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DNA Modification by 4-Aza-3-ene-1,6-diynes: DNA Cleavage, pH-Dependent Cytosine-Specific Interactions, and Cancer Cell Cytotoxicity†

Wirote Tuntiwechapikul, Wendi M. David, Dalip Kumar, Miguel Salazar,* and Sean M. Kerwin*

College of Pharmacy, University of Texas at Austin, PHR 4.220, Austin, Texas 78712-1074

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ABSTRACT: The (*Z*)-hex-1,5-diyne-3-ene reactive core common to the enediyne antitumor antibiotics undergoes a Bergman cyclization after proper activation to afford reactive diradical intermediates that are responsible for initiating DNA cleavage. Direct modification of the enediyne core has been proposed as a method to permit cancer cell-specific triggering of the diradical-generating cyclization. For example, 3-aza-3-ene-1,5-diynes undergo an aza-Bergman cyclization to afford the fleeting 2,5-didehydropyridine diradicals. While protonation of these aza-enediynes can afford products of diradical trapping, the hydrolytic instability of the 3-aza-3-ene-1,5-diyne moiety prevents its use in pH-triggered DNA cleaving anticancer agents. Recently, more hydrolytically stable systems incorporating the 4-aza-3-ene-1,6-diyne moiety were developed. We report here studies of the 4-aza-3-ene-1,6-diyne-containing benzimidazolium salt AZB002 [1-methyl-2-(phenylethynyl)-3-(3-phenylprop-2-ynyl)-3*H*-benzimidazolium tetrafluoroborate] and two structurally related heterocycles that lack the aza-enediyne functionality, AZB016 [1,3-dimethyl-2-(phenylethynyl)-3*H*-benzimidazolium triflate] and AZB004 [3-methyl-2-(phenylethynyl)benzothiazolium triflate]. The interaction of these compounds with supercoiled DNA, a double-stranded DNA fragment, and a short DNA duplex oligonucleotide was investigated. There are three distinct DNA interactions exhibited by AZB002: a frank strand scission leading to the relaxation of supercoiled DNA and formation of at least two different DNA adducts, one of which leads to cytosine-specific cleavage after piperidine/heat treatment. In contrast, analogues lacking the aza-enediyne functionality either fail to interact with DNA (AZB016) or cleave DNA at guanine residues, presumably through alkylation of the N-7 position (AZB004). We also investigated the cytotoxicity of AZB002 and the related heterocyclic compounds AZB004 and AZB016 and find that only the DNA interactive compounds AZB002 and AZB004 display significant cytotoxicity. In particular, AZB002 is cytotoxic against a wide range of cancer cell lines.

Enediyne anticancer antibiotics have become the focus of intensive research due to their potent biological activity and their intricate modes of action (*1*). Despite their potent antitumor activity, some members of this group suffer from delayed toxicity, which precludes their use as anticancer agents (*2*). Consequently, a current major goal of research in this area centers on the development of enediyne analogues that retain their biological potency while simultaneously exhibiting enhanced selectivity toward cancer cells.

The generally accepted mechanism of action of the enediyne antibiotics involves the formation of a reactive diradical intermediate upon Bergman cyclization of the common (*Z*)-1,5-diyne-3-ene reactive core (*3*). This reactive diradical can participate in DNA cleavage reactions, leading to cytotoxicity (*1*, *4*−*5*). While an alternative mechanism of cytotoxicity involving quinone generation from the enediyne core has recently been proposed (*6*), failure to detect the quinone derived from enediyne natural products indicates that this alternative mechanism may not be important in their mechanism of action (*7*). Previous attempts to harness the diradical-initiated DNA cleavage of enediynes have focused largely on the modification of the periphery of the enediyne core (*3*, *8*−*10*). In a contrasting approach, our efforts have targeted the direct modification of the enediyne core to permit cancer cell-specific triggering of the diradical-generating cyclization. For example, we recently reported the first example of a 3-aza-3-ene-1,5-diyne derivative that undergoes an aza-Bergman cyclization to afford the fleeting 2,5-didehydropyridine diradical (*11*). Unfortunately, the hydrolytic instability of the aza-enediyne moiety prevents its use as a DNA cleaving agent (*12*). More recently, we sought to develop a hydrolytically stable heterocyclic system incorporating an 4-aza-3-ene-1,6-diyne moiety that would be physiologically viable (Figure 1) (*13*). The 4-aza-3-ene-1,6-diyne-containing 1-methyl-2-(phenylethynyl)-3-(3-phenylprop-2-ynyl)-3*H*-benzimidazolium tetrafluoroborate (AZB002)[1] represents one such potential multifunctional DNA cleavage

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* Address correspondence to either author. M.S.: e-mail, m-salazar@mail.utexas.edu; tel, 512-471-4551. S.M.K.: e-mail, skerwin@mail.utexas.edu; tel, 512-471-5074.

[1] Abbreviations: bp, base pair; DMSO, dimethyl sulfoxide; EDTA, *N*,*N*,*N*′,*N*′-ethylenediaminetetraacetic acid; FBS, fetal bovine serum; Tris, tris(hydroxymethyl)aminomethane; AZB002, 1-methyl-2-(phenylethynyl)-3-(3-phenylprop-2-ynyl)-3*H*-benzimidazolium tetrafluoroborate; AZB004, 1,3-dimethyl-2-(phenylethynyl)-3*H*-benzimidazolium triflate; AZB016, 3-methyl-2-(phenylethynyl)benzothiazolium triflate.

