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Base Specificity for DNA Interstrand Cross-Linking Induced by Anticancer Agent Bizelesin

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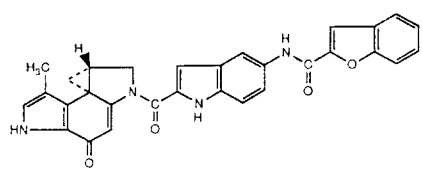
Bizelesin is a promising novel anticancer agent which is known to alkylate N3 of adenine to induce DNA interstrand cross-links (ISC) within 5'-TAATTA and 5'-TAAAAAA. We have investigated the base specificity for DNA ISC induced by bizelesin using oligomers containing the cross-linkable sequence 5'-TAATTN, in which "N" was either A, C, G, or T. An analysis of denaturing polyacrylamide gel showed that bizelesin is able to induce DNA ISC in the duplex oligomer containing sequences 5'-TAATTA and 5'-TAATTG. The formation of interstrand cross-linking did not occur in the sequences 5'-TAATTC and 5'-TAATTT. DNA strand cleavage assay to determine the cross-linking site within 5'-TAATTG sequence showed that bizelesin alkylates guanine. These results demonstrate that bizelesin is able to induce DNA ISC at guanine but not at cytosine or thymine. In addition, guanine adducts have been found to be susceptible to DNA strand cleavage by exposure to hot piperidine. The extent of DNA strand cleavage, however, was not 100% efficient in either neutral pH buffer or hot piperidine.

Key words : Anticancer drug, Bizelesin, DNA interstrand cross-links.

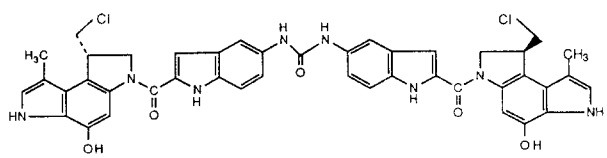
INTRODUCTION

Bizelesin (Fig. 1), an analogs of the antitumor antibiotic CC-1065, is a synthetic DNA interstrand cross-linker that consists of two DNA-reactive cyclopropylpyrroloindole moieties linked by a rigid bis (indolecarboxylic acid) linker (Mitchell *et al.*, 1991; Hanka *et al.*, 1978; Hurley *et al.*, 1984). Bizelesin displays promising antitumor efficacy both *in vitro* and *in vivo*, and it does not show delayed toxicity associated with CC-1065 (Mitchell *et al.*, 1991; Lee and Gibson, 1991; McGovren *et al.*, 1984). Bizelesin is currently being developed for clinical trials by the U.S. National Cancer Institute in conjunction with The Upjohn Co.

Like monofunctional DNA alkylating agent CC-1065, bizelesin reacts with the N3 position of adenine to induce monofunctional adducts (MA) or DNA interstrand cross-links (ISC) (Lee and Gibson, 1993a; Ding and Hurley, 1992). Using oligonucleotides of defined sequence bizelesin was found to induces two distinct types of DNA ISC. One DNA ISC spans 6-nucleotides and mimics the sequence selectivity of CC-1065-induced DNA MA, where the preferred target sequence contains two 5'-TTA sequence within 5'-TAATTA* (where asterisk indicates the site of alkylation and underline T indicates the location of adenine alkylation on the opposite strand). The second DNA ISC spans 7-nucleotides and is found to prefer intrinsically bent adenine tracts (Lee and Gibson, 1993a). Bizelesin has been found to react with guanine and cytosine in addition to adenine using oligomers containing 5'-TTTTTN, in which "N" was either A, G, C, or T (Sun and Hurley, 1993; Sun *et al.*, 1993). Recently, the intracellular alkylation sites of bizelesin have been mapped in single copy genes in



ADOZELESIN



BIZELESIN

Fig. 1. Structures of CC-1065 and bizelesin

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human carcinoma cell lines by means of ligation-mediated polymerase chain reaction (Lee *et al.*, 1994). It has been found that the alkylation pattern of bizelesin in human cellular DNA are similar to that observed in purified cell-free DNA, but non-adenine bases were not observed as alkylation sites for bizelesin.

