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DNA-Protein Cross-Linking by Pyrrolizidine Alkaloids

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DNA-PROTEIN CROSS-LINKING BY PYRROLIZIDINE ALKALOIDS

by

Gail L. Drew

A thesis submitted in partial fulfillment
of the requirement for the degree

of

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in

Toxicology

Approved:

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1997

ABSTRACT

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Major Professor: Dr. Roger A. Coulombe, Jr.
Program: Toxicology

Pyrrolizidine alkaloids (PAs) are natural plant compounds found in hundreds of plant species worldwide and are reported to have cytotoxic, carcinogenic, antimitotic, and genotoxic activity. PAs are metabolized by the cytochrome P450 (CYP) system to the pyrrole or the N-oxide form. The pyrroles are bifunctional electrophilic alkylators that bind cellular nucleophiles such as DNA and proteins and disrupt normal cell processes, including DNA replication and gene transcription, and can cause megalocytosis. The pyrroles dehydrosenecionine (DHSN) and dehydromoncrotaline (DHMO) are among the most potent PA cross-linkers and inducers of megalocytosis. DHSN and DHMO-induced cross-links in cultured normal (MDBK) and neoplastic (MCF7) cells were analyzed by SDS-PAGE and Western blot and both were found to contain the protein actin. Actin is crucial to DNA replication and is known to be involved in cross-links induced by *cis*-dichlorodiammine platinum II (cisplatin), a well known cross-linking drug used for the treatment of cancer. Actin cross-linking may explain the antimitotic, megalocytotic, and anticarcinogenic effects of PAs.

Since protein cross-linking is an important mode of actionfor PAs, we were interested in what characteristics of the protein might make it a good nucleophilic target.

Thus, further research was undertaken based on the hypothesis that cysteine residues, and specifically free sulfhydryl groups, are attractive targets for the bifunctional electrophilic alkylators DHSN and DHMO. Nucleophiles were selected for their abundance in the cell, their cysteine content, and their relationship to the documented side effects of PAs. Actin, glutathione (GSH), metallothionein, topoisomerase II, and cysteine were all found to cross-link with DHSN and DHMO *in vitro* while methionine, with no free sulfhydryl groups, did not cross-link.

Our results support the hypothesis that cysteine residues are a key characteristic of proteins that are cross-linked by PAs. The cross-links could have negative effects to the cell as in the case of binding actin or topoisomerase II to alter normal DNA processes and replication, or beneficial effects such as binding to electrophilic scavengers like GSH or metallothionine as a detoxifying mechanism. The nucleophiles we tested *in vitro* and found to form cross-links with DHSN and DHMO may help to explain the antimitotic, carcinogenic, and anticarcinogenic effects of PAs.

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Gail Leslie Drew

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ABBREVIATIONS

CNS	Central nervous system
CP	*cis*-dichlorodiammine platinum II
CYP	cytochrome proteins
DHMO	Dehydromonocrotaline
DHSN	Dehydrosenecionine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
GSH	L-γ-glutamyl-L-cysteinylglycine; glutathione
HBS	Hanks Balanced Salts
MCF7	Human breast carcinoma
MDBK	Madin Darby bovine kidney
MEM	Minimum Essential Medium Eagles
MMC	mitomycin C
NMR	Nuclear magnetic resonance
PA	Pyrrolizidine alkaloid
PBS	Phosphate balanced salts
PMSF	Phenylmethyl-sulfonyl flouride
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

CHAPTER I

LITERATURE REVIEW

PYRROLIZIDINE ALKALOIDS: OCCURRENCE AND TOXICITY

Pyrrolizidine alkaloids (PAs) are natural plant compounds found worldwide in an estimated 6,000 plant species largely in the genera *Senecio, Crotalaria, Heliotropium,* and *Symphytum*. Some commonly known plants containing PAs include Tansy ragwort, Borage, Common groundsel, Dusty Miller, and Hounds-tongue (Cheeke 1989, Knight *et al.* 1984, Smith and Culvenor 1981). These alkaloids are toxic, carcinogenic, antimitotic, and anticarcinogenic (Culvenor 1968, King *et al.* 1987, Kuhara *et al.* 1980, Mattocks 1986). Animals are exposed to PAs through grazing, and humans are exposed through ingestion of herbal remedies or contaminated grain, milk, or honey (Smith and Culvenor 1981, Stillman *et al.* 1977). PAs cause liver damage, including veno-occlusive disease in humans, and megalocytosis, cirrhosis, and hepatoma in both humans and animals (McLean 1970). In addition, PAs cause CNS disorders and gastrointestinal lesions in horses and pulmonary hypertension in horses and rats (Culvenor 1983, Mattocks and Legg 1980, McLean 1970). PAs cause significant losses to the livestock industry as well as many human deaths. PAs are also anticarcinogenic due to their ability to cross-link DNA and impede mitosis in rapidly dividing tumor cells, and some PAs have been examined as anti-tumor agents through clinical trials (Hincks *et al.* 1991, King *et al.* 1987, Kim 1994, Letendre *et al.* 1981).

Human illness and death due to PAs have been due largely to ingestion of traditional herbal remedies or folk medicines, including comfrey tea, Russian comfrey, coltsfoot, chaparral, Betony, and *Gordoloba Yerba* (Culvenor 1983, Huxtable 1980, Mattocks 1986, Smith and Culvenor 1981). In many cases the remedies were given to a child or infant or taken by a pregnant mother. In the United States, several infant deaths

have been attributed to *Gordolobo Yerba*, a PA-containing herbal remedy often used by Mexican-Americans to treat coughs and colds (Bergeson 1993, Stillman *et al.* 1977). In one case an infant was born with veno-occlusive disease because the mother took *Tussilago farfara* (Coltsfoot) tea, which contained the PA senecionine, throughout her pregnancy (Roulet *et al.* 1988). An 18-month-old child, who had been given an herbal tea mixture containing *Alpendost* (*Adenostyles alliariae*) that contains the PA seneciphylline since 3 months of age, was diagnosed with portal hypertension with severe ascites (Sperl *et al.* 1995). Symptoms of acute PA poisoning include nausea, emesis, abdominal pain, and distention, and finally veno-occlusive disease (Stillman *et al.* 1977).

PA-containing foods have also been responsible for widespread poisonings of people in Russia, Afghanistan, South Africa, Jamaica, and the West Indies (Cheeke 1989, Mattocks 1986, McLean 1970). Grains and cereals that have been contaminated with PA-containing seeds are important routes of exposure in these countries. Cows that forage and bees that pollinate PA-containing plants produce contaminated milk or honey (Smith and Culvenor 1981). PA exposure through foods and herbals is a risk factor for liver disease and deaths, especially in developing nations where foods may be contaminated and in areas where unregulated herbal remedies substitute for medical treatments.

CHEMICAL STRUCTURE AND METABOLISM OF PAS

Pyrrolizidine alkaloids have been classified into four structural groups: α, β unsaturated macrocyclic diesters (senecionine, seneciphylline, riddelline, retrorsine); saturated macrocyclic diesters (monocrotaline); open diesters (heliosupine, latifoline); and necine bases (retronicine). Structures of representative PAs and their metabolites are shown in Figure I-1. Two structural components seem to be crucial to biological toxicity: the α,β unsaturation of the necic acid ester and C1-C2 unsaturation of the necine base (Hincks *et al.* 1991, Kim *et al.* 1995, 1996, Mattocks 1986).

Pyrrolizidine alkaloids themselves are not toxic *per se* but are metabolized by cytochrome P450 (CYP) mixed-function oxidases to highly toxic pyrroles or less toxic N-oxides. Significant species differences exist in the pattern of metabolite formation. For example, resistant species such as sheep, goats, rabbits, and guinea pigs produce mainly the less toxic N-oxide, while susceptible species such as horses, cows, rats, mice, and hamsters produce more of the pyrrole (White *et al.* 1973). Chemical conversion to the pyrrole occurs by allylic oxidation followed by CYP-mediated dehydration (Fig. I-2) (Mattocks 1968, Mattocks and White 1971). Once the pyrrole is formed, this reactive species can alkylate at the C7 or C9 position, cellular nucleophiles such as DNA or proteins. Thus, pyrroles are bifunctional electrophilic alkylators that can covalently bind essential cellular nucleophiles and lead to alterations in cellular function (Mattocks 1968, Robertson 1982).

