DNA Interstrand Cross-Links Induced by the Cyclopropylpyrroloindole Antitumor Agent Bizelesin Are Reversible upon Exposure to Alkali†

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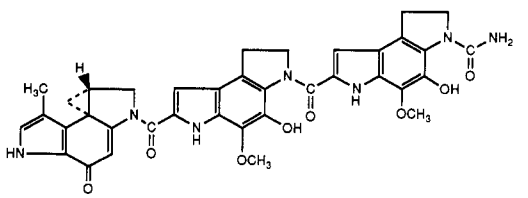
ABSTRACT: Bizelesin, a cyclopropylpyrroloindole (CPI) antitumor agent, has been shown to alkylate and cross-link DNA within A/T-rich tracts. Previous studies have shown that covalent reaction of the CPI adozelesin with DNA was reversible [Warpehoski, M. A., Harper, D. E., Mitchell, M. A., & Monroe, T. J. (1992) *Biochemistry 31*, 2502–2508]. That is, the monofunctional adduct could be lost from DNA, thus restoring the fidelity of DNA. In this study, we demonstrate that covalent DNA adducts induced by bizelesin at the adenine N3 position undergo two subsequent competing reactions: one which causes DNA strand cleavage, via depurination, and one which proceeds through loss of the DNA adduct (adduct reversal with restoration of DNA integrity). Our results were obtained by studying the chemical stability of synthetic DNA oligonucleotides which contained either a distinct DNA monofunctional adduct or DNA interstrand cross-links. Quantification of adduct reversal was performed on the basis that drug-modified DNA, upon exposure to heat followed by hot piperidine treatment, was resistant to strand cleavage at the site of alkylation. The rate of adduct reversal was found to increase with increasing temperature and was found to be maximum at 70–80 °C. The rate of adduct reversal was also found to increase with increasing pH and ionic strength. In contrast, the rate of depurination and subsequent DNA strand cleavage decreased as pH and ionic strength were increased. Adduct reversal was favored in DNA containing interstrand cross-links, whereas rapid depurination occurred preferentially within monofunctionally alkylated DNA.

CC-1065 (Figure 1) is a very potent antitumor antibiotic capable of alkylating the adenine N3 position in a sequence-selective manner (Hurley et al., 1984, 1988, 1990; Reynolds et al., 1985; Boger et al., 1988, 1990, 1991a–c). Bizelesin, a synthetic bifunctional analog of CC-1065, contains two DNA-reactive cyclopropylpyrroloindole (CPI)[1] subunits connected with a rigid bis(indolecarboxylic acid) linker (Mitchell et al., 1991). Recently, bizelesin has been shown to induce a DNA interstrand cross-link (ISC) which spans six or seven nucleotides, which is dependent upon the intervening sequence, within A/T-rich DNA (Ding & Hurley, 1991; Lee & Gibson, 1993).

An attempt, however, to detect the presence of DNA ISCs by the technique of alkaline elution in human carcinoma cells at pharmacologically relevant concentrations was not successful (Lee & Gibson, 1991). This may be due to the fact that CPI-induced DNA adducts (including DNA interstrand cross-links) are not covalently fixed. For instance, adozelesin-induced monofunctional DNA adducts can be reversed, a process which restores the fidelity of DNA (Warpehoski et al., 1992). Such a phenomenon does not appear to be restricted to the CPI class of molecule, as HPLC analysis of oligonucleotides cross-linked by the antitumor agent mitomycin C indicates that DNA cross-links are stable to heat at neutral pH, but are reversed by treatment in hot piperidine (Borowy-Borowski et al., 1990). Taken together, this suggests that DNA adducts induced by bizelesin may be unstable to the alkaline conditions of the elution assay and it is for this reason that we failed to detect DNA interstrand cross-links within cells.



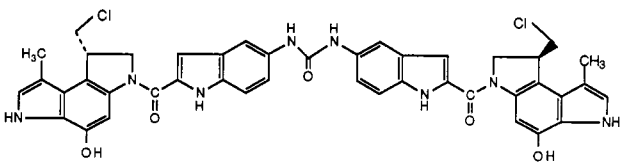


FIGURE 1: Structures of CC-1065 and bizelesin.

In terms of the chemistry of alkylation of DNA by the CPIs, it is known that the monofunctional adenine N3 adduct induced by CC-1065 is susceptible to depurination, with limited cleavage of the phosphodiester backbone occurring upon exposure to heat (Reynolds et al., 1985). Subsequent piperidine treatment of thermally depurinated CC-1065-modified DNA completes a β-elimination reaction, generating a fragment with a 5′-phosphate adjacent to the apurinic site. The resultant product displays an electrophoretic mobility identical to that seen with the Maxam and Gilbert sequencing reactions (Reynolds et al., 1985). CC-1065-induced adenine N3 adducts were not susceptible to DNA strand cleavage by treatment in hot 1 M piperidine (Reynolds, 1984). This is in

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[1] Abbreviations: DNA ISC, DNA interstrand cross-links; DNA MA, monofunctionally alkylated single-stranded DNA; CPI, cyclopropylpyrroloindole.

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contrast to the susceptibility of the guanine N7 adducts induced by nitrogen mustard and/or aziridinylbenzoquinones, which undergo piperidine-mediated DNA strand cleavage (Mattes et al., 1986; Lee et al., 1992).