In this study, we have determined the ability of bizelesin to form DNA ISC at nonadenine bases using 21-bp duplex oligomers containing a defined cross-linkable sequence 5'-TAATTN*, in which "N" was either A, G, C or T. The results have shown that bizelesin induces DNA alkylation only at guanine among nonadenine bases. In addition, guanine adducts were found to be less susceptible to heat-induced DNA strand cleavage than adenine adducts.

MATERIALS AND METHODS

Chemicals and reagents

Bizelesin was generously supplied by The Upjohn Company, Kalamazoo, MI, U.S.A. Drug was dissolved in dimethyl sulfoxide, and the concentration was determined using the extinction coefficient E_{340}= 35000. [γ-^{32}P]ATP was purchased from Amersham. T4 polynucleotide kinase was from Promega and Spin X centrifuge filter units (0.22 m nitrocellulose) was from Costar. A series of oligonucleotides (Fig. 2) were synthesized on an automated DNA synthesizer and fully deprotected with hydroxyl groups on both the 5'- and 3'-ends.

Purification of 5' end-labeled oligonucleotides

Each oligonucleotide was 5'-end-labeled with 7 units of T4 polynucleotide kinase and 40 μCi of [γ-^{32}P]ATP. After removal of unincorporated [γ-^{32}P]ATP by ethanol precipitation, DNA was purified by running a 20% denaturing polyacrylamide gel until the xylene cyanol marker had migrated 10 cm. After the gel was exposed to X-ray film, labeled DNA was excised, crushed, soaked, filtered through Spin X centrifuge filter unit, and precipitated with ethanol and sodium acetate.

NAME	SEQUENCES (5' to 3')
A	GGTCTGACG**TAATTA**CGTCAG
	GACTGC**A**TTAATGCAGTCCCA
G	GGTCTGACG**CAATTA**CGTCAG
	GACTGC**G**TTAATGCAGTCCCA
C	GGTCTGACG**GAATTA**CGTCAG
	GACTGC**C**TTAATGCAGTCCCA
T	GGTCTGACG**AAATTA**CGTCAG
	GACTGC**T**TTAATGCAGTCCCA

Fig. 2. Sequences of duplex oligonucleotides used in this study. Potential cross-linking sequences are shown in bold and potential cross-linking bases were underlined on the bottom strand of duplexes.

Identification of the DNA ISC formation induced by bizelesin

An equal amount of complementary strand was added to 5'-end-labeled oligonucleotide. The mixture was put in 70°C water and then cooled to room temperature to form annealed duplex in 30 μl of 60 mM NaCl, 6 mM Tris-HCl, pH 8.0. Half of a microliter of 1 mM bizelesin was added to annealed duplex and incubated at 37°C overnight. After ethanol precipitation of drug-treated DNA, sample was resuspended in 15 μl of tracking dye containing 80% formamide and 1 mM EDTA and then subjected to a 20% denaturing polyacrylamide gel electrophoresis. The DNA ISC formation was visualized by autoradiograpy and purified using the "crush and soak" procedure described above.

Determination of base involved in the DNA ISC formation

Aliquots of purified DNA ISC were heated in 40 μl of 10 mM phosphate buffer, pH 7.0, at 92°C for 20 min. A subsequent β-elimination reaction, which hydrolyze the phosphate backbone, was achieved by adding 4.4 μl of 10 M piperidine and then heating the mixture at 92°C for additional 20 min. Some of the aliquots were heated in 40 μl of 1 M piperidine at 92°C for 20 min to determine the susceptibility of DNA strand breaks to hot piperidine.

Purine-specific Maxam-Gilbert sequencing reaction

Thirty μl of 88% formic acid was added to 20 μl of purified single-stranded DNA in distilled water and the mixtures were incubated at 37°C for 20 min. The formic acid reaction was terminated with ethanol precipitation. The chemically modified DNA duplex was resuspended in 40 μl of 1 M piperidine, heated at 92°C for 30 min, and then lyophilized overnight.

Sequencing gel electrophoresis

Samples were lyophilized and resuspended in 10 μl of tracking dye containing 80% formamide, 1 mM EDTA, and xylene cyanol. After heating to 90°C for 2 min and quick cooling in an ice-water bath, an equal amounts of DNA samples were loaded onto a 20% denaturing polyacrylamide gel (mono : bis acrylamide ratio=29 : 1, 8 M urea). Electrophoresis was per-

formed at 50°C and 2800 V until the xylene cyanol had migrated 8 cm. Drug alkylation site was identified by reference to Maxam-Gilbert purine-specific chemical reactions on unmodified single-stranded DNA.