PA INTERACTION WITH CELLULAR NUCLEOPHILES

The biological activity of PAs seems to be related to the ability of PAs to cross-link DNA or cellular proteins, which can lead to clinical toxicity as well as the observed antimitotic effect. Cross-links may be DNA-DNA or DNA-protein associated (Hincks *et al.* 1991, Kim *et al.* 1993, Petry *et al.* 1986, Reed *et al.* 1988). Dehydromonocrotaline has been shown to alkylate mostly at its C9 position directly to a nitrogen atom on the nucleoside or nucleotide (Niwa *et al.* 1991). Others have shown pyrroles to most commonly alkylate the N^2 of deoxyguanosine residues at the 5′d(CG) sequence (Robertson 1982, Woo *et al.* 1992). Macrocyclic diester PAs with α, β unsaturation (senecionine, seneciphylline, ridelline, retrorsine) appear to be the most potent cross-linkers as well as antimitotics, inducers of megalocytosis and inhibitors of cell growth in mammalian cell cultures (Hincks *et al.* 1991, Kim *et al.* 1993, Mattocks 1968, Mattocks and Legg 1980). Kim *et al.* (1993) found about 50% of the cross-links induced by PAs in cultured cells to be

protein associated. Those authors also found that the electrophoretic pattern of the PA-induced DNA-protein complexes in these cells were similar to those formed by other bifunctional alkylating agents such as the anticancer chemotherapeutics mitomycin C and *cis*-dichlorodiammine platinum (II) (cisplatin). Bulky DNA-protein cross-links are significant to the cell because they may be ineffectively repaired during DNA replication and cause deletion errors or mutations (Cosma *et al.* 1988, Oleinick *et al.* 1987). Cell processes may be altered by agents that form DNA-DNA or DNA-protein cross-links because proteins bound in the cross-link may be essential to cell function or replication.

Kim *et al.* (1995) found that the proteins associated with PA-induced cross-links in cultured bovine kidney epithelial cells are acidic in nature and in the molecular weight range of 40-60 kD. These authors postulated that actin may be one of the proteins involved in the cross-linking because actin (45 kD) is a cellular target for DNA-protein cross-links by similar compounds, although no conclusive data was shown. Actin is a nuclear, non-histone protein but not normally bound to DNA and is important to cytokinesis and transcription (Rungger *et al.* 1979, Scheer *et al.* 1984). Actin is crucial to the final step of mitosis by enabling the mitotic spindle to contract in the center and separate the newly replicated cell (Voet and Voet 1990). Megalocytosis, or large cells which have replicated but are unable to divide, is a typical symptom of PA exposure (Fig. I-3). Miller *et al.* (1991) found actin to be the major protein in nearly 20% of the DNA-protein cross-links induced by cisplatin or potassium chromate in Chinese hamster ovary cells. Actin-DNA cross-linking may help explain the antimitotic and anticarcinogenic side effects of PAs. One focus of this research is to determine if actin is involved in PA-induced cross-links in normal and cancer cells.

Integration of amino acids and/or proteins into DNA-protein complexes is a mode of action of some carcinogens. For example, trivalent chromium integrates residual peptides and amino acids normally associated with DNA into DNA-protein cross-links

formed in human oesteosarcoma cells *in vitro* (Salnikow *et al.* 1992). Histidine, cysteine, and tyrosine were the predominant amino acids involved in the DNA cross-links formed by trivalent chromium and proteins or peptides with a high proportion of these amino acids such as BSA and actin which were readily complexed into cross-links (Salnikow *et al.* 1992).

Glutathione (GSH; L-γ-glutamyl-L-cysteinylglycine) is a tripeptide which in liver is the most predominant nonprotein intracellular thiol (Meister and Anderson, 1983). GSH contains nucleophilic, free sulfhydryl groups, which efficiently react with the bifunctional electrophilic PAs. Reed *et al.* (1992) found that adding GSH to a mixture of rat liver microsomes and the PA senecionine yielded the dehydrosenecionine-GSH conjugate. These workers concluded that GSH conjugation was a possible detoxification pathway for pyrrolic PAs, formed by the microsomal conversion of senecionine to the highly reactive dehydrosenecionine. Other researchers have found that dietary cysteine protects against PA toxicity by increasing hepatic GSH concentrations (Kim and Jones 1982, White 1976). Nigra and Huxtable (1992) found an association between hepatic GSH concentrations and released dehydromonocrotaline in perfused rat livers and concluded that GSH was involved in forming adducts with the PA metabolite. Researchers have also found that dehydro-monocrotaline reacts with cysteine and GSH at both acidic and physiological pH *in vitro* (Robertson *et al.* 1977). Borges and Wetterhahn (1989) found that chromium-induced cross-linking of glutathione or cysteine to DNA to form DNA-protein complexes *in vitro*. The study described in this thesis will explore the role of several candidate nucleophiles, containing various proportions of cysteine or thiol groups, amino acids, and tripeptides such as GSH as possible targets for PA-induced DNA-protein cross-links *in vitro*.

PAS AND CANCER

Pyrrolizidine alkaloids have been examined as possible antitumor agents in clinical phase I and phase II trials but hepatoxicity has limited their usefulness (King *et al.* 1987, Letendre *et al.* 1981, Poster *et al.* 1981). The antimitotic and antitumor properties of PAs have been linked to their DNA cross-link activity (Hincks *et al.* 1991). Well-known antitumor agents such as cisplatin and mitomycin C form DNA-protein associated cross-links and the proteins from these cross-links have a similar electrophoretic migration pattern to that seen from PA-exposed cells (Kim 1994). Mitomycin C has two alkylating sites and has a molecular geometry similar to that of macrocyclic PAs (Hopkins *et al.* 1991, Woo *et al.* 1992). Since PAs have similarities to anticancer cross-linking agents such as cisplatin and mitomycin C, a more direct investigation of the nature of PA-induced protein cross-links in actual cancer cells would be important to discerning the mechanism of action of these compounds as well as their potential development as anticarcinogens. Indeed, there is an acute need for more anticancer agents. Cisplatin, commonly used to treat ovarian, testicular, head and neck, bladder, and small cell lung carcinomas, is often ineffective in the long term due to acquired cell resistance (Zhen *et al.* 1992). Resistant human ovarian cancer cells have an increased ability to repair the interstrand DNA crosslink induced by cisplatin (Zhen *et al.* 1992). Other potential causes for resistance include elevated levels of intracellular GSH and metallothionein (Kelly *et al.* 1988, Hamilton *et al.* 1985).

Metallothionein is a small protein located in the cytoplasm and in the nucleus and is thought to be the major storage protein for copper as well as a carrier for zinc and cadmium. It also may play a role as an antioxidant since it contains reduced thiolate metal bonds (Da Costa Ferreira *et al.* 1993). Overexpression of metallothionein confers resistance to anticancer drugs *in vitro* (Kelly *et al.* 1988). Metallothionein is a likely target for cross-linking PAs because it contains 28% cysteine residues by weight (Nordberg *et al.* 1972). There is a clear need for more effective cancer chemotherapeutics. Previous work

in Dr. Roger Coulombe's laboratory at Utah State University has shown that the α, β unsaturated macrocyclic diester PAs are strong antimitotics yet relatively moderate cytotoxins (Hincks *et al.* 1991). Some PAs may prove to be attractive antitumor agents.