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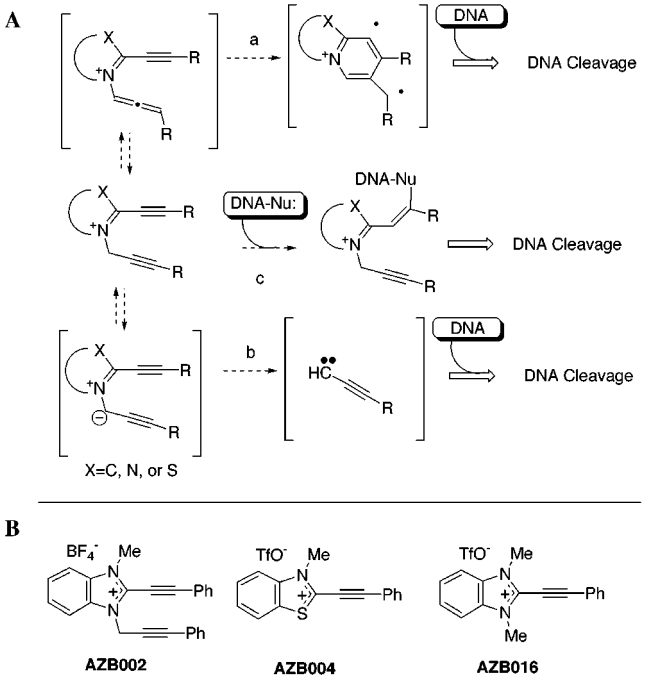

FIGURE 1: Structure and design rationale for heterocyclic 4-aza-3-ene-1,6-diynes. (A) Potential reaction pathways for heterocyclic 4-aza-3-ene-1,6-diynes leading to DNA cleavage including aza-Myers cyclization (a), carbene generation (b), and DNA alkylation (c). (B) Structures of heterocyclic 4-aza-3-ene-1,6-diyne AZB002 and analogues AZB004 and AZB016.

warhead that may give rise to a variety of reactive intermediates capable of DNA damage. AZB002 may undergo pH-triggered isomerization to generate an *N*-allenyl, 2-alkynyl species. The aza-enyne allene moiety may be capable of an aza-Myers type cyclization (*14*), thereby generating diradical intermediates persistent enough to interact with DNA under physiological conditions. *N*-Propargylbenzimidazolium salts similar to AZB002 have been proposed to afford carbene intermediates under basic conditions (*15*). There are reports of DNA cleavage mediated by photochemically generated carbenes (*16*, *17*). Alternatively, electrophilic chemistry may occur via the direct addition of a DNA nucleophile at the 2-alkyne position of AZB002. The resulting alkylated DNA lesion could also give rise to spontaneous DNA cleavage (*18*) or afford DNA cleavage products after base/heat treatment (*19*−*21*). The particular mode of DNA interaction exhibited by heterocyclic 4-aza-3-ene-1,6-diynes such as AZB002 is expected to depend on reaction conditions, particularly pH. The extracellular pH is lower in tumors than in normal tissue, whereas the intracellular pH is similar in normal and cancer cells (*22*) or elevated in the case of drug-resistant cancer cells (*23*). *N*-Propargyl, 2-alkynyl heterocycles such as AZB002 have the potential of being tunable DNA cleavage agents; varying the heterocyclic framework and the propargyl and alkyne substituents may modulate the preferred mechanism and conditions of DNA reactivity such that a suitable antitumor drug candidate can be developed.

We report here studies of the aza-enediyne AZB002 and two related heterocycles that lack the aza-enediyne functionality, AZB016 and AZB004 (Figure 1). The interaction of these compounds with supercoiled DNA, a double-stranded DNA fragment, and a short DNA duplex oligonucleotide was investigated. We find three distinct DNA interactions exhibited by AZB002: a frank strand scission leading to the relaxation of supercoiled DNA and formation of at least two different DNA adducts, one of which leads to cytosine-specific cleavage after piperidine/heat treatment. In contrast, analogues lacking the aza-enediyne functionality either fail to interact with DNA (AZB016) or cleave DNA at guanine residues (AZB004), presumably through alkylation of the N-7 position. We also investigated the cytotoxicity of AZB002 and the related heterocyclic compounds AZB004 and AZB016 and find that only the DNA interactive compounds AZB002 and AZB004 display significant cytotoxicity. In particular, AZB002 is cytotoxic against a wide range of cancer cell lines.

MATERIALS AND METHODS

Materials. Phosphoramidites and solid supports (Glenn Research), dNTP's (Pharmacia), [γ-^{32}P]ATP (Amersham), T4 polynucleotide kinase (New England Biolabs), and Taq DNA polymerase (Promega) were used as purchased without purification. Supercoiled ϕX174 DNA was obtained from Sigma. AZB002, AZB004, and AZB016 were prepared as previously reported (*13*, *24*) and prepared as stock solutions in DMSO (Aldrich) immediately before use.

Supercoiled DNA Relaxation. Solutions of compound in DMSO (4 μL) were added to solutions of supercoiled ϕX174 DNA (26 μL, 50 μM bp) in 50 mM Tris buffer, pH 8.0. The solutions were incubated for 20 h in a 37 °C water bath. After incubation, the samples were briefly centrifuged, mixed with 5 μL of loading dye, and then subjected to electrophoresis at 80 V for 2 h (0.8% agarose). Alternatively, the samples were heated in a 70 °C water bath for 90 s prior to electrophoresis. The gels were stained with ethidium bromide, and the DNA products were visualized and quantified using a Fluorimager with ImageQuant software. The degree of cleavage of form I DNA was determined using the equation:

$$\text{percent cleavage} = \frac{2([\text{form III}] + [\text{form II}])}{2([\text{form III}] + [\text{form II}] + [\text{form I}])} \times 100 \quad (1)$$

The reported, normalized percent cleavage accounts for cleavage in control samples under the reaction conditions employed, and this was calculated according to the equation:

$$\text{normalized percent cleavage} = \frac{\%\ \text{cleavage (drug)} - \%\ \text{cleavage (control)}}{100 - \%\ \text{cleavage (control)}} \quad (2)$$

The values of normalized percent cleavage of DNA presented in Table 1 represent the mean plus or minus one standard deviation from two separate determinations.

