DNA Cross-Linking by Dehydromonocrotaline Lacks Apparent Base Sequence Preference

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Pyrrolizidine alkaloids (PAs) are ubiquitous plant toxins, many of which, upon oxidation by hepatic mixed-function oxidases, become reactive bifunctional pyrrolic electrophiles that form DNA-DNA and DNA-protein cross-links. The anti-mitotic, toxic, and carcinogenic action of PAs is thought to be caused, at least in part, by these cross-links. We wished to determine whether the activated PA pyrrole dehydromonocrotaline (DHMO) exhibits base sequence preferences when cross-linked to a set of model duplex poly A-T 14-mer oligonucleotides with varying internal and/or end 5′-d(CG), 5′-d(GC), 5′-d(TA), 5′-d(CGCG), or 5′-d(GCGC) sequences. DHMO-DNA cross-links were assessed by electrophoretic mobility shift assay (EMSA) of 32P end labeled oligonucleotides and by HPLC analysis of cross-linked DNAs enzymatically digested to their constituent deoxynucleosides. The degree of DNA cross-links depended upon the concentration of the pyrrole, but not on the base sequence of the oligonucleotide target. Likewise, HPLC chromatograms of cross-linked and digested DNAs showed no discernible sequence preference for any nucleotide. Added glutathione, tyrosine, cysteine, and aspartic acid, but not phenylalanine, threonine, serine, lysine, or methionine competed with DNA as alternate nucleophiles for cross-linking by DHMO. From these data it appears that DHMO exhibits no strong base preference when forming cross-links with DNA, and that some cellular nucleophiles can inhibit DNA cross-link formation.

Key Words: dehydromonocrotaline; DNA cross-linking; sequence specificity.

Pyrrolizidine alkaloids (PAs) are natural hepatotoxins and carcinogens found in hundreds of plant species (Coulombe, 2003). People are exposed to PAs by consumption of traditional medicines and herbal teas made from PA-containing plants, such as comfrey (Symphytum officinale). After ingestion, PAs are oxidized by cytochromes P-450 (CYP), primarily CYP 3A4, to reactive, pyrrolic bifunctional electrophiles that are potent DNA cross-linkers, a reputed event critical in PA-induced toxicity and carcinogenesis (Fig. 1). Some PAs have also been investigated as potential cancer chemotherapeutic agents (Culvenor, 1968). In previous studies we demonstrated that PAs form nearly equal proportions of both DNA interstrand and DNA-protein cross-links in vitro (Hincks et al., 1991), and that the cytotoxic, antimitotic, and megalocytic activity of PAs correlate with cross-linking (Kim et al., 1993). Structural features, most notably the presence of α, β-unsaturation and a macrocyclic diester, confer potent cross-link activity to PAs in cellular systems (Kim et al., 1993, 1999). We identified actin as the major protein involved in PA-induced DNA-protein cross-links in human breast carcinoma (MCF-7) and in Madin-Darby bovine kidney (MDBK) cells (Coulombe et al., 1999; Kim et al., 1995). Dehydrosecionine (DHSN) and dehydromonocrotaline (DHMO) have also been shown to inhibit amplification of a segment of pBR322, implying that cross-linking by activated PAs is functionally significant in the cell (Kim et al., 1999).

Activated PAs share a common pyrrolic substructure with reductively activated bifunctional mitomycins, such as mitomycin C, which preferentially cross-link 5′-CG sequences within DNA (Woo et al., 1993). There is conflicting evidence whether activated PAs share similar cross-linking base or DNA sequence specificity. Several DNA bases have been shown to be involved in covalent interactions and/or cross-links by pyrrolic PAs. For example, dehydroretronecine reacts with purine and pyrimidine nucleosides such as the N2 of deoxyguanosine (dG) and N6 of deoxyadenosine (dA). The O2′ sites of uridine and deoxythymidine or thymidine (dT) have also been identified as targets (Robertson, 1982; Wickramanayake et al., 1985). Dehydromonocrotaline and dehydroretorsine preferentially cross-linked dG to dG at a 5′-CG sequence in synthetic duplex DNA (Weidner et al., 1990). Dehydromonocrotaline was shown to cross-link at the N7 position of guanine in a 35 bp fragment of pBR322 with a preference for 5′-GG and 5′-GA sequences (Pereira et al., 1998). However, because these authors examined only alklylation at guanyl residues, no light was shed on whether DHMO alkylated at other sites.