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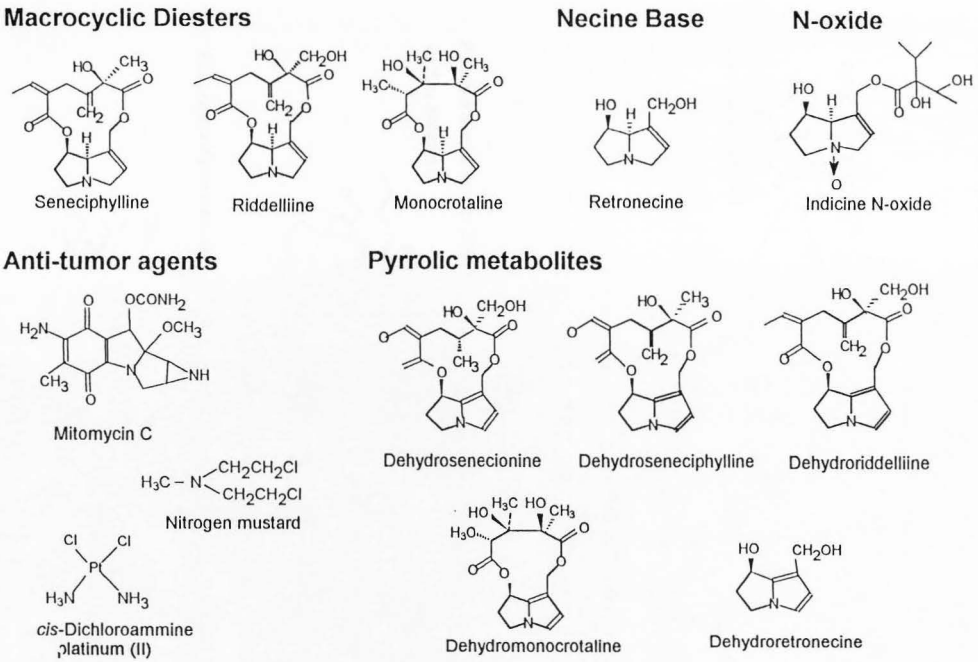
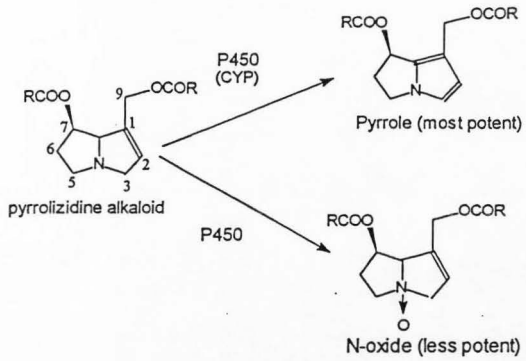


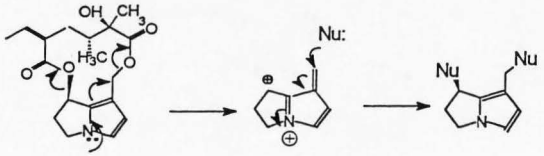
FIG. I-1. Chemical structures of some pyrrolizidine alkaloids and other known DNA cross-linkers.

Metabolism of pyrrolizidine alkaloids



Possible mechanism of DNA cross-link formation by PAs

via Sn_1 Solvolysis:



For unsaturated esters, alkylation may also occur via Michael Addition:

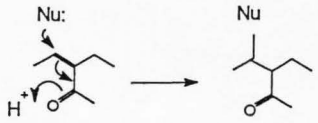


FIG. I-2. Metabolic pathways of pyrrolizidine alkaloids.

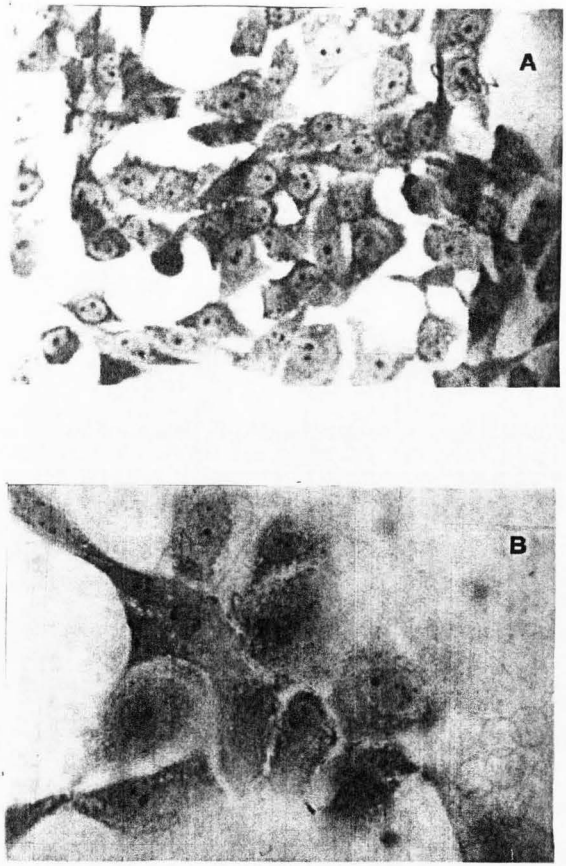


FIG. I-3. Phase-contrast photomicrographs of normal and pyrrolizidine alkaloid-treated MDBK cells showing megalocyte formation. Untreated control cells are shown in A and cells exposed to 500 µm dehydrosenecionine (DHSN) are shown in B. X 400.

CHAPTER II

DNA-PROTEIN CROSS-LINKING INDUCED BY DEHYDRO-MONOCROTALINE AND DEHYDROSENECIONINE IN NORMAL (MDBK) AND NEOPLASTIC (MCF7) CELLS

ABSTRACT

Pyrrolizidine alkaloids (PAs), which are natural compounds found in plants worldwide, are reported to be cytotoxic, carcinogenic, antimitotic, and genotoxic *in vivo* and *in vitro*. PAs are bifunctional electrophilic alkylators that bind cellular nucleophiles including DNA and proteins, and disrupt normal cell processes. Documented effects on cells treated with PAs is disruption of cell replication and megalocytosis. The macrocyclic PA metabolites, dehydrosenecionine (DHSN) and dehydromonocrotaline (DHMO), are among the most potent cross-linkers and inducers of megalocytosis. DHMO and DHSN-induced cross-links in normal (MDBK) and neoplastic (MCF7) cells were analyzed by SDS PAGE and Western blot and both were found to contain the protein actin. In addition, mitomycin C, a DNA cross-linker used as a control in this experiment, also contained actin in the cross-links induced in both cell types. To our knowledge, actin cross-linking by mitomycin C has not been previoulsly documented. Researchers have shown other anticarcinogenic cross-linking drugs such as cisplatin to incorporate actin into nearly 20% of their cross-links. Actin cross-linking may explain the antimitotic, megalocytotic, and anticarcinogenic effects of PAs because actin is crucial to DNA replication.

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are natural plant compounds found worldwide in an estimated 6,000 plant species largely in the genera *Senecio, Crotalaria, Heliotropium,* and *Symphytum*. PAs are toxic, carcinogenic, antimitotic, and anticarcinogenic (Culvenor

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are natural plant compounds found worldwide in an estimated 6,000 plant species largely in the genera *Senecio, Crotalaria, Heliotropium,* and *Symphytum*. PAs are toxic, carcinogenic, antimitotic, and anticarcinogenic (Culvenor 1968, King *et al.* 1987, Kuhara *et al.* 1980, Mattocks 1986) and cause liver damage, including veno-occlusive disease in humans, and megalocytosis, cirrhosis, and hepatoma in both humans and animals (McLean 1970). In addition, PAs cause CNS disorders and gastrointestinal lesions in horses and pulmonary hypertension in horses and rats (Culvenor 1983, Mattocks and Legg 1980, McLean 1970). PAs are also anticarcinogenic due to their ability to cross-link DNA and impede mitosis in rapidly dividing tumor cells, and some PAs have been examined in clinical trials as anti-tumor agents (Hincks *et al.* 1991, King *et al.* 1987, Kim 1994, Letendre *et al.* 1981).

Pyrrolizidine alkaloids have been classified into four structural groups: α, β unsaturated macrocyclic diesters (senecionine, seneciphylline, riddelline, retrorsine); saturated macrocyclic diesters (monocrotaline); open diesters (heliosupine, latifoline); and necine base (retronicine) (see Fig. I-1). Two structural components seem to be crucial to biological toxicity: the α,β unsaturation of the necic acid ester and C1-C2 unsaturation of the necine base (Hincks *et al.* 1991, Kim *et al.* 1995; Mattocks 1986). Pyrrolizidine alkaloids themselves are not toxic *per se* but are metabolized by cytochrome P450 (CYP) mixed-function oxidases to highly toxic pyrroles or less toxic N-oxides. Chemical conversion to the pyrrole occurs by allylic oxidation followed by CYP-mediated dehydration (see Fig. I-2) (Mattock 1968, Mattocks and White 1971). Once the pyrrole is formed, this reactive species can alkylate at the C7 or C9 position, cellular nucleophiles such as DNA or proteins. Thus, pyrroles are bifunctional electrophyllic alkylators that can covalently bind essential cellular nucleophiles and lead to alterations in cellular function (Mattocks 1968, Robertson 1982). Macrocyclic diester PAs with α, β unsaturation,

Pyrrolizidine alkaloids have been examined as possible antitumor agents in clinical phase I and phase II trials but hepatoxicity has limited their usefulness (King *et al.* 1987, Letendre *et al.* 1981, Poster *et al.* 1981). The antimitotic and antitumor properties of PAs have been linked to their DNA cross-link activity (Hincks *et al.* 1991). Well-known antitumor agents such as cisplatin and mitomycin C form DNA-protein associated cross-links, the proteins from which have a similar electrophoretic migration pattern to that seen from PA-exposed cells (Kim 1994). Mitomycin C has two alkylating sites positioned at nearly the same distance apart as that of the two alkylating sites of macrocyclic PAs (Hopkins *et al.* 1991, Woo *et al.* 1992). Since PAs have similarities to anticancer cross-linking agents such as cisplatin and mitomycin C, a more direct investigation of the nature of PA-induced protein cross-links in actual cancer cells would be important to discerning the mechanism of action of these compounds as well as their potential development as anticarcinogens.