Preparation of Oligonucleotides. The oligonucleotides and primers were synthesized on a Perseptive Biosystems Expedite 8909 DNA synthesizer and purified by denaturing polyacrylamide gel electrophoresis. The concentrations of DNA were determined by UV absorption at 260 nm. The 5′-end-labeled oligonucleotides were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The short duplexes were prepared by adding 3 molar equiv of the complementary unlabeled strand followed by purification by 20% nondenaturing PAGE.

Table 1: Cancer Cell Cytotoxicity and DNA Cleavage Ability of Aza-enediynes and Analogues

	IC_{50} (μM)		
compound	A549 cells[a]	MCF-7 cells[a]	DNA cleavage (%)[b]
AZB002	0.8 ± 0.5	6.0 ± 1	42 ± 1
AZB004	19 ± 1	6.5 ± 0.5	80 ± 1
AZB016	25 ± 4	16 ± 5	0
mitomycin	0.035 ± 0.005	0.075 ± 0.02	ND[c]

[a] Inhibition of cancer cell growth as determined by alamarBlue assay, mean $\pm$ SD ($n = 2$). [b] Normalized percent cleavage of supercoiled ϕX174 DNA as determined by agarose gel electrophoresis after incubation of the DNA (50 μM bp) with 100 μM compound in pH 8.0 Tris buffer, 37 °C for 20 h, mean $\pm$ SD ($n = 2$). [c] Not determined.

Preparation of the 5′ Singly End-Labeled 229 Base Pair DNA Fragment. First, 40 pmol of the R1400 primer (dCTACC AGCGG TGGTT TGTTT) was 5′-labeled with [γ-^{32}P]ATP (Amersham) using T4 polynucleotide kinase. The labeled R1400 primer was then purified by elution through a Bio-Rad Biospin column. The purified radiolabeled primer was then used directly in the PCR reaction by adding 40 pmol of the L1172 primer (dGTCTT GAGTC CAACC CGGTA), 10 ng of pUC19 (New England Biolabs) DNA template, 5 μL of 10× buffer (100 mM Tris, pH 9.0, 0.5 M KCl, 15 mM $MgCl_2$, and 1% Triton X-100), 0.2 mM dNTPs, and 2.5 units of Taq DNA polymerase. The volume was adjusted with H_2O to 50 μL in a 200 μL PCR tube, and PCR was then performed in an Eppendorf Master Cycler Personal Thermocycler instrument. After initial denaturation at 95 °C for 5 min, the cycling conditions were as follows: 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min, for 25 cycles. The final extension was done at 72 °C for another 5 min. The single amplified product was then easily purified with a Microcon PCR spin column (Millipore), which separates the PCR product from its primers, the dNTP's, and the polymerase. The residual radiolabeled primer was then washed by adding washing buffer (1 mM Tris, pH 8.0, 0.1 mM EDTA) and centrifuging twice at 1000*g* for 15 min.

Base Preference Studies. The 20 μL reaction mixture consisted of 25000 cpm of 5′-radiolabeled DNA fragments (approximately 10 μM bp), 500 μM indicated compounds, and 10 mM indicated buffer. The samples were incubated at either 37 °C for 1 h or room temperature for 2 h. The reaction mixtures were diluted with 100 μL of precipitation buffer (10 μg of glycogen and 0.45 M sodium acetate, pH 5.2). The DNA substrates were purified by extraction into phenol/chloroform and were ethanol precipitated. The pellets were washed twice with 70% ethanol. One set of samples was subjected to heat treatment with 1 M piperidine at 95 °C for 15 min while another set was not. The samples were then redissolved in formamide loading dye and were analyzed by 6% denaturing PAGE. The images were captured and visualized by PhosphorImager (Molecular Dynamics model 445 S1).

Cytotoxicity Assays. Cell culture cytotoxicity assays were performed using the alamarBlue method, as previously described (*25*). Cell lines were obtained from the American Type Culture Collection and cultured in minimum essential medium supplemented with 10% FBS (MCF-7, SK-N-MC, SK-MEL-5), RPMI 1640 supplemented with 10% FBS (DLD-1, PC-6, U937, H33HJ-J1), RPMI 1640 supplemented with 20% FBS (HL-60), RPMI 1640 supplemented with 20% FBS and 0.2 IU of bovine insulin/mL (T47D), McCoy's medium supplemented with 10% FBS (HT-29), F-12 nutrient mixture (Ham) supplemented with 10% FBS (PC-3), F-12K nutrient mixture (Kaighn's modification) supplemented with 10% FBS (A549), or Dulbecco's modified Eagle's medium supplemented with 10% FBS (MIA PaCa-2, PANC-1). Briefly, aliquots of 100 μL of cell suspension [$(1.0-2.5) \times 10^3$ cells] were placed in 96-well microtiter plates in an atmosphere of 5% CO_2 at 37 °C. After 24 h, 100 μL of culture medium and 2 μL of compound in DMSO or DMSO alone were added, and the plates were incubated an additional 72 h. Compounds were evaluated in duplicate at final concentrations ranging from 0.01 to 100 μM. After the culture media had been removed from each well, 200 μL of fresh media and 20 μL of 90% alamarBlue reagent were added, followed by an additional 6 h incubation. The fluorescent intensity was measured using a SpectrafluorPlus plate reader with excitation at 530 nm and emission at 590 nm. Results are reported as IC_{50} values, the average concentration required to produce a decrease of fluorescent intensity of 50% relative to vehicle-treated controls in two separate determinations.