RESULTS AND DISCUSSION

Determination of the bizelesin-induced DNA ISC formation within 5'-TAATTN* sequences by a denaturing gel electrophoresis

Since the preferred sites of interstrand cross-linking by bizelesin was found to occur within 5'-TAATTA* sequence, we have synthesized four kinds of 21-bp duplex oligomers containing a defined cross-linkable sequence 5'-TAATTN*, in which "N" was either T, C, G, or A (Lee and Gibson, 1993a). A 21-bp oligomer was labeled either on the top strand or the bottom strand and duplex oligomers were reacted with bizelesin overnight. The reaction was terminated by ethanol precipitation and product mixtures were analyzed using a 20% denaturing polyacrylamide gel electrophoresis. DNA ISC are not able to denature and migrate with reduced electrophoretic mobility, thus DNA ISC can be separated from DNA MA as shown in Fig. 3.

When the top duplex strand was end-labeled (upper panel of Fig. 3), we observed the formation of DNA ISC in the oligomers containing the sequences 5'-TAATTA (lane A) and 5'-TAATTG (lane G). By contrast, the oligomers containing the sequence 5'-TAATTC (lane C) and 5'-TAATTT (lane T) did not allow the formation of DNA ISC but did allow the formation of DNA MA. To confirm this result, we have used the bottom strand labeled DNA and analyzed the DNA ISC formation. As shown in the lower panel of Fig. 3, identical results have been observed in that DNA ISC was formed in oligonucleotides containing the sequences 5'-TAATTA (lane A) and 5'-TAATTG (lane G). Thus, it can be concluded that bizelesin is

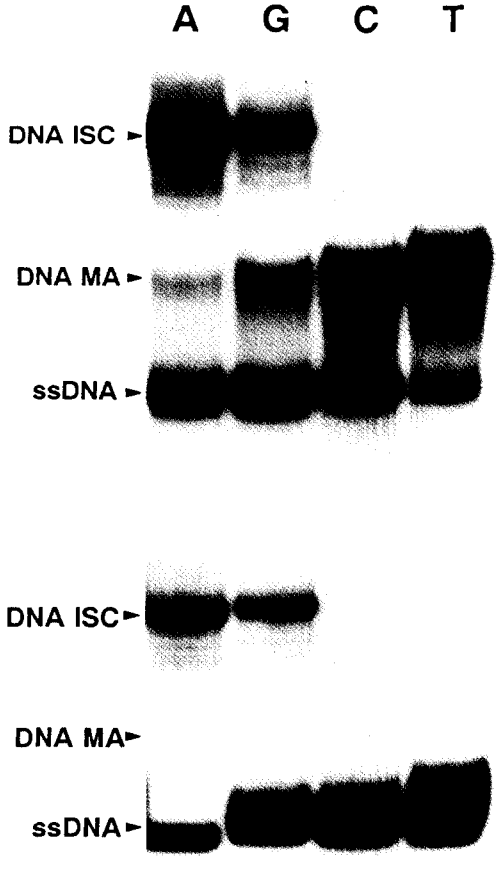

Fig. 3. Autoradiogram of a 20% denaturing acrylamide gel showing that DNA interstrand cross-links induced by bizelesin are formed in oligomers A and G. The upper panel of autoradiogram is obtained from the top strand labeled oligomers and the lower panel is obtained from the bottom strand labeled oligomers.