Pyrrolic PAs cross-link cellular proteins. For example, Kim *et al.* (1993) found approximately 50% of the cross-links induced by PAs in cultured cells are protein associated. Bulky DNA-protein cross-links are significant to the cell because they may be ineffectively repaired during DNA replication and cause deletion errors or mutations (Cosma *et al.* 1988, Oleinick *et al.* 1987). Kim *et al.* (1995) found that the proteins associated with PA-induced cross-links in cultured bovine kidney epithelial cells to be acidic in nature and in the molecular weight range of 40-60 kD. These authors postulated that actin (45 kD) may be one of the proteins involved in the cross-linking because this protein is a cellular target for DNA-protein cross-links by similar compounds. Actin is a nuclear, nonhistone protein but not normally bound to DNA and is important to cytokinesis and transcription (Rungger *et al.* 1984, Scheer *et al.* 1984). Actin is crucial to the final step of mitosis by enabling the mitotic spindle to contract in the center and separate the newly replicated cell (Voet and Voet 1990). Megalocytosis, or large cells which have replicated

but are unable to divide, are typical symptoms of PA exposure (see Fig. I-3). Miller *et al.* (1991) found actin to be the major protein in nearly 20% of the DNA-protein cross-links induced by cisplatin or potassium chromate in Chinese hamster ovary cells. Actin-DNA cross-linking may help explain the antimitotic and anticarcinogenic side effects of PAs. The purpose of this study is to determine if actin is involved in PA-induced cross-links in normal and cancer cells.

MATERIALS AND METHODS

Chemicals and Reagents

Human breast cancer cells (MCF7) and Madine Darby bovine kidney epithelial cells (MDBK) were purchased from the American Type Culture Collection (Rockville, MD). Senecionine was kindly provided by Russell J. Molyneux, U.S.D.A.-A.R.S., Albany, CA. Monocrotaline, minimum essential medium eagle (MEM), Hanks balanced salts (HBS), sodium pyruvate, anhydrous dimethylsulfoxide (DMSO), Tween 20, cisplatin, mitomycin C, α-skeletal actin, monoclonal anti-actin reactive against all actin isoforms (mouse ascites fluid clone AC-40), and normal goat serum were purchased from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum was obtained from Summit Biotechnology (Ft. Collins, CO). Sodium dithionite ($N_2S_2O_4$) was purchased from Eastman Kodak Company (Rochester, NY). DNAse I and SDS-PAGE molecular weight markers were purchased from Promega Corporation (Madison, WI). Laemmli sample buffer, secondary antibody (blotting grade affinity purified goat anti-mouse IgG horseradish peroxidase conjugate) and the silver staining kit were purchased from Bio-Rad (Hercules, CA). The chemiluminescent detection kit (ECL) was obtained from Amersham (Arlington Heights, IL), the nitrocellulose membrane (Nitrobind) was purchased from Micron Separations Inc. (Westborough, MA) and the reflection autoradiograph film from Dupont (Boston, MA).

Cell Culture and Treatment

Cells were grown in MEM containing 1 mM sodium pyruvate and 10 % iron-supplemented fetal bovine serum in an atmosphere of 95% air and 5% CO_2 at 37°C. Confluent MCF7 cells from passages 154-156 and MDBK cells from passages 122-130 were rinsed in PBS, removed by scraping, and counted (Model FN counter, Coulter Electronics, Hialeah FL). Equal numbers of cells were used for each treatment; for MCF7 cells, 2.2 x 10^7 ; and for MDBK cells, 6 x 10^7 were used. Viability of the cells was checked by trypan blue exclusion.

Isolation of Nuclei

Nuclei were isolated by a modification of the method of Ausubel *et al.* (1996). Briefly, cells were harvested, rinsed in PBS, then aliquoted into treatment tubes, centrifuged (1850 x g, 10 min, 4°C), and the pellet was resuspended in ice-cold hypotonic buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethyl-sulfonyl flouride, 0.5 mM dithiothreitol). This suspension was again centrifuged (1850 x g, 5 min, 4°C) and the pellet was resuspended in hypotonic buffer and allowed to swell for 10 min on ice and then homogenized by 10 strokes on a Dounce homogenizer and centrifuged (2700 x g, 18 min, 4°C) to yield isolated nuclei for treatment.

Preparation of Pyrrolic PAs

Pyrroles (DHMO and DHSN) were prepared from their parent compounds by the method of Mattocks *et al.* (1989) Briefly, 100 mg of the parent compound in 15 ml of anhydrous DMSO was mixed with 125 mg of tetrachloro-1,2-benzoquinone in 15 ml anhydrous DMSO in a separatory funnel. Two ml of a base solution (1.4 g KOH, 40 mg $NaBH_4$ in 2 ml cold H_2O) was added and the mixture was shaken vigorously for 20 sec. The organic phase was filtered through activated charcoal through a single no. 595 filter paper (Schleicher and Schuell Inc., Keene, NH) into a beaker containing 0.5 g anhydrous

Na_2SO_4. The solution was swirled for 1 min, filtered again to remove the Na_2SO_4, and then evaporated under a stream of N_2 gas. The resulting pyrrole was resuspended in anhydrous DMSO, capped with N_2, and stored in the dark at -80°C. Pyrrole purity was checked by ^1H NMR.

Treatment of Nuclei and Isolation of DNA-Protein Cross-Links

Nuclei were resuspended in HBS and treated with either DMSO, 1 mM DHMO, 1 mM DHSN, 4 mM cisplatin, or 400 μM mitomycin C. Chemicals were dissolved in DMSO and added to cultures at no more than 1% of the incubation volume. DMSO, DHMO, DHSN, or cisplatin was added directly to the nuclear suspension and then shaken gently for 4 hr at 37° C. Mitomycin C was added to the nuclei suspension and then reductively activated by a modification of the method of McGuinness *et al.* (1991). Briefly, 40 μl of a fresh, ice-cold, 25 mM solution of $N_2S_2O_4$ in HBS was added to the treatment in five increments every 5 min (5 x 8 μl) and kept under a stream of N_2 for 30 min on ice. The nuclei were incubated with activated mitomycin C for 3.5 hr on a shaker at 37°C.

All cross-linking reactions were stopped by centrifugation (1850 x g, 5 min, 4°C), then rinsing the pellet twice in a solution of ice-cold PBS with 0.1 mM PMSF. Cross-linked DNA was separated from non-cross-linked DNA by a modification of the method of Banjar *et al.* (1984). Briefly, the treated nuclei were resuspended in 5 ml of lysing buffer (2% SDS, 50 mM Tris-HCl, 0.1 mM PMSF, pH 8.0) then mixed on a rotary stirrer for 7 hr at room temperature and centrifuged at 100,000 x g for 17 hr at 22° C. The lysis and centrifugation steps were repeated, and the resulting DNA pellet was resuspended and homogenized (Dounce homogenizer) in 2 mM Tris-HCL with 0.1 mM PMSF, pH 7.0.