RESULTS AND DISCUSSION

Cleavage of Supercoiled DNA by Aza-enediyne AZB002 and Analogues. We have previously reported the DNA cleavage ability of the heterocyclic aza-enediyne AZB002 as determined by supercoiled plasmid DNA nicking experiments. The extent of DNA nicking by AZB002 was shown to be dependent upon AZB002 concentration, pH, incubation time, and incubation temperature but to be insensitive to brief heat treatment of the samples after incubation (*13*). Approximately 50% normalized DNA cleavage was obtained after supercoiled DNA was incubated in the presence of 100 μM AZB002 at pH 8 for 20 h at 37 °C. To compare DNA cleavage activity of the heterocyclic aza-enediyne AZB002 and analogues under identical reaction conditions, each of the compounds, AZB002, AZB004, and AZB016, was incubated at 100 μM concentration with supercoiled ϕX174 DNA (50 μM base pairs) in pH 8.0 Tris buffer at 37 °C for 20 h. Following incubation, the extent of DNA cleavage was determined by agarose gel electrophoresis of the DNA products. As we have previously reported (*13*), the aza-enediyne AZB002 demonstrates significant relaxation (42% normalized cleavage) of supercoiled DNA under these conditions (Figure 2), and the amount of cleavage is unchanged upon mild heat treatment (70 °C, 90 s) of the samples after incubation (Figure 2). Both AZB002 and the corresponding triflate salt demonstrated equivalent DNA relaxation ability (data not shown). In contrast, the corresponding *N*,*N*′-dimethylbenzimidazolium triflate salt AZB016 does not relax supercoiled DNA at all under these conditions (Figure 2). Because AZB016 lacks an aza-enediyne moiety, this result suggests that the presence of an aza-enediyne in these benzimidazolium salts is necessary for DNA cleavage. Our previous work has demonstrated that in a series of 2-alkynylbenzothiazolium salts, such as AZB004, the 2-alkynyl substituent is much more susceptible to nucleophilic attack than in a series of 2-alkynyl-substituted benzimidazolium salts, such as AZB002. For example, methanolic solutions of 2-alkynylbenzothiazolium salts convert quantitatively to methanol addition products over the course of

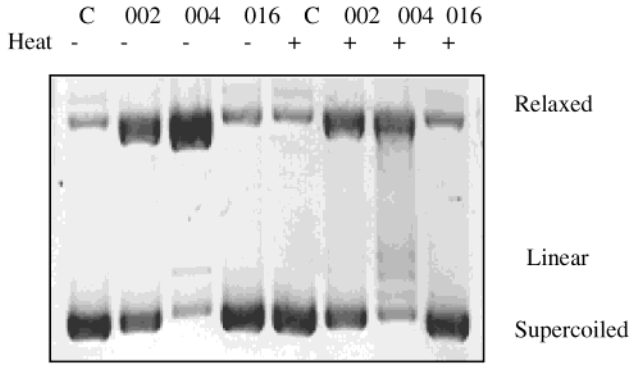

FIGURE 2: DNA cleavage by compounds AZB002, AZB004, and AZB016. Supercoiled ϕX174 phage DNA (50 μM base pairs) was incubated alone (C) or with 100 μM AZB002 (002), AZB004 (004), or AZB016 (016) (pH 8, 50 mM Tris buffer, 13% v/v DMSO, 20 h at 37 °C) and analyzed by gel electrophoresis (0.8% agarose, ethidium bromide stain) before or after incubation at 70 °C for 90 s (heat). A representative gel of two separate experiments is shown.

minutes to hours at room temperature (*24*), whereas benzimidazolium salts are stable indefinitely in methanolic solutions (*13*). When supercoiled DNA is incubated with 100 μM AZB004, nearly complete relaxation (80% normalized cleavage) of the DNA is observed along with a very minor amount (ca. 1%) of linear DNA product (Figure 2). Experiments performed at 50 μM concentration confirmed the increased DNA nicking ability of AZB004 (60% normalized cleavage) over that of AZB002 (21% normalized cleavage) (data not shown). In contrast to the results with AZB002, there is significantly more DNA cleavage observed upon brief heat treatment of the DNA cleavage reactions run in the presence of AZB004, as evidenced by the appearance of linear DNA products (ca. 30% of product DNA) (Figure 2). The ability of 2-alkynylbenzothiazolium salts to serve as electrophiles may explain the DNA cleavage ability of the benzothiazolium salt AZB004, which lacks an aza-enediyne moiety (*24*). Alkylation of DNA by AZB004, particularly at purine bases, would lead to depurination and subsequent DNA strand scission (*19*). Heat treatment facilitates these processes and results in increased DNA cleavage.