Prior to continuing extensive studies of CPI-induced DNA damage within cells, we wished to determine the chemical and thermal stability of purified DNA ISC and monofunctionally alkylated DNA (DNA MA) induced by bizelesin within defined DNA duplex oligonucleotides. Our results suggest that purified bizelesin DNA adducts can undergo two competing reactions, namely, depurination and adduct reversal. Adduct reversal produces DNA which is resistant to the thermally induced depurination which would normally occur at CPI-induced alkylation sites. Depurinated DNA is subsequently susceptible to alkaline hydrolysis of the phosphate backbone, and thus thermally induced apurinic sites can be separated from alkylation sites which have undergone adduct reversal. Bizelesin-induced DNA ISCs were found to be relatively stable to depurination and thus capable of undergoing adduct reversal, whereas bizelesin-induced DNA MAs were found to rapidly depurinate. We found that the rate of adduct reversal within bizelesin-induced DNA ISCs increased with increasing pH, temperature (up to a maximum of 80 °C), and ionic strength. Our results suggest that the ability to detect CPI-induced alkylations within genomic DNA will thus be dependent upon the conditions used to extract and analyze such damaged DNA.

MATERIALS AND METHODS

Chemicals and Reagents. Bizelesin was generously supplied by The Upjohn Company, Kalamazoo, MI. Ammediol, methylpiperidine, and piperidine were obtained from Sigma. The Spin X centrifuge filter unit (0.22-mm nitrocellulose) was purchased from Costar. T4 polynucleotide kinase was obtained from Boehringer Mannheim. [γ-^{32}P]ATP was obtained from NEN Du Pont, and acrylamide solution was obtained from Fisher.

Preparation of Oligonucleotides. Oligonucleotides (see below for sequence) were synthesized on an automated DNA synthesizer (Applied Biosystems 391) and deprotected with saturated ammonium hydroxide at 55 °C overnight. Each oligonucleotide was purified on a denaturing 20% polyacrylamide gel prior to use. The melting temperature of this duplex DNA was predicted to be 47 °C by a computer program (Rychlik & Rhoads, 1989). Oligonucleotide sequence:

5′-GGTCGTCGATAAAAATCGACG
GCAGCTATTTTTAGCTGCCCA-3′

Purification of Bizelesin-Induced DNA ISC and DNA MA. 5′-End-labeled DNA ISC and MA were isolated on a denaturing 20% acrylamide gel as described previously (Lee & Gibson, 1993).

Determination of Reversal of the Bizelesin-Induced Adenine N3 Adduct. A schematic diagram of the entire experimental protocol to determine the ability of purified DNA ISC and DNA MA to undergo adduct reversal and/or be susceptible to depurination is shown in Figure 2. The stability of purified DNA ISC was determined upon heat and/or alkali exposure (see the caption of Figure 2 for appropriate conditions). Four different reaction products can be observed: (1) DNA MA, (2) depurinated DNA, (3) DNA which has lost an associated adduct (i.e., DNA which has undergone adduct reversal), and (4) DNA strand cleavage products. Upon denaturing gel electrophoresis depurinated DNA and DNA which has lost an associated adduct have similar mobilities (Castaing et al., 1992). Thus a conversion reaction is required to distinguish between depurinated DNA and DNA which has lost a bizelesin-induced DNA adduct. This conversion reaction was performed by heating DNA in 40 μL of 10 mM phosphate (pH 7.0) at 93 °C for 30 min and then reheating in 1 M piperidine at 93 °C for 30 min. This conversion step converts drug-bound or depurinated DNA into DNA strand cleavage products, while DNA which has lost the bizelesin adducts remains unbroken. Consequently, the appearance of a band which has electrophoretic mobility identical to the intact nonmodified DNA represents DNA which has lost a bizelesin-associated adduct and which has thus undergone adduct reversal.

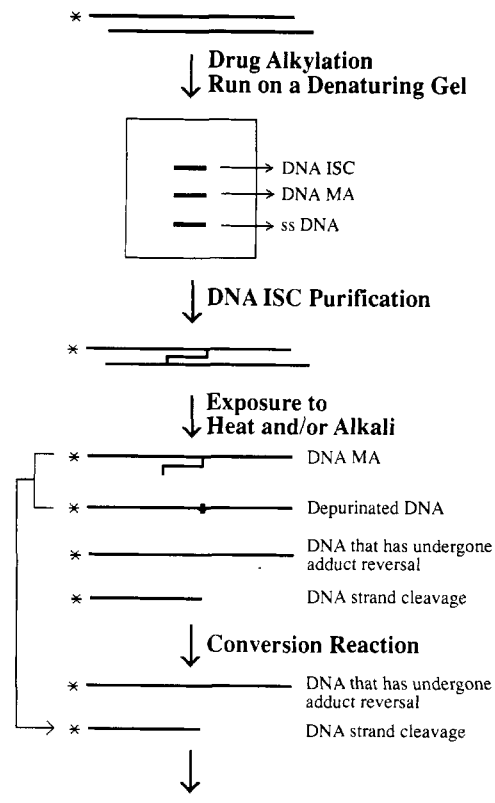

FIGURE 2: Schematic representation of the experimental protocol to determine bizelesin adduct reversal from purified DNA ISC. DNA ISC, showing the slowest migration mobility on a denaturing gel, was purified and exposed to a variety of different incubation conditions (see figure captions). The conversion reaction refers to the fact that only DNA containing either a monoadduct or a site of depurination will be converted to a DNA strand cleavage product. This conversion reaction was required in order to distinguish DNA which had undergone adduct reversal from depurinated DNA and DNA MA. DNA which had undergone adduct reversal and DNA strand cleavage products could then be separated on a 20% denaturing gel.