The cross-linked samples were sonicated 4 times at 20% power (Fisher Sonic Dismembrator, Model 300, Fisher Scientific, St. Louis, MO) for 20 sec each at 0°C to

release proteins cross-linked to DNA. The amount of DNA was estimated spectrophotometrically (λ=260), then the samples were digested with 40 µg DNAse I /mg DNA for 1 hr at 37° C and then dialyzed (Spectra Por, Spectrum Medical Industries Inc., Houston TX) overnight at 4° C against sterile double distilled water. DNA and protein concentrations in each sample were estimated again spectrophotometrically. The samples were aliquoted into tubes, freeze dried, and stored at -80°C. Proteins that were cross-linked to 40 µg of DNA were electrophoresed on an 11% running and 4.5% stacking SDS-PAGE gel. One half of the gel was silver stained and the other half transferred by semi-dry blotter (Buchler Instruments, Kansas City, MO) to a nitrocellulose membrane for immunoblotting.

Immunodetection of Proteins in the DNA-Protein Cross-Links

The nitrocellulose membrane was blocked overnight in a solution of 5% nonfat dry milk (Carnation), 1% normal goat serum and 1% Tween 20 in phosphate buffered saline (PBS). The membrane was washed twice in PBS for 5 min and incubated 2 hr in a 1:500 dilution of the primary antibody (monoclonal anti-actin) in PBS. The membrane was washed four times for 5 min (PBS, PBS with 0.5% Tween 20, PBS with 0.5% Tween 20, PBS) and then incubated 1 hr with the secondary antibody (1:40,000) in 5% nonfat dry milk dissolved in PBS. The membrane was washed again four times for 5 min (PBS, PBS with 0.5% Tween 20, PBS with 0.5% Tween 20, PBS), and then washed in 10 ml of chemiluminescent solution for 2 min, exposed to autoradiographic film and developed. The relative densities of the actin signal were read for each treatment group (DHMO, DHSN, mitomycin C and cisplatin) on a UV densitometer (Multimedia densitometer, Gilford-Corning, Corning, NY).

RESULTS

Characterization of DNA Cross-Linked Proteins from Nuclei

In this experiment, SDS-PAGE was used to separate the proteins associated with DHMO and DHSN-induced cross-links in cell nuclei from both normal (MDBK) and neoplastic cells (MCF7). Figures II-1 and II-2 show the electrophoretic pattern of proteins from DHMO, DHSN, mitomycin C, and cisplatin-induced DNA-protein cross-links for MDBK and MCF7 cells, respectively. The predominant cross-linked proteins from the pyrrole- (DHMO, DHSN) treated nuclei were generally in the 40-60 kD range with major bands at 45-kD and 55-kD (Figs. II-1 and II-2). Proteins from the mitomycin C-induced cross-links exhibited a banding pattern similar to that for pyrrole-induced cross-links, and also had a protein band around 45-kD (Figs. II-1 and II-2). Proteins from the cisplatin-induced DNA-protein cross-links exhibited a slightly different electrophoretic pattern than that of the pyrroles, but like the pyrroles had a protein band at 45-kD. However, the DMSO treatment lane in both gels (Figs. II-1 and II-2) also showed a band in the 45-kD range. Protein banding patterns were nearly identical between normal (MDBK) and neoplastic cells (MCF7) for each treament type (DHMO, DHSN, mitomycin C, cisplatin) (Figs II-1 and II-2).

Immunodetection of Proteins in DNA-Protein Crosslinks in Nuclei

We wished to determine if actin was present in DHSN and DHMO-induced DNA-protein cross-links in normal (MDBK) and neoplastic (MCF7) cell nuclei. The silver-stained gels did reveal bands in the 45-kD range for all treatments for each cell type; however, a similar band at 45-kD was evident in the DMSO (negative control) lane in both gels. We sought to clarify this by using immunodetection, a highly sensitive method. Immunodetection with a polyvalent anti-actin antibody revealed that actin was part of both

DHMO and DHSN-induced DNA-protein cross-links in nuclei from both cell types (Figs. II-3 and II-4). Actin was also detected in the mitomycin C and cisplatin-induced DNA-protein cross-links in these nuclei (Figs. II-3 and II-4). Two immunoreactive signals appeared for the DHMO, DHSN, and mitomycin C and three signals from the MCF7 cells treated with cisplatin. In contrast, the MDBK cells had only one actin signal for the DHMO, DHSN, mitomycin C, and cisplatin treatments. Densitometer scans revealed that the amount of actin cross-linked to DNA was approximately the same between DHMO and DHSN-treated MDBK and MCF7 nuclei (Fig. II-5). The amount of actin cross-linked to DNA in cisplatin treated nuclei was significantly greater than that in DHMO, DHSN, and mitomycin C in both cell types (Fig. II-5). Mitomycin C cross-linked more actin than either of the pyrroles in both cell types. Nuclei treated with the vehicle (DMSO) did not show an actin signal for either cell type.

DISCUSSION

Pyrrolizidine alkaloids are strong bifunctional alkylators of cellular nucleophiles, including DNA and proteins (Hincks *et al.* 1991, Kim *et al.* 1993, Wagner *et al.* 1993). PA-induced DNA-protein cross-linking appears to be crucial to the bioactivity of PAs and a significant portion of these cross-links are protein associated (Kim *et al.* 1995). Formation of DNA-protein cross-links are important to the toxic, antimitotic, and carcinogenic effects of PAs and other known cross-linkers such as cisplatin (Banjar *et al.* 1984) and nitrogen mustard (Thomas *et al.* 1978). DNA-protein complexes induced by pyrrolic PAs have been previously documented in MDBK cells by SDS-PAGE and isoelectric focusing (Kim *et al.* 1995). Kim *et al.* (1995) concluded that the protein actin, (45 kD, 5.4 PI) a nuclear, non-histone protein associated with transcription and cytokinesis (Rungger *et al.* 1984, Voet and Voet 1990), may be one of the proteins associated with the cross-links. The objective of this study was to determine if actin is one of the nucleophilic targets in PA-

induced cross-links in normal (MDBK) and neoplastic (MCF7) cells. We also used two PA types: DHSN, a strong cross-linker, and DHMO, a moderate cross-linker, to illustrate the differences in cross-linking potential. Previous research has shown that DHSN is a stronger cross-linker and more potent inducer of megalocytosis than DHMO (Kim *et al.* 1993).

SDS-PAGE analysis of the DHSN and DHMO-treated nuclei of both cell types showed a prominent protein band at 45 kD. Nuclei, rather than whole cells, were used in this experiment to remove any effect of cytoplasmic detoxifying mechanisms on the pyrrole as well as to focus on the involvement of nuclear actin.

Actin appeared to be a component in the DHMO and DHSN-induced DNA-protein cross-links in both MDBK and MCF7 nuclei. The cross-links in the MDBK nuclei had a single actin signal with the same electrophoretic mobility as the actin standard. The cross-links from the MCF7 nuclei consistently showed a different pattern from the MDBK cells in that there were two actin signals, the additional one having a slightly lower electrophoretic mobility. We believe that the second signal was actin bound in the PA-induced cross-link.

Agents known to cross-link DNA to proteins were used as standards to compare to the PAs used in our study. Cisplatin is known to cross-link actin to DNA (Miller *et al.* 1991). In our system, cisplatin induced actin-DNA cross-links in nuclei from both cell types to a greater extent than the pyrrolic PAs.

Mitomycin C was also used as a positive standard because when reductively activated, it is known to cross-link DNA and is structurally similar to PAs in that the distance between the two electrophilic sites on mitomycin C is similar to the two sites on DHMO (Hopkins *et al.* 1991). In addition, mitomycin C and DHMO have a similar base sequence specificity for binding to DNA bases (Weidner *et al.* 1990). Actin was also strongly present in the mitomycin C-induced cross-links in both the MDBK and MCF7

nuclei. To our knowledge, actin has not been documented as a protein associated with mitomycin C-induced cross-links in cultured cells.

There were significant differences in the amount of actin in the DNA-protein cross-links in nuclei treated with cisplatin, mitomycin C, DHSN, and DHMO, which could indicate that these agents have differing reactivities toward this protein. The order of potency of DNA-actin cross-linking was: cisplatin > mitomycin C >> DHSN ≥ DHMO. It is also possible that the differences observed may relate to the relative stability of these agents in aqueous meduim.