From the data obtained from these supercoiled DNA relaxation experiments, the relative DNA nicking abilities of the three compounds can be determined. Using a Poisson formula, the observed supercoiled DNA relaxation can be related to DNA nicking frequencies, taking into account the "silent" nicking events that occur on already relaxed or linearized DNA species as well as the nicking events on separate strands that lead to linear DNA products (*26*). AZB002 demonstrates 42% normalized DNA cleavage at 100 μM, corresponding to 0.55 DNA nicking events per 5386 base pair plasmid, or 1 nick per 19580 bases. For AZB004, the observed 80% normalized cleavage at 100 μM concentration prior to heat treatment corresponds to 1.7 nicks per plasmid, or 1 nick per 6340 bases. The nicking frequencies determined at 50 μM concentration of compound are approximately one-half the values obtained at 100 μM concentration (50 μM AZB002, 1 nick per 44830 bases; 50 μM AZB004, 1 nick per 12520 bases). After heat treatment, the DNA cleavage due to AZB002 is unchanged, but that due to AZB004 is much greater, as judged from the presence of a distribution of linear DNA fragments in the gel (Figure 2). The presence of these DNA products indicates a large increase in the DNA nicking frequency by AZB004 after heat treatment, which we estimate at 20 nicks/plasmid, or an approximately 10-fold increase in DNA nicking frequency after heat treatment. The lack of supercoiled DNA relaxation observed with compound AZB016 indicates that under these conditions this compound affords less than 0.05 nicking events per plasmid or less than 1 nick per 215440 bases. In summary, the supercoiled DNA nicking assay demonstrates that AZB016 is ineffective at cleaving DNA. AZB002 affords relatively infrequent frank DNA single-strand cleavage events leading to DNA nicking. AZB004 also affords relatively infrequent frank DNA strand breaks, but at a frequency approximately three times greater than that of AZB002. However, heat treatment of the supercoiled DNA reactions reveals a much higher frequency of DNA scission by AZB004, whereas under these conditions no such increase in DNA scission frequency is observed with AZB002.

AZB002 Modifies DNA in a pH-Dependent Manner To Effect Cytosine-Specific Cleavage after Treatment with Hot Piperidine. A 5′-^{32}P-labeled, 229 bp DNA fragment was incubated with 500 μM AZB002 for 1 h at 37 °C in reaction buffer that ranged in pH from 6 to 9. The DNA samples were then purified by phenol/chloroform extraction followed by ethanol precipitation. One set of samples was dissolved in formamide loading dye directly and then subjected to polyacrylamide gel electrophoresis, while another set was treated with hot piperidine prior to dissolution of the samples in formamide loading dye and electrophoresis. In the absence of treatment with hot piperidine, the DNA products showed no signs of cleavage; instead, the DNA products migrated more slowly than control DNA (Figure 3B). Similar low-mobility DNA products have been observed when supercoiled DNA is incubated in the presence of 500 μM AZB002 (*13*). Electrophoresis of the 229 bp DNA-derived reaction products after treatment with hot piperidine revealed DNA strand cleavage product bands (Figure 3A). At pH 6 and 7, the cleavage pattern indicates that AZB002 modifies DNA primarily at the cytosine bases. The cytosine specificity, however, diminishes with increasing pH. At pH 9, for example, there is a streak of breakage products rather than a well-defined DNA cleavage pattern. DNA cleavage reactions run at various concentrations of AZB002 (500 and 1000 μM) and at different times (0.5−20 h) indicated that the DNA cleavage after hot piperidine treatment was time and concentration dependent; incubation of the 229 bp DNA fragment with 500 μM AZB002 for 16 h followed by hot piperidine treatment results in nearly complete digestion of the DNA (data not shown).

The lack of evidence for frank strand scission in the reactions involving labeled oligonucleotides contrasts with the results obtained in the supercoiled DNA relaxation assays. Because the relaxation of supercoiled ϕX174 phage DNA requires only one single-strand cleavage event per 5386 base pair plasmid, the relaxation assay can detect even relatively inefficient DNA cleavage events (vide supra). Furthermore, previous work has shown that the frank supercoiled DNA nicking ability of AZB002 is minimal for incubation times less than 4 h, even at AZB002 concentrations as high as 1 mM (*13*). In contrast, the electrophoresis of DNA products from the 229 bp DNA fragment typically can only detect a few percent cleavage of the ca. 150 bases of DNA that are clearly resolved in the gel, corresponding to about 1 nick

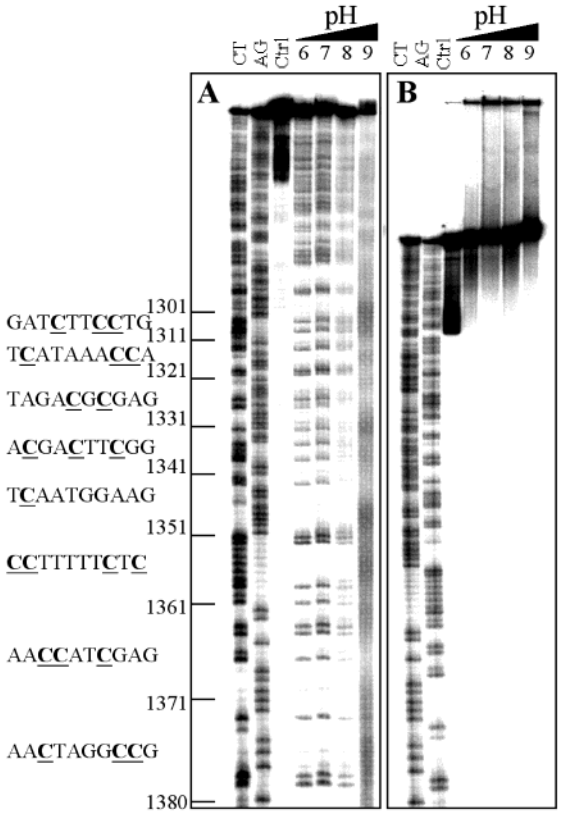

FIGURE 3: DNA cleavage pattern caused by AZB002 at various pH values. A 5′-^{32}P-labeled, 229 bp DNA fragment generated from PCR amplification of base locations 1172−1400 of pUC19 plasmid was incubated with 500 μM AZB002 for 1 h at 37 °C at the indicated pH values. Samples were purified by phenol/chloroform extraction and ethanol precipitated. (A) With subsequent piperidine heat treatment. (B) No subsequent piperidine heat treatment. The numbers denote the base location on the pUC19 plasmid from which the corresponding DNA fragment was derived, with cleaved residues underlined. Lane Ctrl is a control experiment in which ABZ002 was omitted. The AG and CT lanes are Maxam−Gilbert sequencing lanes. A representative gel of two separate experiments is shown.

per 1500 bases. Thus, under the reaction conditions employed for cleavage studies with the 229 bp DNA fragment, one would not expect to see evidence of the relatively inefficient frank DNA strand scission observed in the supercoiled DNA nicking experiments.