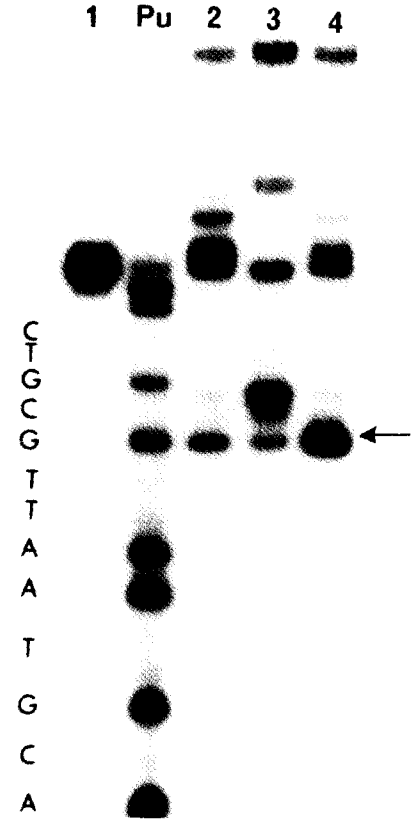

Fig. 4. Determination of cross-linking site in duplex G containing the sequence 5'-TAATTG. Gel-purified DNA ISC were heated at 92°C for 20 min in the presence of 1 M piperidine (lane 2), 10 mM phosphate buffer, pH 7.0 (lane 3) and 10 mM phosphate buffer, pH 7.0 and subsequent 1 M piperidine (lane 4) as described in materials and methods. Lane 1, control DNA; Pu, purine-specific sequencing reaction

able to induce DNA ISC within the sequence 5'-TAATTG in addition to 5'-TAATTA.

Determination of the base involved in the DNA ISC formation within 5'-TAATTG sequence

To determine whether guanine is the site of alkylation involved in the DNA ISC formation within sequence 5'-TAATTG, the bottom strand labeled DNA ISC were subjected to three different strand cleavage assays. The resulting DNA fragments products were then resolved on a 20% sequencing gel in parallel with the Maxam-Gilbert sequencing reaction (Fig. 4).

Lane 1 is the drug unmodified control DNA. Lane 2 is the heat treatment of the sample in 1 M piperidine solution, which showed the band that comigrates with the products of a guanine-specific reaction as defined by the Maxam-Gilbert sequencing reaction (see arrow). It is interesting to note that guanine adducts are susceptible to DNA strand cleavage while adenine adducts undergo a reaction which eliminates the DNA adducts and does not cause DNA strand cleavage in the presence of 1 M piperidine (Lee and Gibson, 1993b; Warpehoski *et al.*, 1992). Lane 3 is the heat treatment of the samples in neutral pH buffer, showing the band that migrates more slowly than a guanine-specific reaction in addition to the band of a guanine-specific reaction. This suggests that strand cleavage occurs to the 3'-side of the guanine cross-linking site (Maxam and Gilbert, 1980; Reynolds *et al.*, 1985). The subsequent 1 M piperidine treatment of samples shown in lane 3, which induces the second β-elimination reaction at apurinic sites, was able to convert these products into a band corresponding to the guanine-specific reaction (lane 4). These results observed in lanes 2, 3, and 4 demonstrate that bizelesin is able to alkylate guanine in the sequence 5'-TAATTG (see arrow in Fig. 4).

Although guanine alkylation has been observed in DNA treated with bifunctional agent bizelesin, the exact alkylation site of guanine is not known. The N3 position of guanine, however, might be the potential alkylation site since bizelesin is known to alkylate the N3 adenine in the minor groove of the opposite strand of DNA. This thought is supported by the fact that duocarmycin A, a structural analogue of CC-1065, alkylates the guanine N3 position (Sugiyama *et al.*, 1993; Mitchell *et al.*, 1993).

Time-dependent DNA strand cleavage at the guanine cross-linking site

Since DNA ISC was not completely converted into DNA strand cleavage (Fig. 4), we have performed