The purpose of this study was to determine whether DHMO shows DNA base preference(s) when cross-linking a set of model 14-base oligonucleotides of differing sequences. Similar defined oligonucleotides are frequently used tools to discern possible sequence specificity of various cross-linking agents such as mitomycin C (Alley et al., 1994; Borowy-Borowski et al., 1990; Esposito et al., 1988; Huang et al., 1995; Sigurdsson et al., 1996).
Weidner et al. (1989, 1990). We present evidence herein that DHMO does not appear to have a strong base sequence preference for any single nucleotide. We also show that certain nucleophiles such as tyrosine, glutathione, cysteine, and aspartic acid inhibit DNA cross-linking by DHMO, presumably by acting as an alternate nucleophilic cross-link target.

**MATERIALS AND METHODS**

**Chemicals and reagents.** Monocrotaline, molecular sieves, GSH, nucleoside HPLC standards, amino acids (except L-tyrosine), and DNase I were all obtained from Sigma Chemical Co. (St. Louis, MO). L-tyrosine (dissodium salt) was obtained from United States Biochemical (Cleveland, OH). Sodium borohydride, HCHO, 3-mercaptopropanol, and urea were from Mallinckrodt (Paris, KY). GSH, nucleosides (except L-tyrosine), and DNase I were all obtained from Worthington Biochemical Co. (Lakewood, NJ). [14C]ATP (3000 Ci/mmol) was from ICN (Costa Mesa, CA). Other chemicals or reagents were obtained from Aldrich (Milwaukee, WI). Phosphodiesterase I and bacterial alkaline phosphatase were from Worthington Biochemical Co. (Lakewood, NJ). [32P]ATP (3000 Ci/mmol) was from ICN (Costa Mesa, CA). Other chemicals or reagents were obtained either from Aldrich (Milwaukee, WI) or Acros (Pittsburgh, PA).

**Preparation of DHMO.** Dehydromonocrotaline was prepared from monocrotaline by chemical oxidation using o-chloranil with slight modification (Kim et al., 1995). After addition of the base to the alkaloid/o-chloranil solution, the suspension was shaken vigorously for 15 s prior to phase separation. DHMO was then suspended in anhydrous DMSO, capped with N₂, and stored at –20°C until use. Pyrrole yield and purity was checked by 1H NMR (Coulombe et al., 1999).

**Thermal denaturation analysis.** Oligonucleotides (5.5 nmol as duplex) were EtOH precipitated, resuspended in 991.2 µl of 0.2 M Tris, pH 7.5, after which 10 µl of DMSO was added. Absorbance (λ = 260 nm) values were read at 5 to 70°C in 1°C increments using a model UV-2101 PC scanning spectrophotometer equipped with temperature controller TCC-260 (Shimadzu, Columbia, MD).

**Oligonucleotide cross-linking and analysis.** Duplex 14-mer oligonucleotides 5'-ATAATCGATATAT-3' ("CG"), 5'-ATATATCGATATAT-3' ("GC"), 5'-ATATATATTATAT-3' ("TA"), 5'-CGATCGCGATATCG-3' ("4-CG"), and 5'-GATCGCGATATCG-3' ("4-GC") were synthesized by Macromolecular Resources (Colorado State University, Ft. Collins, CO). Oligonucleotides were end-labeled with [γ-32P]ATP using T4 polynucleotide kinase according to manufacturer’s protocol (MBI Fermentas, Amherst, NY). Following the labeling step, the reaction solution was diluted to 100 µl in STE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl), and labeled oligonucleotides were separated from unreacted [γ-32P]ATP using Sephadex G-25 or G-50 columns (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated in STE buffer. To insure that the oligonucleotides were in duplex form before cross-linking, an annealing process was performed according to the method of Williams et al. (1997). Unlabeled (0.157 nmol as duplex) and labeled (~600–3000 dpm) oligonucleotide were combined, EtOH precipitated, and resuspended in 56.6 µl of 0.2 M Tris, pH 7.5. The corresponding complement of each strand was hybridized to itself by heating to 75°C for 15 min, cooling to room temperature over 2–2.5 h, then to 4°C for 2 h. Varying concentrations of DHMO (31.4, 94.1, or 157 nmol) and DNA to give 1:200, 1:600, and 1:1000 duplex oligo DNA:DHMO molar ratios, respectively, were incubated at 4°C for 4 h, and allowed to warm to room temperature overnight. Samples of resultant cross-linked DNA containing equal dpm (Model LS-3801, Beckman, Fullerton, CA) were separated from free DNA by 25% PAGE (acylamide:bis-acylamide 19:1) 0.7 M urea, 0.025% TEMED, 0.07% APS) with 1X TBE (pH 8.3) (Weidner et al., 1990). Gels were exposed to...
BioMax MS film and intensifying screen (Kodak, Rochester, NY) for 1–2 days, developed, then the autoradiographs were computer-archived.