The DNA cleavage by AZB002 observed with the 229 bp DNA fragment after hot piperidine treatment is relatively efficient. Quantification of the bands due to DNA cleavage products from the 229 bp DNA fragment incubated with 500 μM AZB002 indicates that the DNA cleavage frequency after piperidine heat treatment is approximately one cleavage per 200 bases after 1 h of incubation. There is also an efficient DNA adduct formation that is evident as low-mobility DNA products observed in the gels prior to hot piperidine treatment of the DNA reaction mixtures (Figure 3B), but the quantification of these low-mobility products is difficult with such a relatively long starting DNA fragment (vide infra). Treatment of the DNA containing these low-mobility products with hot piperidine affords products of DNA cleavage at cytosine residues, for reactions that were carried out at pH 7 or below, or less-specific DNA cleavage, for reactions that were run at pH 8 and above. The supercoiled DNA nicking experiments did not reveal these more efficient DNA

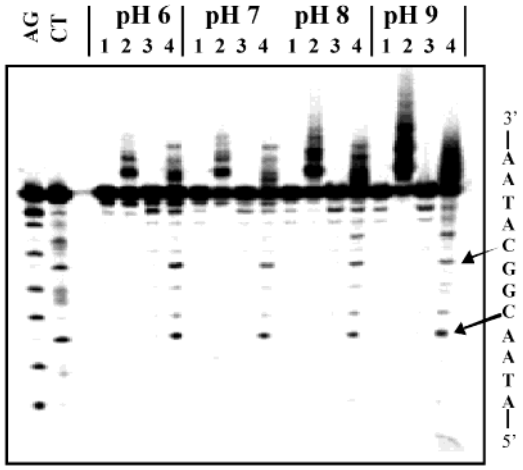

FIGURE 4: Mobility shift and cleavage pattern of a 12-mer duplex caused by AZB002 at various pH values. A 5′-^{32}P-labeled 12-mer duplex was incubated with 500 μM AZB002 for 2 h at room temperature. Samples were lyophilized and were redissolved into formamide loading dye, with (lanes 3 and 4) or without (lanes 1 and 2) a prior piperidine heat treatment. Lanes 1 and 3 are control experiments in the absence of AZB002. The AG and CT lanes are Maxam−Gilbert sequencing lanes. The sequence of the oligomer is denoted on the right. A representative gel of two separate determinations is shown.

interactions (adduct formation and subsequent piperidine-induced cleavage) because (a) at low concentrations of AZB002 (1−100 μM), the adducted plasmid DNA could not be resolved by agarose gel electrophoresis, although evidence of adduct formation was detected at higher AZB002 concentrations (1 mM) (*13*), and (b) the hot piperidine treatment of supercoiled DNA resulted in a high level of background cleavage, which precluded the accurate assessment of cleavage due to prior incubation with AZB002 (data not shown). Thus, there are three distinct DNA interactions exhibited by AZB002: an inefficient frank strand scission and formation of at least two adducts, one of which leads to cytosine-specific cleavage after piperidine/heat treatment.

AZB002 Forms DNA Adducts That Result in Cytosine Cleavage in a pH-Dependent Manner. We performed the experiment described above with a short DNA duplex in order to better monitor DNA adduct formation upon incubation with AZB002. A 5′-^{32}P-labeled 12-mer duplex was incubated with 500 μM AZB002 for 2 h at room temperature in reaction buffers that ranged in pH from 6 to 9. The samples were subjected to phenol/chloroform extraction, lyophilized, and either redissolved directly in formamide loading dye or first subjected to heat treatment with piperidine and then redissolved into formamide loading dye prior to electrophoresis. Figure 4 shows the electrophoretic mobility shift of the 12-mer duplex treated with AZB002 (lanes 2) and the cleavage pattern after heat treatment with piperidine (lanes 4) as a function of pH. AZB002 forms adducts with DNA, leading to a mobility retardation of the adducted strand (lanes 2). The intensity of the shifted bands increases with increasing pH, indicating that this interaction of AZB002 with DNA is more dominant at higher pH. These slow-moving bands diminish simultaneously with the appearance of cleavage fragments upon treatment with hot piperidine (lanes 4). At pH 6 and 7, the resulting DNA fragments correspond to cleavage preferentially at cytosine bases. At pH 9, however, the DNA fragments generated upon heat