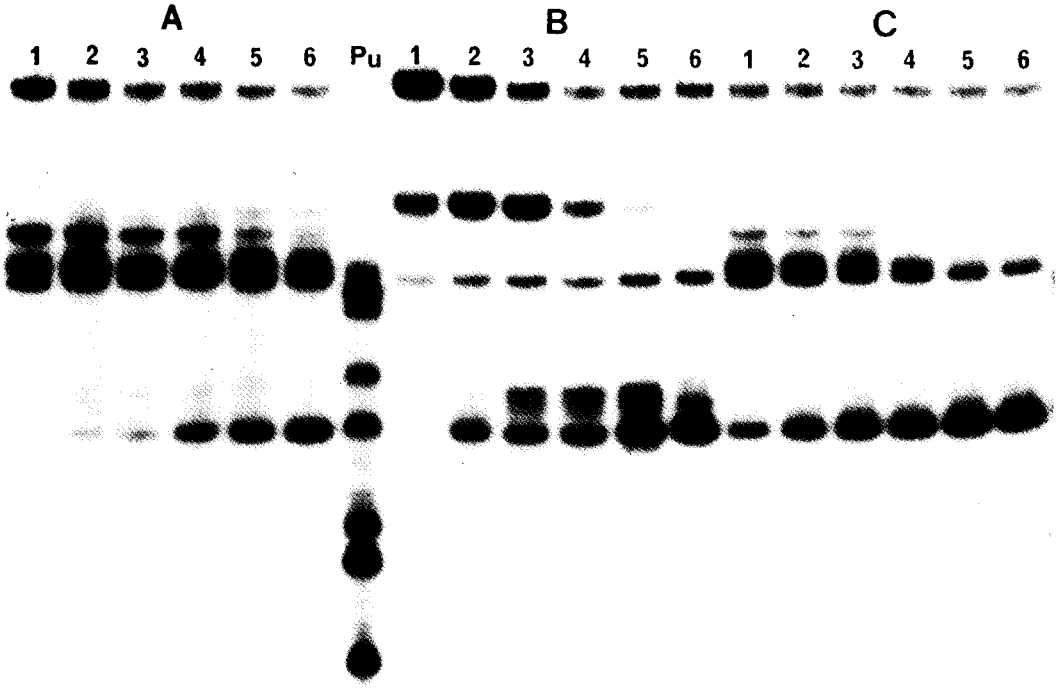

Fig. 5. Time-dependent DNA strand cleavage assay showing that DNA strand cleavage at guanine site is not complete. Gel-purified DNA ISC were treated at 92°C for 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 20 min (lane 4), 40 min (lane 5) and 60 min (lane 6) in the presence of 1 M piperidine (A), 10 mM phosphate buffer, pH 7.0 (B), Part C was obtained by heat treatment at 92°C for 20 min in the presence of 10 mM phosphate buffer, pH 7.0, and subsequent 1 M piperidine at 92°C for 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 20 min (lane 4), 40 min (lane 5) and 60 min (lane 6).

time-dependent DNA strand cleavage assay. As shown in Fig. 5, cross-linked DNA was heated at 92°C for 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 20 min (lane 4), 40 min (lane 5), and 60 min (lane 6) in the presence of 1 M piperidine (part A), or neutral pH buffer (part B). Samples which were heat treated at 92°C for 20 min and then exposed to 1 M piperidine treatment at 92°C for various times are shown in part C of Fig. 5. The results suggest that the extent of DNA strand cleavage (see arow) is directly proportional to the time of incubation. The guanine cross-linking sites, however, were not completely converted to DNA strand breaks under either reaction condition. This low susceptibility of guanine adducts to heat-induced DNA strand cleavage is in contrast to the complete DNA strand cleavage at the CC-1065 site of adenine N3 alkylation or the nitrogen mustard or aziridimylbenzoquinone sites of guanine N7 alkylation (Lee *et al.*, 1991; Mattes *et al.*, 1986).

Recent mapping of bizelesin alkylation sites in human cellular DNA did not show alkylations at guanine sites (Lee *et al.*, 1994). This might be explained by the fact that the sites of guanine alkylation were not converted into DNA strand breaks by the conditions used. It is also possible that the frequency of guanine adducts in human cellular DNA is extremely low to detect in comparison with that of adenine adducts. In conclusion, this work suggests that bizelesin can alkylate and cross-link DNA at adenine or guanine position but not at cytosine or thymine.

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REFERENCES CITED

Berardini, M. D., Souhami, R. L., Lee, C.-S., Gibson, N. W., Butler, J., and Hartley, J. A. Two structually related diaziridinylbenzoquinones preferentially cross-link DNA at different sites upon reduction with DT-diaphorase. *Biochemistry*, 32, 3306-3312 (1993).

Ding, Z.-M., and Hurley, L. H. DNA interstrand cross-linking, DNA sequence specificity, and induced conformational changes produced by a dimeric analog of (+)-CC-1065. *Anti-Cancer Drug Design*, 6, 427-452 (1992).