Actin is the most predominant cytoplasmic protein in eukaryotic cells and can constitute up to 10% of their total protein (Voet and Voet 1990). However, nuclear actin plays an important role in cytokinesis. There was no involvement of cytoplasmic actin because isolated nuclei were used in this study. Therefore, the known role of PAs in mitotic inhibition and megalocyte formation (Kim *et al.* 1993, Hincks *et al.* 1991) may relate to the affinity of this toxin to cross-link DNA to nuclear actin. PA-treated cells are able to produce DNA, RNA, and proteins but are unable to divide (Hoorn *et al.* 1992). Futhermore, PA-induced DNA-DNA and DNA-protein cross-links are resistant to repair and their related antimitotic effects are long lasting (Kim *et al.* 1993, Wagner *et al.* 1993). Therefore, PAs may be of possible use in chemotherapy where drug resistance has occurred due to increased cellular repair of DNA-protein cross-links as in the case of cisplatin resistance in the treatment of ovarian tumors (Zhen *et al.* 1992).

More research about the nature of PA-induced DNA-protein cross-links is needed. If the proteins such as actin discovered in these experiments or other nucleophiles involved in the cross-links are associated with either mitosis or gene regulation, it would help explain the antimitotic, anticarcinogenic, and carcinogenic effects of PAs. Further study should include Western blots probed with antibodies for other proteins or nucleophiles likely to be associated with PA-induced cross-links. The specific amino acid composition

that makes these nucleophiles good targets for the electrophilic PAs and the base pair sequence that constitutes a specific binding site in DNA is the subject of further study currently underway in our laboratory at Utah State University.

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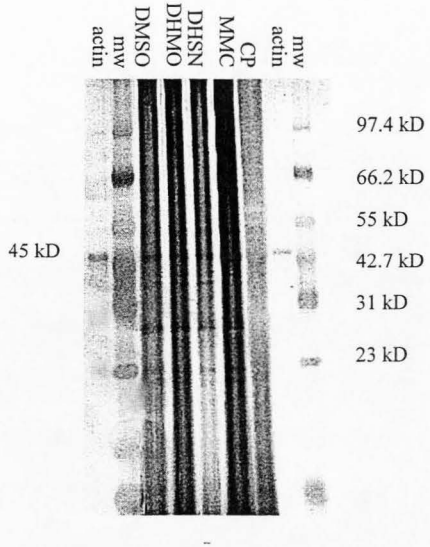

FIG. II-1. Electrophoretic separation of DNA-protein cross-links from MDBK nuclei treated with pyrrolic PAs. DNA-protein cross-links were separated on an 11% SDS-PAGE gel and stained with silver nitrate. All samples were standardized to 40 µg of DNA. These data indicate protein bands mostly in the molecular weight range of 40-60 kD including a protein band at 45 kD for the PA treatments (DHMO and DHSN), mitomycin C (MMC), and cisplatin (CP).

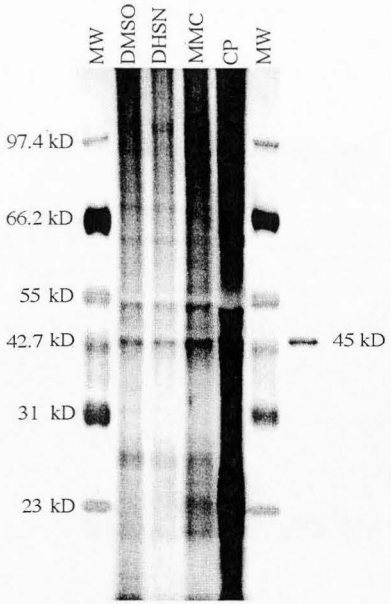

FIG. II-2. Electrophoretic separation of DNA-protein cross-links from MCF7 nuclei treated with pyrrolic PAs. DNA-protein cross-links were separated on an 11% SDS-PAGE gel and stained with silver nitrate. All samples were standardized to 40 μg of DNA. These data indicate protein bands mostly in the molecular weight range of 40-60 kD including a protein band at 45 kD for the PA treatment (DHSN), mitomycin C (MMC), and cisplatin (CP).

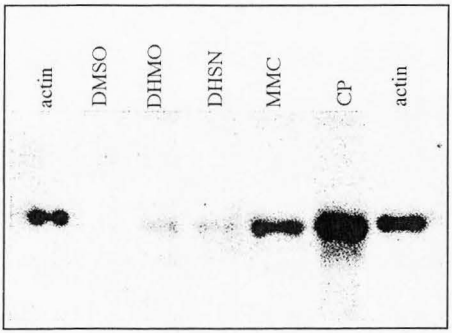

FIG. II-3. Western blot analysis of DNA-protein cross-links from MDBK nuclei treated with pyrrolic PAs. DNA-protein cross-links were separated on an 11% SDS-PAGE gel and the blot was probed with monoclonal anti-actin. All samples were standardized to 40 µg of DNA. These data indicate a strong actin signal for the cisplatin and mitomycin C-treated nuclei and a slight signal for the PA-treated nuclei.

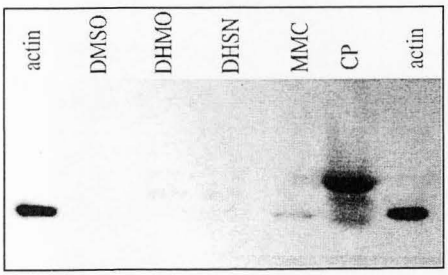

FIG. II-4. Western blot analysis of DNA-protein cross-links from MCF7 nuclei treated with pyrrolic PAs. DNA-protein cross-links were separated on an 11% SDS-PAGE gel and the blot was probed with monoclonal anti-actin. All samples were standardized to 40 μg of DNA. These data indicate a strong actin signal for the cisplatin and mitomycin C-treated nuclei and a slight signal for the PA-treated nuclei.

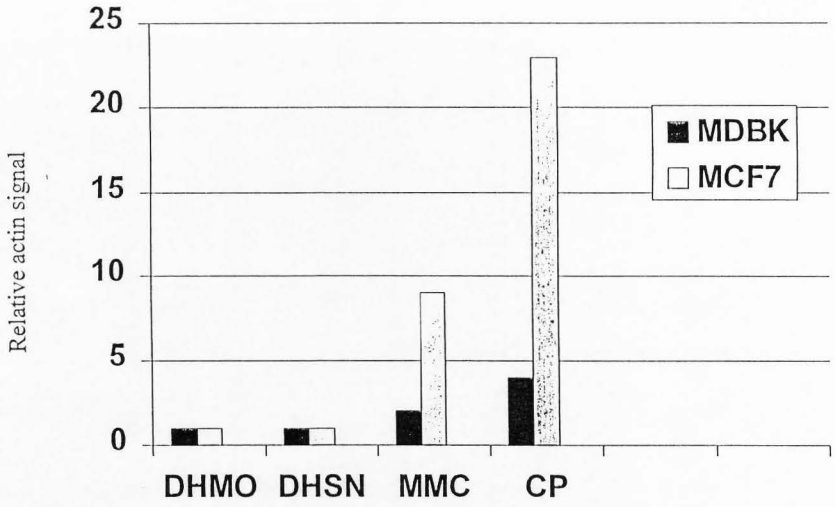

FIG. II-5. Relative density of actin signals on the Western blots for dehydromonocrotaline (DHMO), dehydrosenecionine (DHSN), mitomycin C (MMC), and cisplatin (CP) treatments on MDBK and MCF7 cell nuclei as measured by densitometer. Densities were compared relative to the pyrrole treatments DHMO and DHSN which were equal in density in both cell types. Data indicates that cisplatin has the strongest actin signal at 4-23 times more dense, and the mitomycin C signal 2-9 more dense as the DHMO or DHSN treatment signals.

CHAPTER III

DNA-PROTEIN CROSS-LINKING BY DEHYDROSENECIONINE AND DEHYDROMONOCROTALINE *IN VITRO*

ABSTRACT

Pyrrolizidine alkaloids (PAs), which are found in hundreds of plant species around the world, reportedly have cytotoxic, carcinogenic, antimitotic, and genotoxic activity. PAs are bifunctional electrophilic alkylators that disrupt normal cell processes by binding cellular nucleophiles such as DNA and proteins. The ability of six cellular nucleophiles, including cysteine, methionine, actin, glutathione, metallothionine, and topoisomerase, to bind with dehydrosenecionine (DHSN) or dehydromoncrotaline (DHMO) was tested *in vitro*. All of the nucleophiles except methionine were cross-linked by both DHSN and DHMO as evidenced by the gel shift assay. Common to all the nucleophiles tested was the presence of cysteine residues which would provide sulfhydryl groups for binding by the electrophilic PAs. Methionine, which was not bound by either DHSN or DHMO, contains no free sulhydral groups and thus supports our hypothesis that PAs disrupt normal cell processes such as mitosis by binding crucial proteins based in part on the free sulfhydryl content of these proteins.