Characterization of Depurination and DNA Strand Cleavage. Piperidine (a secondary amine), but not *N*-methylpiperidine (a tertiary amine), has been shown to displace the alkaline ring-opened formamidopyrimidine product of a guanine N7 adduct via a β-elimination event (Mattes et al., 1986). Cleavage of the phosphodiester backbone by alkaline hydrolysis then occurs. In contrast, alkylations at the adenine N3 position are thought to be stable to piperidine, and depurination of such sites is required prior to cleavage of the phosphodiester backbone of piperidine. We have taken advantage of the proposed stability of adenine N3 adducts to piperidine in order to probe the stability of bizelesin-induced adducts to heat, alkali (sodium hydroxide), a secondary amine (piperidine), a tertiary amine (*N*-methylpiperidine), and combinations thereof.

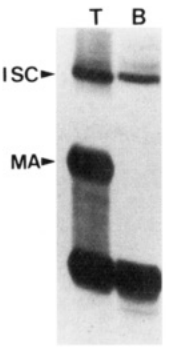

FIGURE 3: Autoradiogram of a 20% denaturing acrylamide gel showing that bizelesin-induced DNA ISC and MA have different electrophoretic mobilities. T, top-strand-labeled DNA; B, bottom-strand-labeled DNA.

The chemistry involved in the DNA fragmentation reaction was probed by comparing the reaction products obtained when purified bizelesin-induced DNA ISC and MA were heated at 92 °C for 30 min in 40 μL of 10 mM phosphate buffer (pH 7.0), piperidine, methylpiperidine, or sodium hydroxide at the concentrations described in each figure. After lyophilization overnight (piperidine or methylpiperidine) or precipitation of DNA (sodium hydroxide), each sample was reexposed to heat and alkali, prior to suspension in 10 μL of tracking dye containing 80% formamide and 1 mM EDTA, and then subjected to 20% denaturing polyacrylamide gel electrophoresis (acrylamide mono:bis ratio = 29:1) in parallel with modified Maxam and Gilbert DNA sequencing reactions (Lee & Gibson, 1993).

RESULTS

Purification of DNA ISC and DNA MA Produced by Bizelesin. DNA ISC and MA were separated from non-alkylated DNA by 20% denaturing polyacrylamide gel electrophoresis (Figure 3). We have previously demonstrated that the slow-mobility band is a DNA ISC and the intermediate-mobility band is a DNA MA (Lee & Gibson, 1993). Sequencing gel analysis identified the cross-linking site of bizelesin as occurring within 5′-TAAAAA*, where the asterisk indicates the cross-linking site on the top strand and T indicates the cross-linking site on the bottom strand (Lee & Gibson, 1993). Sequencing analysis of bizelesin-induced DNA MA suggested a mixture of different products where each adenine indicated could be alkylated at the adenine N3 position (5′-TAA*A*A*A; each asterisk represents a site of monofunctional alkylation on the top strand of the DNA duplex). DNA MA was not observed when the DNA duplex was labeled on the bottom strand (lane B, Figure 3). This is due to the fact that monofunctional alkylation does occur within 5′-TTTT-TA* but DNA MA is converted to a DNA ISC with 100% efficiency.

Characterization of the DNA Strand Cleavage Reaction. The monofunctional adenine N3 adduct induced by CC-1065 is resistant to DNA strand cleavage in hot 1 M piperidine (a secondary amine) (Reynolds, 1984). We have thus studied the susceptibility of DNA modified by bizelesin to DNA strand cleavage induced by other representative alkalis such as sodium hydroxide and *N*-methylpiperidine (a tertiary amine) (Figure 4). Lane 1 shows the products obtained after a hot 1M piperidine treatment of a purified DNA ISC induced by bizelesin. The major band observed represents DNA which is resistant to cleavage, with the minor band representing a small amount of DNA cleavage. In contrast, thermal treatment of this purified bizelesin-induced DNA ISC in 10 mM phosphate buffer (pH 7.0) produced one major band, representing fragmentation of DNA immediately 5′ to the apurinic site (band Y, Figure 4), with two minor bands representing the products of the initial thermal depurination reaction (band X, Figure 4). Although the identities of the products designated as band X are unknown, they presumably represent the 2′,3′-unsaturated sugar and its rearrangement product (Bailly & Verly, 1987; Jones et al., 1968), with the phosphate backbone being cleaved at the 3′ side of the sugar, and are thus incomplete products of DNA degradation.