**HPLC analysis of cross-linking.** Unlabeled oligos (1.875 nmol as duplex) were cross-linked with DHMO (1500 nmol) for a molar ratio of 1:800 (DNA:DHMO) in 0.2 M Tris, pH 7.5 with 1% DMSO (total volume of 676 µl) as above. Resultant complexes were then digested using a modified protocol of Borowy-Borowski et al. (1990) wherein cross-linked or native DNAs were EtOH precipitated, digested by 0.43 U phosphodiesterase I (4.3 U/ml final conc.) and 0.21 U bacterial alkaline phosphatase (2.1 U/ml final conc.) for 4.5 h at 45°C. Preliminary trials showed that addition of DNase I (0.3 U) did not increase the efficiency of DNA hydrolysis (data not shown). Enzyme was then separated from resulting free deoxynucleosides and cross-linked oligonucleotides by filtration (Ultrafree MC 30,000, Millipore, Bedford, MA). The filtrates were separated by HPLC (Model 5000 Liquid Chromatograph, Varian-Varicrom UV Detector, Varian Associates, Palo Alto, CA) with Beckman System Gold software (Fullerton, CA), and a Waters Nova-Pak C18 4µm × 3.9 × 300 mm column (Milford, MA). Each sample was eluted by an isocratic solvent program consisting of 96% triethylamine (pH 7.0 with HOAc) and 4% acetonitrile for 30 min at a flow rate of 0.9 ml/min at 24°C. Peaks were detected at 254 nm. Elution times for dC, dG, dA, and dT were approximately 3, 5.2, 6.2, and 9.8 min, respectively. Authentic deoxynucleoside standards were used to determine retention times.

**Competition of amino acids and GSH with pyrroles for DNA cross-linking.** The CG oligonucleotide was end-labeled and purified as in Oligonucleotide Cross-Linking and Analysis. Following the labeling step, the reaction solution was diluted to 100 µl in STE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl), and labeled oligonucleotides were separated from unreacted [γ-32P]ATP. DNA was then incubated with pyrroles in 1% DMSO (without Tris buffer) and ~7.4 × 105 dpm of 32P-labeled at 37°C for 1.75 h with varying amounts of Asp, Cys, GSH, Lys, Met, Phe, Ser, Thr, or Tyr. The order of reagents added in the 100 µl reaction (with H2O) was H2O, DNA, DMSO, amino acid or GSH, then DHMO. In some experiments, reaction tubes were kept at 37°C after the 1.75 h incubation, while small aliquots were counted. Each tube was briefly mixed, then aliquots containing equal dpm were electrophoretically separated. Gels were exposed to X-ray film, developed, and autoradiographs were computer-archived. Electrophoretic migration of oligonucleotides was not affected by the addition of those nucleophiles above (data not shown).

**Data analysis.** Autoradiographs were computer-archived and intensities of autoradiographic bands were computed using a Nucleovision 920 Imaging Workstation and GelExpert software (Nucleotech Corporation, San Carlos, CA). Densitometric results were analyzed by one-way analysis of variance followed by post-hoc Tukey’s t-test. A level of p < 0.05 was considered significant.

**RESULTS**

Potential solvent effects on cross-linking efficiency were evaluated in preliminary experiments using varying concentrations of DMSO. Because DMSO concentrations >1% (~2.5% tested) inhibited cross-linking of GC (data not shown), all subsequent cross-linking reactions had a DMSO concentration of 1%.

We verified that the oligonucleotides used in our cross-linking experiments were predominately in duplex form by thermal melting point analysis. Melting points (Tm) of CG, GC, and TA were 43, 44, and 37°C, respectively, while those of 4-CG and 4-GC were 40 and 38°C, respectively (data not shown).