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are natural plant compounds found worldwide in an estimated 6,000 plant species largely in the genera *Senecio, Crotalaria, Heliotropium,* and *Symphytum*. These alkaloids are toxic, carcinogenic, antimitotic, and anticarcinogenic (Culvenor 1968, King *et al.* 1987, Kuhara *et al.* 1980, Mattocks 1986). Animals are exposed to PAs through grazing, and humans are exposed through ingestion of herbal remedies or contaminated grain, milk, or honey (Smith and Culvenor 1981, Stillman *et al.*

1977). PAs cause liver damage, including veno-occlusive disease in humans, and megalocytosis, cirrhosis, and hepatoma in both humans and animals (McLean 1970). PAs are also anticarcinogenic due to their ability to cross-link DNA and impede mitosis in rapidly dividing tumor cells, and some PAs have been examined as anti-tumor agents through clinical trials (Hincks *et al.* 1991, King *et al.* 1987, Kim 1994, Letendre *et al.*1981).

Pyrrolizidine alkaloids themselves are not toxic *per se* but are metabolized by cytochrome P450 (CYP) mixed-function oxidases to highly toxic pyrroles or less toxic N-oxides (see Fig. I-1). Two structural components seem to be crucial to biological toxicity: the α, β unsaturation of the necic acid ester and C1-C2 unsaturation of the necine base (Hincks *et al.* 1991, Kim *et al.* 1995, Mattocks 1986). Chemical conversion to the pyrrole occurs by CYP-mediated dehydration followed by allylic oxidation (see Fig. I-2) (Mattocks 1968, Mattocks and White 1971). Once the pyrrole is formed, this reactive species can alkylate at its C7 or C9 position cellular nucleophiles such as DNA or proteins. Thus, pyrroles are bifunctional electrophilic alkylators that can covalently bind essential cellular nucleophiles and lead to alterations in cellular function (Mattocks 1968, Robertson 1982).

The biological activity of PAs appears to be related to the ability of PAs to cross-link DNA or cellular proteins, which can lead to clinical toxicity as well as the observed antimitotic effect. Cross-links may be DNA-DNA or DNA-protein associated (Hincks *et al.* 1991, Kim *et al.* 1993, Petry *et al.* 1986, Reed *et al.* 1988). One study found about 50% of the cross-links induced by PAs in cultured cells to be protein associated (Kim *et al.* 1993). Bulky DNA-protein cross-links are significant to the cell because they may be ineffectively repaired during DNA replication and cause deletion errors or mutations (Cosma *et al.* 1988, Oleinick *et al.* 1987). Cell processes may be altered by agents that form DNA-DNA or DNA-protein cross-links because proteins bound in the cross-link may

be essential to cell function or replication.

We were interested in identifying some nucleophiles that might be likely targets for the electrophilic pyrroles, the characteristics of these nucleophiles that enable the PA-induced cross-link to form, and the possible consequences to the cell. Thus we selected several nucleophiles based on their abundance in the cell, our hypothesis that sulfhydryl groups are important to formation of the cross-link, and the resulting consequences to the cell if these nucleophiles were involved in a pyrrole-induced cross-link.

Integration of amino acids and/or proteins into DNA-protein complexes is a mode of action of some carcinogens. For example, trivalent chromium integrates residual peptides and amino acids normally associated with DNA into DNA-protein cross-links formed in human oesteosarcoma cells *in vitro* (Salnikow *et al.* 1992). Histidine, cysteine, and tyrosine were the predominant amino acids involved in the DNA cross-links formed by trivalent chromium, and proteins or peptides with a high proportion of these amino acids such as BSA and actin were readily complexed into cross-links (Salnikow *et al.* 1992). Dehydromonocrotaline reacts with cysteine and GSH at both acidic and physiological pH *in vitro* (Robertson *et al.* 1977). We chose to test cysteine for its ability to react as a nucleophile with two strong PA cross-linkers, dehydromonocrotaline (DHMO) and dehydrosenecionine (DHSN), based on previous research and the presence of sulfhydryl groups. Research in our laboratory has shown that DHSN is among the strongest PA cross-linkers due to the α,β unsaturation of the necic acid ester (see Fig. I-1) (Kim *et al.* 1993). Dehydromonocrotaline, with only one unsaturated position in the necic acid ester (see Fig. I-1), is a good cross-linker but weaker than DHSN (Kim *et al.* 1993). This experiment illustrated the relative cross-linking abilities of these two PA metabolites with various nucleophiles.

Previous research in our laboratory has shown that actin is strongly suspected to be

present in pyrrole-induced cross-links in cultured cells. Actin is also a cellular target for DNA-protein cross-links by cross-linkers such as cisplatin and potassium chromate (Miller *et al.* 1991). Actin (45 kD) is a nuclear, nonhistone protein containing cysteine residues and is important to cytokinesis and transcription (Rungger *et al.* 1986, Scheer *et al.* 1984). Actin is crucial to the final step of mitosis by enabling the mitotic spindle to contract in the center and separate the newly replicated cell (Voet and Voet 1990). Megalocytosis, or large cells which have replicated but are unable to divide, is a typical symptom of PA exposure (see Fig. I-3). Actin-DNA cross-linking may help explain the antimitotic and anti-carcinogenic side effects of PAs and thus was tested in our study for its ability to cross-link with DHMO and DHSN.

We also chose to test glutathione (GSH; L-γ-glutamyl-L-cysteinylglycine) because it is abundant in cells and contains a nucleophilic, free sulfhydryl group that has been observed to efficiently react with the bifunctional electrophilic PAs (Robertson *et al.* 1977). GSH is a tripeptide, which in the liver is the most predominant nonprotein intracellular thiol (Meister and Anderson, 1983). Reed *et al.* (1992) found that adding GSH to a mixture of rat liver microsomes and the PA senecionine yielded the dehydrosenecionine-GSH conjugate and they concluded that GSH conjugation was a possible detoxification pathway for pyrrolic PAs. Other researchers have found that dietary cysteine protects against PA toxicity by increasing hepatic GSH concentrations (Kim and Jones 1982, White 1976). Nigra and Huxtable (1992) found an association between hepatic GSH concentrations and released dehydromonocrotaline in perfused rat livers and concluded that GSH was involved in forming adducts with the PA metabolite. Borges and Wetterhahn (1989) found that the cross-linker chromium induced cross-linking of glutathione or cysteine to DNA to form DNA-protein complexes *in vitro*. Thus, formation of a GSH-PA cross-link could be a detoxification pathway for the PA metabolites and could help explain the observed anticarcinogenic effect.

We also tested metallothionein because it is thought to operate somewhat like GSH as an electrophile scavenger. It is a small protein located in the cytoplasm and in the nucleus and is the major storage protein for copper as well as a carrier for zinc and cadmium. It also may play a role as an antioxidant since it contains reduced thiolate metal bonds (Da Costa Ferreira *et al.* 1993). Overexpression of metallothionein confers resistance to anticancer drugs *in vitro* (Kelly *et al.* 1988). Metallothionein contains 28 % cysteine residues by weight (Nordberg *et al.* 1972) and therefore could be a likely target for cross-linking PAs.

The observed antimitotic and megalocytotic effect of PAs on cells may involve cross-linking proteins such as topoisomerase II (approx. 400 kD), an enzyme crucial to the formation of DNA by catalyzing negative supercoiling. When topoisomerase II is inhibited by drugs such as novobiocin, DNA replication cannot proceed (Voet and Voet, 1990). If bifunctional alkylating agents such as DHMO and DHSN can bind topoisomerase II, they may be able to impede DNA replication and alter normal DNA behavior. In summary, this study attempts to explain some of the observed effects of PA cross-linking by exploring the role of several candidate nucleophiles, containing various proportions of cysteine or thiol groups, as possible targets for PA-induced DNA-protein cross-links *in vitro*.