Hanka, L. J., Dietz, A., Gerpheide, S. A., Kuentzel, S. L., and Martin, D. G. CC-1065 (NSC-298223), a new antitumor antibiotic, production, in vitro biological activity, microbiological assay and taxonomy of the producing microorganism. *J. Antibiot.*, 31, 1211-1217 (1978).

Hurley, L. H., Reynolds, V. L., Swenson, D. H., Petzold, G. L., and Scahill, T. A. Reaction of the antitumor antibiotic CC-1065 with DNA. Structure of a adduct with DNA sequence specificity. *Science*, 226, 843-844 (1984).

Lee, C.-S., and Gibson, N. W. DNA damage and differential cytotoxicity produced in human carcinoma cells by CC-1065 analogues, U-73,975 and U-77,779. *Cancer Res.*, 51, 6586-6591 (1991).

Lee, C.-S., and Gibson, N. W. Nucleotide preference for DNA interstrand cross-linking induced by cyclopropylpyrroloindole analogue, U-77,779. *Biochemistry*, 32, 2592-2600 (1993a).

Lee, C.-S., and Gibson, N. W. DNA interstrand cross-links induced by the cyclopropylpyrroloindole antitumor agent U-77,779 are reversible upon exposure to alkali. *Biochemistry*, 32, 9108-9114 (1993b).

Lee, C.-S., Pfeifer, G. P., and Gibson, N. W. Mapping of DNA alkylation sites induced by adozelesin and bizelesin in human cells by ligation-mediated polymerase chain reaction. *Biochemistry*, 33, 6024-6030 (1994).

Lee, C.-S., Sun, D., Kizu, R., and Hurley, L. H. Determination of the structural feature of (+)-CC-1065 that are responsible for bending and winding of DNA. *Chem. Res. Toxicol.*, 4, 203-213 (1991).

Mattes, W. B., Hartley, J. A., and Kohn, K. W. DNA sequence selectivity of guanine-N7 alkylation by nitrogen mustards. *Nucleic Acids Res.*, 14, 2971-2987 (1986).

Maxam, A., and Gilbert, W. Sequencing end-labeled DNA with base-specific chemical cleavage. *Methods Enzymol.*, 65, 499-560 (1980).

McGovren, J. P., Clarke, G. L., Pratt, E. A., & DeKoning, T. F. Preliminary toxicity studies with the DNA-binding antibiotic CC-1065. *J. Antibiot.*, 37, 63-70 (1984).

Mitchell, M. A., Kelly, R. C., Winnienski, N. A., Hatzenbuhler, N. T., and Williams, M. G. Synthesis and DNA cross-linking by a rigid CPI dimer. *J. Am. Chem. Soc.*, 113, 8994-8995 (1991).

Mitchell, M. A., Weiland, K. L., Aristoff, P. A., Johnson, P. D., and Dooley, T. P. Sequence-selective guanine reactivity by duocarmycin A. *Chem. Res. Toxicol.*, 6, 421-424 (1993).

Reynolds, V. L., Molineux, I. J., Kaplan, D. H., and Hurley, L. H. Reaction of the antitumor antibiotic CC-1065 with DNA. Location of the site of thermally induced strand breakage and analysis of DNA sequence specificity. *Biochemistry*, 24, 6228-6237 (1985).

Sugiyama, H., Ohmori, K., Chan, K. L., Hosoda, M.,

Asai, A., Saito, H., and Saito, I. A novel guanine N 3 alkylation by antitumor antibiotic duocarmycin A. *Tetrahedron Lett.*, 34, 2179-2182 (1993).

Sun, D., and Hurley, L. H. Analysis of the monoalkylation and cross-linking sequence specificity of bizelesin, a bifunctional alkylation agent related to (+)-CC-1065. *J. Am. Chem. Soc.*, 115, 5925-5933 (1993).

Sun, D., Park, H-J., and Hurley, L. H. Alkylation of guanine and cytosine in DNA by bizelesin. Evidence for a covalent immobilization leading to a proximity-driven alkylation of normally unreactive bases by a (+)-CC-1065 cross-linking compound. *Chem. Res. Toxicol.*, 6, 889-894 (1993).

Warpehoski, M. A., Harper, D. E., Mitchell, M. A., and Monroe, T. J. Reversibility of the covalent reaction of CC-1065 and analogs with DNA. *Biochemistry*, 31, 2502-2508 (1992).