomes more effective in competing for the [^{14}C] EA_1 label, but these concentrations also result in a shift in the DNA structure buoyant density. Unphotolyzed [^{14}C] EA_1, and presumably EBr, were removed completely from the DNA as shown by control experiments (Figure 2). It must be assumed, therefore, that the residual small amount of covalent binding under conditions of EBr excess either represents a unique class of binding sites or provokes a covalent change in DNA structure. These [^{14}C] labeling experiments provide a direct means for determining the extent of interaction of the azide analogs.

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