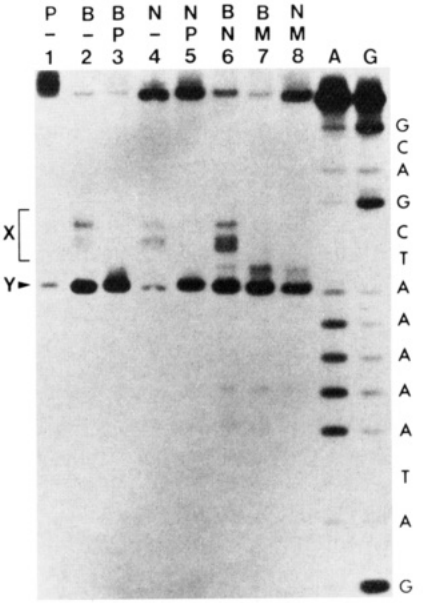

FIGURE 4: Stability of purified bizelesin-induced DNA ISC to alkali and heat. The top-strand-labeled purified DNA ISC was heated with 40 μL of 1 M piperidine (lane 1), 10 mM phosphate buffer (pH 7.0) (lanes 2, 3, 6, and 7), or 10 mM sodium hydroxide (lanes 4, 5, and 8) at 92 °C for 30 min. Subsequent exposure of the DNA, which had undergone thermal depurination in phosphate buffer (lane 2), to 1 M piperidine (lane 3), 10 mM sodium hydroxide (lane 6), or 1 M methylpiperidine (lane 7) is shown. Subsequent exposure of the DNA, which had first undergone exposure to 10 mM sodium hydroxide (lane 4), to 10 mM phosphate buffer (lane 5), 1 M piperidine (lane 6), or 1 M methylpiperidine (lane 8) is also shown. A, adenine-specific sequencing reaction; G, guanine-specific sequencing reaction.

Exposure of purified bizelesin-induced DNA ISC, previously depurinated under neutral thermal conditions, to 1 M piperidine (pH 12.5) or 1 M *N*-methylpiperidine (pH 12.0) converts, via a β-elimination reaction, the initial thermal depurination products to a species which represents the DNA fragment containing a 5′-phosphate adjacent to the apurinic site (lanes 3 and 7, respectively). This DNA fragment has an identical electrophoretic mobility to the Maxam and Gilbert adenine sequencing reaction product and thus facilitates the assignment of the site of adenine alkylation (Hurley et al., 1984). Exposure of this thermally depurinated DNA ISC to a mixture of 10 mM sodium hydroxide and 10 mM phosphate buffer results in incomplete β-elimination and thus incomplete DNA fragmentation (see above description for band X and band Y, Figure 4). In contrast, exposure to 0.1 M piperidine, methylpiperidine, or sodium hydroxide makes the pH of the incubation mixture sufficiently alkaline for complete β-elimination and thus complete DNA fragmentation to occur (data not shown).

Treatment of purified bizelesin-induced DNA ISC with 10 mM sodium hydroxide (pH 12.0; Figure 4, lane 4) produced as the major product DNA which was resistant to cleavage, with 20% of the reaction constituting the products of complete and incomplete DNA fragmentation. Exposure of the DNA

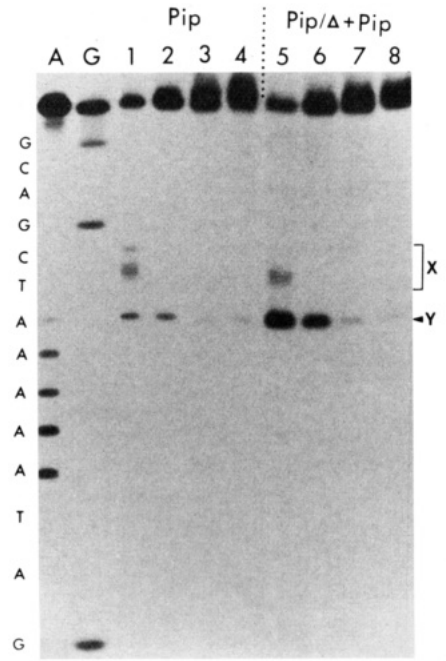

FIGURE 5: Piperidine-induced reversal reaction of DNA ISC. The top-strand-labeled DNA ISC was heated in 40 μL of increasing concentrations of piperidine at 92 °C for 30 min. After lyophilization of piperidine overnight, duplicate samples were subjected to the conversion reaction as described in Material and Methods: 0.001 M piperidine (lanes 1 and 5), 0.01 M piperidine (lanes 2 and 6), 0.1 M piperidine (lanes 3 and 7), and 1 M piperidine (lanes 4 and 8).

ISC to piperidine (lane 5) or *N*-methylpiperidine (lane 8) after exposure to 10 mM sodium hydroxide did not convert significantly the DNA which was resistant to cleavage, but converted all the incomplete DNA fragmentation products to a single DNA fragment containing a 5′-phosphate adjacent to the apurinic site (band Y, lanes 5 and 8).

Piperidine Induces Bizelesin Adduct Reversal. In order to investigate the phenomenon that alkali induces loss of the bizelesin-induced DNA ISC by adduct reversal, we have determined the dependence of this process upon the concentration of piperidine. Treatment of purified bizelesin-induced DNA ISC with different concentrations of piperidine (lanes 1 to 4, Figure 5) was subsequently followed by the conversion reaction described in Materials and Methods (lanes 5–8 in Figure 5). As the concentration of piperidine was increased, DNA cleavage decreased. The appearance of products representing both complete and incomplete DNA fragmentation was found to decrease as the concentration of piperidine increased. Subsequent exposure of these samples to heat followed by piperidine converted all minor reaction products to the end product of the β-elimination reaction, a DNA fragment which contains a 5′-phosphate adjacent to the apurinic site. Exposure of purified DNA ISC to increasing concentrations of sodium hydroxide produced similar results to piperidine exposure (data not shown). These results suggest that exposure of purified DNA ISC to alkali results in DNA which is subsequently resistant to cleavage by heat and alkali and thus represents DNA which has lost an associated DNA adduct.