![FIG. 2. Autoradiographs from EMSA showing DHMO-induced cross-linking of model duplex oligonucleotides termed “CG” (5’-dATATATCGATATAT-3’), “GC” (5’-dATATATGCATATAT-3’), “TA” (5’-dATATATTAATATAT-3’) (A), “4-CG” (5’-dCGATCGCGATATCG-3’) and “4-GC” (5’-dGCATGCATGCATGC-3’) (B). Experiments were conducted as described in Materials and Methods. For each oligonucleotide, four reactions with increasing DHMO to give molar ratios of 1:0, 1:200, 1:600, and 1:1000 (DNA:DHMO) are shown. MW standards showed that cross-linked complexes were greater than 18.8 kD (data not shown). At least four independent experiments were conducted, of which this is representative.](image-url)
Since cross-linking experiments were carried out at temperatures lower than these melting points, we presumed that DNAs were predominately double stranded. With the oligonucleotide targets used in this study, DHMO induced DNA cross-links with a molecular weight greater than 18.8 kD, the size of the largest standard (data not shown). Dehydromonocrotaline cross-linked all DNA targets used in this study in a dose-dependent manner. Cross-linking was clearly observed by the appearance of the high molecular weight complex coinciding with increased dose of the pyrrole. The highest dose of DHMO induced complete cross-linking, in that all of the DNA in the lane was in high molecular weight form at the top of the gel (Figs. 2A and 2B). As can be observed from autoradiographs of EMSA gels, there was no obvious sequence preference with respect to cross-link formation by DHMO (Figs. 2A and 2B). The one exception to this observation was that at a DNA:DHMO ratio of 1:600, there was a slight preference \((p = 0.048)\) in cross-linking of the GC compared to the CG oligonucleotide (Fig. 3A). The lack of obvious cross-linking sequence preference between oligonucleotide targets is especially evident when the densitometric intensities of the signals from the autoradiograms were compared (Figs. 3A and 3B).

Cross-linking was also assessed by HPLC separation of cross-linked oligonucleotides digested to the deoxynucleoside level by phosphodiesterase and alkaline phosphatase. Because of the inherent resistance of DNA cross-links to enzymatic digestion, a detectable reduction in the amount of free deoxynucleoside base is observed if that base were preferentially involved in a DNA cross-link (Warren and Hamilton, 1996). Peaks corresponding to oligonucleotides from digested native oligonucleotides (Fig. 4A) were then compared with those of the digested cross-links (Fig. 4B). These results are in general agreement with the EMSA data in that there was a reduction in all the constituent deoxynucleoside peaks with no obvious decrease occurring in any deoxynucleoside(s) as would be expected if some sequence preference for cross-linking had occurred.

We used the standard EMSA to determine which cellular nucleophiles, if any, compete with DNA for DHMO-induced DNA cross-links. Addition of compounds that act as alternate nucleophiles caused a sequential disappearance of the large molecular weight cross-link complex with a concomitant reappearance of the native DNA. A representative autoradiogram clearly shows that GSH caused a sequential disappearance of the large molecular weight cross-linked species with a concomitant reappearance of the original native oligonucleotide, while Lys did not affect the extent of DHMO-induced cross-linking (Fig. 5A). Tyrosine most effectively competed with DNA for DHMO, followed by GSH, Cys, and Asp, while Lys, Met, Phe, Ser, and Thr did not act as competitors with DNA (Fig. 5B).

**DISCUSSION**

The interactions of cross-linking agents with DNA are complex giving rise to lesions that can underlie toxicity, mutagenesis, and carcinogenesis. One of the potential variables in such interactions is the sequence of bases in available regions of the target DNA. Upon chemical or enzymatic oxidation to their respective bifunctional pyrrolic (dehydro) derivatives, pyrrolizidine alkaloids readily cross-link cellular nucleophiles such as amino acids, proteins, and DNA (Kim et al., 1995; Robertson, 1982; Woo et al., 1993; Woynarowski et al., 1999).