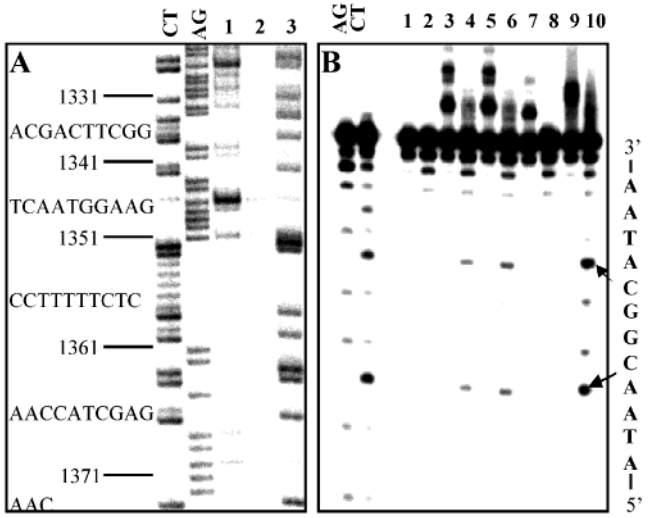

FIGURE 5: Mobility shift and cleavage pattern of DNA caused by various aza-enediyne analogues at pH 6. (A) A 5′-^{32}P-labeled 229 bp DNA fragment was incubated with 500 μM AZB004 (lane 1), AZB016 (lane 2), or AZB002 (lane 3) at 37 °C for 1 h. Samples were purified by phenol/chloroform extraction and ethanol precipitation, treated with piperidine at 95 °C for 15 min, and subjected to electrophoresis. (B) A 12-mer duplex was incubated with compounds (500 μM) at room temperature for 2 h with (even lanes) or without (odd lanes) subsequent piperidine heat treatment. Lanes 1 and 2: control. Lanes 3 and 4: AZB002. Lanes 5 and 6: triflate salt of AZB002. Lanes 7 and 8: AZB016. Lanes 9 and 10: AZB004. A representative gel of two separate determinations is shown.

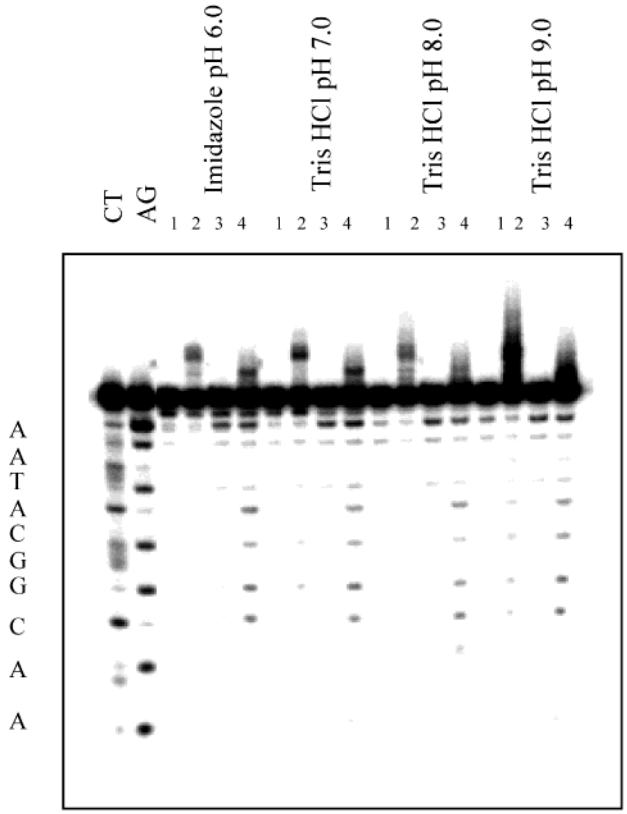

FIGURE 6: Mobility shift and cleavage pattern of a 12-mer duplex caused by AZB004 at various pH values. A 5′-^{32}P-labeled 12-mer duplex was incubated with 500 μM AZB002 for 2 h at room temperature. Samples were lyophilized and were redissolved into formamide loading dye, with (lanes 3 and 4) or without (lanes 1 and 2) a prior piperidine heat treatment. Lanes 1 and 3 are control experiments in the absence of AZB004. The AG and CT lanes are Maxam−Gilbert sequencing lanes. The sequence of the oligomer is denoted on the left. A representative gel of two separate determinations is shown.

treatment with piperidine show diminished cytosine specificity, and some of the slow-moving bands remain unaffected by heat treatment with piperidine. The persistence of these slower moving products can be rationalized on the basis of one or both of the following: (a) incomplete piperidine-based hydrolysis of products generated from the reaction of AZB002 or (b) the presence of nonhydrolyzable DNA adducts generated from the reaction of AZB002 with the DNA (*27*).

Cytosine-Specific Cleavage Does Not Occur with the Structurally Related Compounds AZB004 and AZB016. We further probed the structural origin of the cytosine-specific DNA cleavage by AZB002 by comparing AZB002 with the structurally related compounds AZB004 and AZB016. We also tested AZB002 with two different counterions, triflate and tetrafluoroborate, to investigate whether the counterion has any effect on the DNA cleavage reaction. Figure 5A shows that the cytosine-specific cleavage induced by heat treatment with piperidine is unique to the aza-enediyne AZB002 (lane 3, Figure 5A) among the three compounds tested. The *N*,*N*′-dimethyl-substituted benzimidazolium salt, AZB016, affords no noticeable DNA cleavage after heat treatment with piperidine (lane 2, Figure 5A). On the other hand, AZB004, a benzothiazolium derivative, shows enhanced reactivity toward guanine bases (lane 1, Figure 5A). DNA cleavage reactions with a 12-mer duplex (Figure 5B) show that the counterion plays no significant role in the cytosine-specific reactivity of AZB002. The tetrafluoroborate (lanes 3 and 4) and triflate (lanes 5 and 6) AZB002 salts give the same cytosine-specific cleavage products. AZB016 (lanes 7 and 8) affords no DNA cleavage after heat treatment with piperidine, which is consistent with the results with the longer 229 bp DNA substrate (Figure 5A, lane 2). There is a difference in the base preference of DNA cleavage by AZB004 with the 229-mer duplex (Figure 5A) and the 12-mer duplex (Figure 5B). With the longer duplex, AZB004 cleaves preferentially at guanines, but with the shorter duplex, the DNA cleavage pattern is less base-specific. It is possible that the differences in the cleavage patterns for AZB004 in Figure 5 result from the diminished stability of the shorter duplex. AZB004 may cleave more readily at residues other than guanine if these residues are, at least transiently, not Watson−Crick hydrogen bonded. We also examined the pH dependence of DNA cleavage of the 12-mer duplex by AZB004 (Figure 6). We found that AZB004 exhibited a pH dependence for DNA alkylation, wherein increasing pH corresponded with increasing alkylation, as evidenced by an increase in the lower mobility DNA products before treatment with hot piperidine (Figure 6, lanes 2). Also, a very small amount of cleavage of the 12 bp DNA oligonucleotide can be detected before treatment with hot piperidine (Figure 6, lanes 2). After hot piperidine treatment, the amount of the DNA cleavage products increases, and the pattern of DNA cleavage is the same across all values of pH tested (i.e., pH 6−9), with cleavage products occurring mainly at G, C, and A residues.