Temperature Dependency of Bizelesin Adduct Reversal. Purified bizelesin-induced DNA ISC was exposed to either 10 mM sodium hydroxide [Figure 6A, (A)] or 10 mM piperidine [Figure 6A, (B)] under conditions of increasing temperature. As the temperature was increased from 37 to 80 °C, the rate of DNA adduct reversal increased while DNA strand cleavage decreased. Densitometric analysis of this data

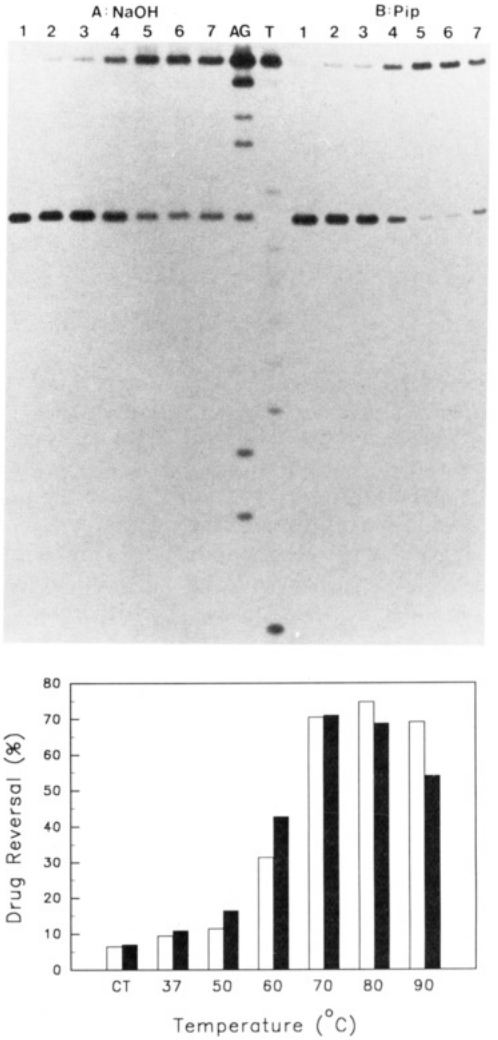

FIGURE 6: Temperature dependency of bizelesin adduct reversal. Panel A (top): Autoradiogram of 20% denaturing acrylamide gel. The top-strand-labeled bizelesin-induced purified DNA ISC was heated in 40 μL of 10 mM sodium hydroxide (A) or 10 mM piperidine (B) at 25 °C (lane 1), 37 °C (lane 2), 50 °C (lane 3), 60 °C (lane 4), 70 °C (lane 5), 80 °C (lane 6), or 90 °C (lane 7) for 30 min. After DNA precipitation, the conversion reaction was performed as described in Materials and Methods. Panel B (bottom): Densitometric analysis of the autoradiogram shown in panel A. Open bars represent samples treated with 10 mM sodium hydroxide, and closed bars represent samples treated with 10 mM piperidine.

is shown in panel B of Figure 6. Maximum adduct reversal was obtained at 80 °C in the presence of sodium hydroxide and at 70 °C in the presence of piperidine.

Sequence and pH Dependency of Bizelesin Adduct Reversal. The level of bizelesin adduct reversal from purified DNA ISC was not sequence specific. Heat treatment of the top-strand-labeled [Figure 7, (6A–T)] or the bottom-strand-labeled [Figure 7, (6A–B)] DNA ISC did not show any difference in the level of DNA which was resistant to thermal cleavage at various pH's (Figure 7, panels A and C). Subsequent exposure of these products to the conversion reaction caused an increase in the DNA strand cleavage products and a decrease in the DNA with a mobility representing full-length single-stranded DNA (Figure 7, panels B and D). When the ionic strength of the buffers was kept constant, an increase in the amount of bizelesin adduct reversal was observed with increasing pH. When, however, ammediol buffers (lanes 4, 5 and 6) were substituted for phosphate buffer to adjust for appropriate alkaline pH's, the amount of bizelesin adduct reversal appeared inconsistent with the data described above.

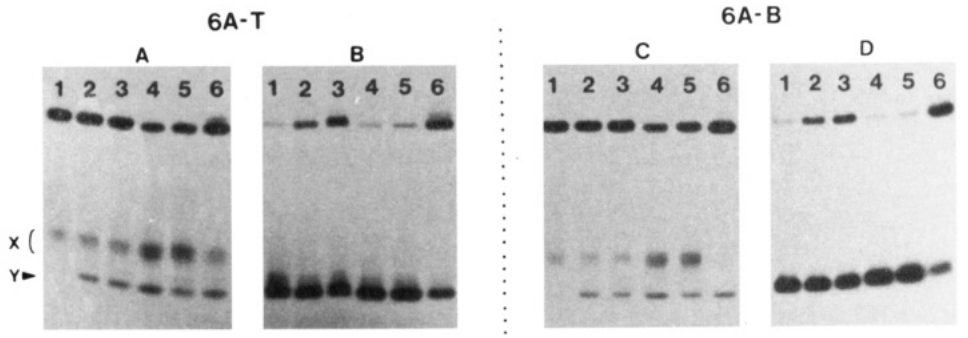

FIGURE 7: Sequence and pH dependency of bizelesin adduct reversal. The top- (6A-T) or bottom-strand-labeled (6A-B) purified bizelesin-induced DNA ISC was heated at different pH's at 90 °C for 30 min (panels A and C). After DNA precipitation, the conversion reaction was performed as described in Materials and Methods (panels B and D). Exposures were as follows: 10 mM phosphate buffer, pH 7 (lane 1), pH 8 (lane 2), and pH 9 (lane 3); 10 mM ammediol buffer, pH 10 (lane 4), pH 11 (lane 5), and pH 12 (lane 6).