In total, our data indicate that DHMO did not appear to have a strong base sequence preference when cross-linked to a set of self-complimentary, duplex poly A-T 14-mer oligonucleotides with varying internal and/or end 5'-d(CG), 5'-d(GC), 5'-d(TA), 5'-d(CGCG), or 5'-d(GCGC) sequences. This apparent lack of sequence preference was observed when cross-linking was
measured by either EMSA or by HPLC of digested, cross-linked complexes. Panels of similar self-complimentary, duplex A-T based oligonucleotides with differing central base sequences have been commonly used to discern possible sequence effects of a variety of DNA cross-linking agents such as mitomycin C (Borowy-Borowski et al., 1990; Weidner et al., 1990; Woo et al., 1993), psoralen (Esposito et al., 1988), bizelesin (Thompson et al., 1995), and formaldehyde (Huang et al., 1992).

The observation of a slight preference for cross-linking of the oligonucleotide with a central GC must be interpreted with caution due to the semi-quantitative nature of densitometry, that it was not observed at dose ratios other than 1:600 (DNA:DHMO), and that it was not substantiated by HPLC analysis of digested cross-linked complex. In any event, the degree of cross-linking was affected consistently by the amount of DHMO, but not the base sequence of the oligonucleotide DNA targets.

Our results are in contrast to two studies showing some sequence preference involving dG by PA pyroles. For example, DHMO and dehydroretorsine preferentially formed dG-to-dG cross-links at 5'-d(CG) to 5'-d(GC) sequences in 17-mer synthetic duplex oligonucleotides (Weidner et al., 1990). However, those workers focused on DNAs with central 5'-d(CG) or 5'-d(GC) sequences, and did not examine possible cross-linking in duplexes comprised of only dA and dT. In another study, DHMO cross-linked 5'-d(GG) and 5'-d(GA) sequences in a 375 bp fragment of an EcoRI digest of BR322 (Pereira et al., 1998). However, because this group relied on a protocol utilizing hot piperidine that detects alkylation only at guanyl residues, cross-linking at other non-guanyl sites could not be evaluated.

The mechanistic reason for this apparent lack of base preference of DHMO is uncertain. The potent hepatotoxic, hepatocarcinogenic, anti-mitotic, and other biological activities of PAs may be related to their relatively indiscriminant pattern of DNA cross-linking. Our conclusion that DHMO-induced DNA cross-linking lacks sequence specificity must be tempered with the recognition that a limited set of model oligonucleotides were used in this study. Indeed, nearly all covalently bonding DNA-reactive compounds show some degree of DNA sequence specificity, although some have very modest base preference (Hurley, 1989). Thus, we cannot rule out the possibility that some type of DNA sequence specificity may be identified using a larger set of DNA targets or under a different experimental protocol than used in this study.

Many herbal supplements and traditional folk remedies are made from plants such as Comfrey (Symphytum officinale), Common Hound’s Tongue (Cynoglossum officinale), Heliotrope (Heliotropium arborescens), Coltsfoot (Tussilago farfara), Ragwort and Groundsel (Senecio sp.) that may contain as much as 0.3% dry weight of PAs (Roeder, 1995). Persons regularly consuming these products may be at heightened risk to cancer in addition to the other well-known adverse health effects of PAs (Huxtable, 1989). It is for this reason that Germany and Canada have banned the sale of comfrey (Coulombe, 2003).

We have shown that a substantial portion of PA-induced DNA cross-links in cells are protein-associated (Hincks et al., 1991; Kim et al., 1993, 1995) and that actin is a significant constituent...
in DNA-protein cross-links induced by PAs both in normal and transformed mammalian cells (Coulombe et al., 1999). Cellular components that can become incorporated in such DNA-protein cross-links are therefore of interest. Reduced thiols such as GSH and Cys have a strong affinity for pyrroles when added to in vitro crosslink reactions with HindIII-digested λ-phage DNA (Coulombe et al., 1999). The present data confirms these findings in that Tyr, GSH, and Cys dramatically reduced DNA cross-linking induced by DHMO. Lin et al. (1992) similarly found that Cys and Tyr (in addition to Thr and Ser) were more frequently involved in chromate-induced DNA-protein cross-links in Chinese hamster ovary cells compared to other amino acids. Another possible significance of this finding is that these molecules may act as protective scavengers of reactive pyrroles in the cell. Indeed, animals given dietary supplements of Cys showed reduced symptoms of PA-induced hepatotoxicity compared to control animals (Miranda et al., 1982). Alternatively, proteins especially rich in Tyr, Cys, and Asp may act as alternate cellular targets for pyrrolic PAs, although at least one form of human actin is not especially enriched in Tyr (Harata et al., 1999).

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