We have previously reported that 2-alkynyl-substituted benzothiazolium salts are electrophilic species that cleave DNA, presumably through alkylation of DNA bases, fol-

Table 2: Cancer Cell Cytotoxicity of AZB002

cell line	IC_{50} (μM)[a]	cell line	IC_{50} (μM)[a]
PC-6	2.0 ± 1.5	DLD-1	2.0 ± 1
HL-60	2.8	SK-N-MC	4.0 ± 1
T-47D	5.0 ± 1	MIA PaCa-2	15 ± 8
U-937	1.8 ± 1	PANC-1	13 ± 6
H33HJ-JA1	3.0 ± 2	PC-3	11 ± 9
HT-29	10 ± 2	SK-MEL-5	8.0 ± 4

[a] Inhibition of cancer cell growth as determined by alamarBlue assay, mean $\pm$ SD ($n = 2$).

lowed by depurination and subsequent DNA strand scission (*24*). The demonstration of guanine-selective DNA cleavage by AZB004 shown here supports this alkylation mechanism, in that guanine is recognized as the primary site of attack by a number of electrophilic species (*19*). By comparison to the corresponding benzothiazolium analogues, 2-alkynyl-substituted benzimidazolium salts are less prone to nucleophilic attack at neutral pH but do undergo nucleophilic attack under more basic conditions (*13*). The increase in DNA adducts observed for reactions with the benzimidazolium AZB002 at pH 9 may be the result of nucleophilic attack by DNA on AZB002 in a manner analogous to that for the benzothiazolium AZB004. Alternatively, deprotonation of AZB002 at high pH followed by α-elimination would give rise to a carbene intermediate (*15*) that may form adducts with DNA (*17*). However, the cytosine-specific cleavage due to AZB002 at pH 6 and 7 probably occurs via a different mechanism. Recently, 2-hydroperoxytetrahydrofuran (THF-OOH) has been reported to react with DNA to afford heat-labile adducts affording DNA cleavage preferentially at cytosine residues (*28*, *29*). Although the mechanistic details of the cytosine-specific cleavage by THF-OOH have yet to be elucidated, it has been proposed that modification at N3 of cytosine by a free radical intermediate is involved (*29*). This proposal was supported by the results from ESR studies, in which carbon-centered radicals are the dominant species trapped during the decomposition of THF-OOH (*28*). It remains to be determined if a similar free radical-based mechanism is operative in the cytosine-selective DNA modification of DNA by AZB002 reported here. In support of such a mechanism, we find that free radical scavenging agents such as DTT (1 mM) and ascorbic acid (10 mM) significantly inhibit the cytosine-specific cleavage by AZB002 (data in Supporting Information).

Cancer Cell Cytotoxicity of AZB002 and Analogues Reflects the Differential Ability of These Compounds To Interact with DNA. The heterocyclic salts AZB002, AZB004, and AZB016 were tested for in vitro cytotoxicity against two human cancer cell lines. The non-DNA-cleaving benzimidazolium salt AZB016 is 2.6−30 times less cytotoxic than the DNA-cleaving benzimidazolium salt AZB002 (Table 1). The benzothiazolium salt AZB004, which also cleaves DNA, has comparable cytotoxicity against MCF-7 cells as AZB002 but is significantly less cytotoxic to A549 cells (Table 1). Additional testing of AZB002 against other cancer cell lines (Table 2) demonstrates that this aza-enediyne is moderately cytotoxic against most of the cell lines examined.

CONCLUSIONS

In summary, the results presented here provide support for at least three different covalent DNA interactions by AZB002: (a) a process leading to relaxation of supercoiled DNA that involves an inefficient frank DNA strand scission; (b) an alkylation reaction that occurs preferentially at high pH and is independent of the aza-enediyne functionality; and (c) an unusual cytosine-specific reaction at low pH that has parallels with the organic radical-mediated, cytosine-specific cleavage of DNA by THF hydroperoxide. Work is underway to further define the mechanisms of these DNA interactions and to explore their relative role in the distinct cancer cell cytotoxicity of AZB002.

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SUPPORTING INFORMATION AVAILABLE

Time course of cytosine-specific DNA cleavage of the 229 bp DNA fragment by AZB002, inhibition of cytosine-specific DNA cleavage of the 229 bp DNA fragment by AZB002 in the presence of ascorbic acid, and inhibition of DNA adduct formation and cytosine-specific cleavage of the 12-mer duplex by AZB002 by DTT. This material is available free of charge via the Internet at http://pubs.acs.org.

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