Within this buffer, however, an increase in the amount of adduct reversal was apparent as the pH was increased. These results suggest that bizelesin adduct reversal from purified DNA ISC was dependent upon not only pH but also buffer composition. In addition, no sequence-dependent variation in the level of DNA ISC reversibility between 5′-TAAAAA* (6A–T) and 5′-TTTTTA* (6A–B) sequences was observed.

Ionic Strength Dependency of Bizelesin Adduct Reversal. Since bizelesin adduct reversal from purified DNA ISC was sensitive to buffer composition, the effect of ionic strength upon adduct reversal was studied. As shown in Figure 8, bizelesin adduct reversal from DNA ISC was increased as the concentration of sodium chloride was increased from 0 to 400 mM, while the pH of the ammediol buffer (pH 11) was kept constant. When similar incubations were performed in 10 mM phosphate buffer (pH 8.0), a similar increase in bizelesin adduct reversal from purified DNA ISC was observed as the ionic strength was increased (data not shown).

Time Dependency of Bizelesin Adduct Reversal. Figure 9 shows the time dependence of bizelesin adduct reversal when purified DNA ISC was incubated in 100 mM NaCl and 10 mM phosphate buffer (pH 8.0) at 37 °C (panel A) and 55 °C (panel B). Little increase in bizelesin adduct reversal was observed at 37 °C during a 5-day incubation, suggesting that DNA ISC is very stable at 37 °C in aqueous solution. However, the level of bizelesin adduct reversal was increased when the purified DNA ISC was held at 55 °C for 24 hours. An additional 4 days of incubation resulted in no further increase in the level of bizelesin adduct reversal.

Stability of Bizelesin-Induced DNA MA. Figure 10 shows a comparison between the thermal stability of purified DNA ISC and purified DNA MA when each lesion was incubated for 10 min in 20 μL of tracking dye containing 80% formamide and 1 mM EDTA at various temperatures. Purified DNA MA was observed to rapidly depurinate, even at room temperature (lane 1, MA-T), with more than 60% of the DNA MA being converted either to an apurinic site or to DNA which had lost a drug-associated adduct. In contrast, most of the purified bizelesin-induced DNA ISC remains stable at room temperature (lane 1, ISC-T and ISC-B).

As the temperature was gradually increased in 10 °C increments, there was little difference in the pattern of depurination observed with DNA MA (Figure 10, lanes 1–5, MA-T). At 80 °C, however, purified DNA MA was found to be converted to products which represent the thermal decomposition products associated with an apurinic site and the incomplete products of DNA fragmentation (lane 6, MA-T; see description of band X above). As the temperature of incubation was increased, thermal conversion of the purified

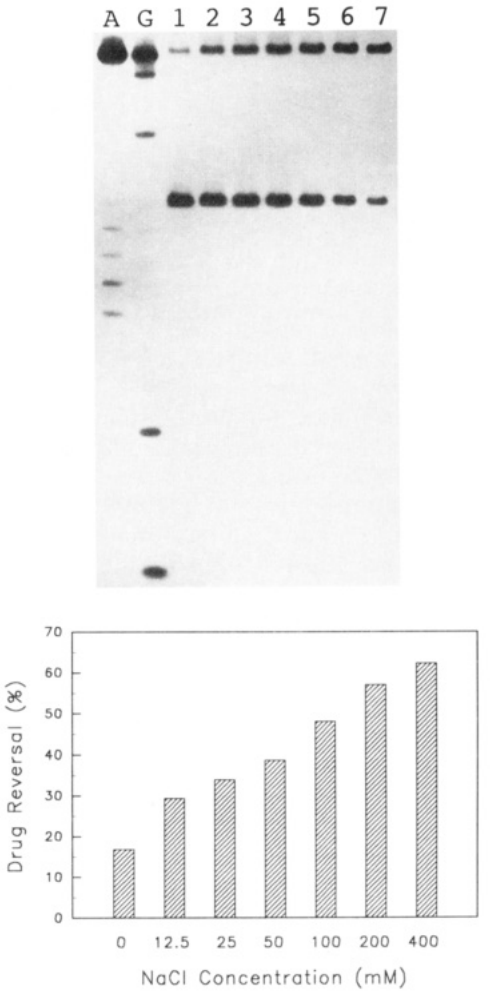

FIGURE 8: Ionic strength dependency of bizelesin adduct reversal. Panel A (top): Autoradiogram of 20% denaturing acrylamide gel. The top-strand-labeled purified bizelesin-induced DNA ISC was heated in 40 μL of 10 mM ammediol buffer (pH 11) at 80 °C for 30 min with the following concentrations of sodium chloride: 0 (lane 1), 12.5 (lane 2), 25 (lane 3), 50 (lane 4), 100 (lane 5), 200 (lane 6), and 400 mM (lane 7). After DNA precipitation, the conversion reaction was performed as described in Materials and Methods. Panel B (bottom): Densitometric analysis of the autoradiogram shown in panel A.

DNA ISC to products which represented DNA MA, unmodified DNA, and incomplete products of DNA fragmentation (i.e., band X) was increased (lanes 1–5, ISC-T and ISC-B). In general, the higher the temperature, the greater the degree of degradation of DNA ISC observed (compare patterns observed in lanes 1–6). Lane 7 shows the product expected if complete DNA fragmentation at the alkylation

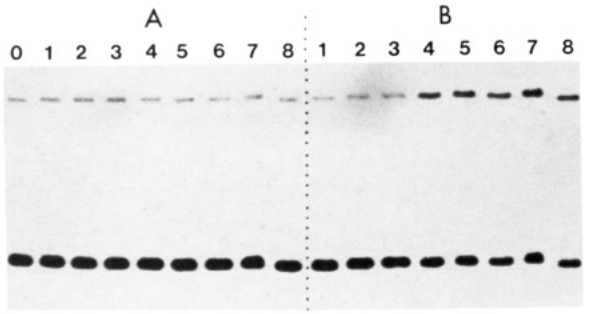

FIGURE 9: Time dependency of bizelesin adduct reversal. The bottom-strand-labeled purified bizelesin-induced DNA ISC was incubated at 37 (A) or 55 °C (B) for 2 (lane 1), 4 (lane 2), 8 (lane 3), 24 (lane 4), 48 (lane 5), 72 (lane 6), 96 (lane 7), and 120 h (lane 8) in 100 mM NaCl and 10 mM phosphate buffer (pH 8). After DNA precipitation, the conversion reaction was performed as described in Materials and Methods.

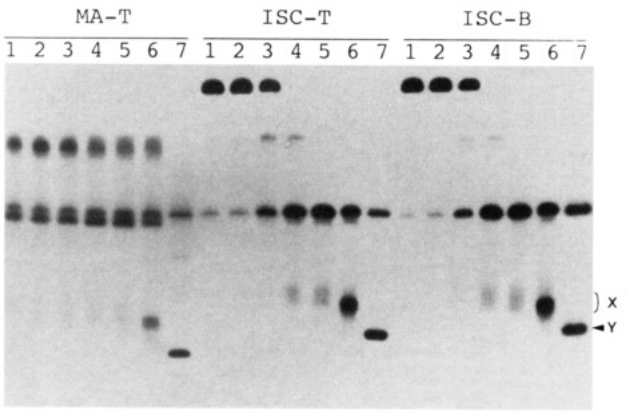

FIGURE 10: Chemical stability of purified bizelesin-induced DNA ISC and DNA MA. Top-strand-labeled DNA ISC, (ISC-T), bottom-strand-labeled DNA ISC (ISC-B), and top-strand-labeled DNA MA (MA-T) were heated in 20 μL of tracking dye containing 80% formamide and 1 mM EDTA for 10 min at 25 (lane 1), 37 (lane 2), 50 (lane 3), 60 (lane 4), 70 (lane 5), or 80 °C (lane 6). Samples were also heated at 85 °C for 30 min in 10 mM phosphate (pH 7.0), which was followed by 1 M piperidine treatment at 90 °C for 30 min (lane 7).

site had occurred.

DISCUSSION

The results described in this paper, when taken together with the previously published data regarding the reversibility of CPI-induced DNA adducts (Warpehoski et al., 1992), provide additional insight into the chemical and thermal stability of these adducts. The use of sequencing gel electrophoresis coupled with a controlled conversion reaction allowed us to characterize the frequency of the reversal of bizelesin-induced DNA adducts. Figure 11 highlights the chemical mechanism which may be responsible for the two reactions observed within, namely, depurination and adduct reversal (with adduct reversal resulting in restoration of DNA integrity). Table I summarizes the preferred conditions for either adduct reversal or the depurination reaction. The most favorable conditions for adduct reversal, particularly within purified DNA ISC, were high temperature (70–80 °C), high pH, and high ionic strength. Further, depurination within the DNA ISC was found to occur preferentially at higher temperatures (100 °C), under conditions of low pH and low ionic strength. In contrast, the purified DNA MA was susceptible to depurination even at room temperature. This suggests that in order to interpret data obtained during the study of CPI-induced DNA alkylation products, careful

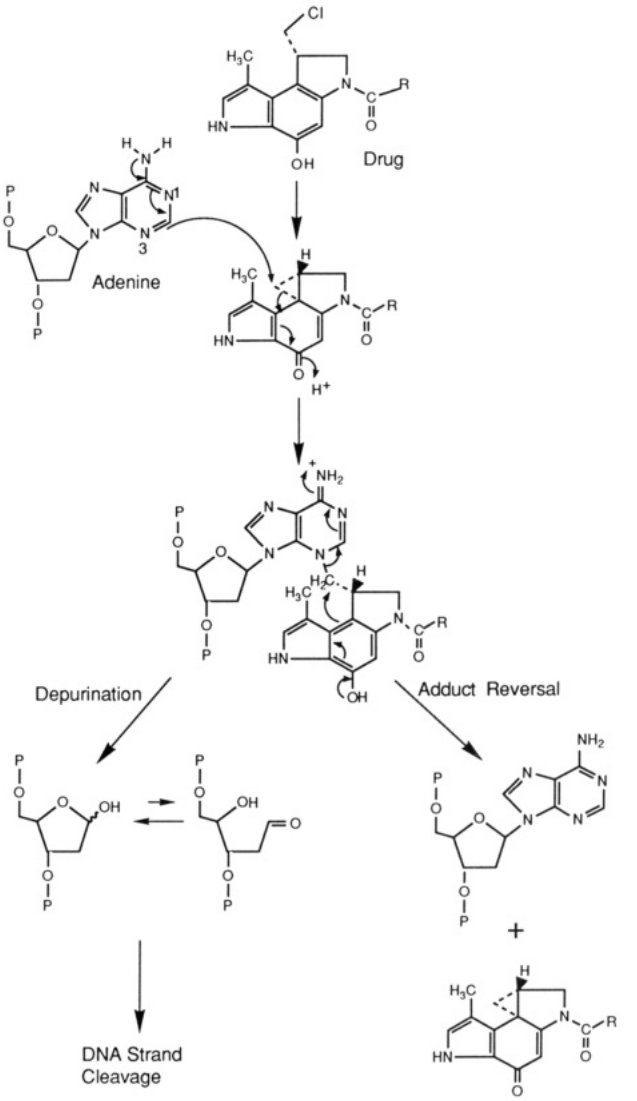

FIGURE 11: Scheme showing the chemical mechanisms involved in CPI-induced DNA alkylation, depurination, and adduct reversal at the adenine N3 position. Depurination of DNA–CPI adduct occurs very rapidly in single-stranded DNA, while adduct reversal occurs preferentially in double-stranded DNA.

Table I: Summary of Preferred Reaction Conditions for Adduct Reversal and the Depurination Reaction at the N3 Adenine Alkylation Site

	adduct reversal	depurination reaction
pH	high	low
temp (°C)	70–80	100
ionic strength	high	low
DNA	dsDNA	ssDNA

consideration must be given to the experimental conditions employed.

Warpehoski and colleagues (Warpehoski et al., 1992) have hypothesized that reversal of CPI-induced DNA adducts may occur preferentially in double-stranded DNA. The following data generated within are consistent with such a thought. First, DNA MA, when purified, exists in single-stranded form, and such adducts have been found to undergo depurination in preference to adduct reversal. Next, high ionic strength, a condition which is known to stabilize the DNA double helix, results in increased levels of adduct reversal. Moreover, the maximum yield of adduct reversal occurred at temperatures of 70–80 °C, with depurination being predominant at temperatures of 90 °C or higher. This presumably reflects

the fact that DNA melting is occurring and that the characteristics of the purified DNA ISC are more like those of single-stranded DNA than double-stranded DNA.

Reversal of adozelesin-induced DNA adducts has been proposed to occur via a retrohomologous Michael reaction (Warpehoski et al., 1992). In this study, we have shown that adduct reversal is dependent upon high pH regardless of the nature of the alkali. Indeed, the presence of a primary or secondary amine is not required for adduct reversal. This is unlike the mechanism of DNA strand cleavage associated with guanine N7 alkylations where a primary or secondary amine is required in order to displace the ring-opened base from its corresponding sugar (Mattes et al., 1986). Thus the chemistry involved in the cleavage (and adduct reversal) of adenine N3 adducts is distinct from that involved in the cleavage of guanine N7 alkylations. We have also determined the reversibility of 3-methyladenine adducts induced within DNA by dimethyl sulfate (data not shown). Dimethyl sulfate modified DNA was treated with increasing concentrations of piperidine or sodium hydroxide and then subjected to the conversion reaction. The disappearance of adenine-specific cleavage, indicative of a reversible reaction, was not observed as the concentration of piperidine or sodium hydroxide was increased. These results suggest that adduct reversal at adenine N3 sites did not occur, at least when the adduct was 3-methyladenine, and was thus specific for CPI-induced DNA damage.

The findings reported within may also help explain the inability of numerous investigators (including ourselves) to detect CPI-induced DNA ISC and DNA MA within cellular DNA at pharmacologically relevant concentrations. We have previously failed to detect, utilizing the alkaline elution technique (pH 12.1), DNA ISC when human tumor cells were exposed to bizelesin (Lee & Gibson, 1991). Further, no CC-1065 DNA adducts were detected within cellular DNA when alkaline sucrose gradient analysis was performed (pH 13.0) (Zsido et al., 1991). The failure to detect such CPI-induced DNA adducts may reflect the fact that adduct reversal is favored under alkaline conditions, especially when the alkylated cellular DNA is exposed to such pH's for as long as 15 h. Moreover, the apparent resistance of CPI-induced adenine N3 DNA adducts to strand cleavage by hot 1 M piperidine treatment (Reynolds, 1984) can now be explained by the fact that such conditions favor adduct reversal.

In conclusion, the data presented within this paper highlight the inherent complexities involved in the analysis of CPI-induced DNA adducts. This is true whether cell-free or cellular DNA is used as the target for alkylation. Our data suggests that in order to detect the presence of CPI-induced DNA ISC, conditions of neutral pH, room temperature, and low ionic strength are favored in the extraction of modified DNA.

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