MATERIALS AND METHODS

Chemicals and Reagents

Hind III-digested λ-phage DNA was purchased from New England Biolabs (Beverly, MA) and senecionine was generously provided by Russell J. Molyneux, U.S.D.A.- A.R.S. (Albany, CA). Monocrotaline, α-skeletal actin, glutathione, cysteine, methionine, and metallothionine were purchased from Sigma Chemical Co. (St Louis, MO). Topoisomerase II was obtained from Promega Corporation (Madison, WI).

Preparation of Pyrrolic PAs

Pyrroles (DHMO and DHSN) were prepared from parent compounds by the method of Mattocks *et al.* (1989). Briefly, 100 mg of the parent compound in 15 ml of anhydrous DMSO was mixed with 125 mg of tetrachloro-1,2-benzoquinone in 15 ml anhydrous DMSO in a separatory funnel. Two ml of a base solution (1.4 g KOH, 40 ml $NaBH_4$ in 2 ml cold H_2O) was added and the mixture was shaken vigorously for 20 sec. The organic phase was filtered through activated charcoal through a single no. 595 filter paper (Schleicher and Schuell Inc., Keene, NH) into a beaker containing 0.5 g of anhydrous Na_2SO_4. The solution was swirled for 1 min, filtered again to remove the Na_2SO_4, and then evaporated under a stream of N_2 gas. The resulting pyrrole was resuspended in anhydrous DMSO, capped with N_2,and stored in the dark at -80° C. Pyrrole purity was checked by ^1H NMR.

DNA Cross-Link Studies

Various amino acids, tripeptides, or proteins were investigated for their ability to compete with DNA as a nucleophile in the pyrrole-induced cross-link as assessed by gel-shift analysis. This method is based on the retarded mobility of DNA-DNA or DNA protein complexes compared to untreated, non-cross-linked DNA separated by agarose gel electrophoresis. Nucleophile competition was assessed at DNA:pyrrole:nucleophile ratios of 1:2.5:0, 1:2.5:0.5, 1:2.5:1, 1:2.5:2, and 1:2.5:4 for DHMO and DHSN. The DNA was added first to the reaction, the nucleophile second, and finally the pyrrole. DNA: pyrrole:nucleophile reactions were mixed by vortexing, centrifuged briefly (13,600 x g, 10 sec, 22° C), capped with nitrogen, incubated for 2 hr at 0° C, and then directly separated on a 0.8% agarose gel electrophoresed at 50 V and stained with ethidium bromide (1μg/ml). DNA bands were then visualized on a transilluminator, then photographed on a Polaroid MP-4 camera system.

RESULTS

DNA-Protein Cross-Links in Hind III-digested λ- Phage Plasmid DNA

The pyrroles dehydrosenecionine (DHSN) and dehydromonocrotaline (DHMO) induced cross-links between Hind III-digested λ -phage DNA and nucleophiles *in vitro* as shown in the agarose gel-shift assay (Figures III-1 through III-12). In these experiments, the cross-linking is apparent by a reduction in the electrophoretic mobility of the DNA bands compared to those of the control. Complete cross-linking of the λ-phage DNA was seen as a single intense band at the top of the gel (Fig. III-11 and III-12, lanes 3-7). Increasing amounts of nucleophile were added to the DNA and pyrrole reaction to determine if a cross-linking preference was shown to either the DNA or the nucleophile in the reaction.

Neither DMSO (lane 1 for all figures) nor the nucleophile alone (lane 2 for all figures) influenced the electrophoretic mobility of the DNA fragments (Figs. II-1 through II-12). The pyrrole with DNA alone clearly induced a cross-link (lane 3, Figs. II-1 through II-12) as shown by the reduced electrophoretic mobility of the DNA as compared to control. As the ratio of nucleophile to DNA was increased in the reaction, the retarded shift induced by the DHSN or DHMO was partially overcome as illustrated by a progressive increase in electrophoretic mobility of the DNA fragments and reappearance of the DNA fragments. Cysteine was a readily cross-linked nucleophile by both DHSN (Fig. III-1) and DHMO (Fig. III-2). For DHMO, cysteine was preferred over the plasmid DNA even at the lowest ratio of 1:0.5 (DNA : cysteine), causing a complete reappearance of the plasmid DNA (Fig. III-2, lane 4). Glutathione (Fig. III-3 and Fig. III-4) and topoisomerase (Fig. III- 9 and Fig. III-10) were also favorable targets for both DHSN and DHMO in this system. Slightly less favorable but still cross-linked by DHSN and DHMO

were actin (Fig. III-5 and Fig. III-6) and metallothionein (Fig. III-7 and Fig. III-8). Methionine, with no free sulfhydryl groups, was not cross-linked by either DHSN (Fig. III-11) or DHMO (Fig. III-12) even at the highest ratio of DNA to methionine (1:4). We confirmed that DHSN is a more potent DNA cross-linker than DHMO because a smaller amount of the nucleophile was required to overcome the cross-linking and produce a gel shift effect for DHMO (Lanes 4-6, Figs. III-1 through III-12).

DISCUSSION

The antiproliferative, antimitotic, and carcinogenic effects of PAs may be explained by the fact that PAs could form bulky cross-links with proteins crucial to DNA replication, gene transcription, and cell replication. The purpose of this study was to test several cellular nucleophiles for their ability to cross-link and compete with DNA in the PA-induced cross-linking reaction *in vitro*. The nucleophiles were chosen on the basis of their reported nucleophilic properties and their association with either mitosis, gene transcription, DNA replication, or carcinogenesis. We hypothesized that the relative number of cysteine residues in the nucleophile and thus the number of sulhydryl groups available for binding the electrophilic PA metabolites DHSN and DHMO was important to the ability of a protein to be cross-linked. We used the two PA metabolites DHSN and DHMO because both are known to cross-link DNA, but DHSN is a stronger cross-linker and more potent inducer of megalocytosis than DHMO (Kim *et al.* 1993). It was of interest to see if these two PA metabolites had different cross-linking preferences to the nucleophiles we tested.

Our results showed that the relative cross-linking ability of the nucleophiles tested with both DHMO and DHSN was cysteine > GSH > actin ≥ topoisomerase ≥ metallothionein > methionine. The molecular weight and relative number of sulhydryl

groups available for binding was important to the formation of the cross-link as well as the observed effect in the gel shift assay. Cysteine was easily cross-linked by DHMO and DHSN. Cysteine contains a free sulfhydryl group, which presumably provides a good nucleophilic target for the electrophilic pyrroles DHMO and DHSN. To verify this hypothesis, we tested the amino acid methionine, which does not contain a free sulfhydryl group and found that it did not cross-link with DHMO or DHSN even at the highest methionine:DNA ratio (4:1).

Salnikow *et al.* (1992) tested chrominum, a known DNA-protein cross-linker and found that the cross-linking activity of chromium was associated most frequently with cysteine and tyrosine compared to the other amino acids. Examining the involvement of actin, BSA, and histones, these workers postulated that chromium reacts with protein first, the selection determined in part by the amino acid composition of the proteins such as cysteine, tyrosine, or histidine, and then binds to DNA. Lin *et al.* (1992) found chromium- and nickel-induced cross-links were most often associated with cysteine and histidine. Kim *et al.* (1995) found cysteine to be an important factor when they demonstrated that DHSN cross-linked the most easily with BSA, which contains a higher proportion of cysteine, over actin and myosin which have fewer cysteine residues. The number of cysteine residues and thus the number of free sulhydryl groups available for binding could be critical to the formation of the PA-induced protein cross-link.

In this experiment we attempted to explain some of the observed cellular effects of PAs in terms of their known mode of operation, binding cellular nucleophiles. Based on our results that PAs bind cysteine but not methionine, we selected nucleophiles that contain free sulfhydryls, are abundant in the cell, and if bound in a PA-induced cross-link could induce some of the observed effects of PAs. To help explain the antimitotic and carcinogenic effects of PAs, we tested actin and topoisomerase II and found them both to

be cross-linked by DHMO and DHSN. In chapter II we demonstrated that actin is present in the DNA-protein cross-links induced by the pyrroles DHMO and DHSN and the standard cross-linkers cisplatin and mitomycin C in both normal (MDBK) and neoplastic (MCF7) cells. Miller *et al.* (1991) found actin in nearly 20% of the cross-links induced by cisplatin in cultured Chinese hamster ovary cells. Actin is a nuclear, nonhistone protein, containing approximately 2% cysteine residues by number and thus could be an attractive target for PAs. In addition, actin is not normally bound to DNA and is important to cytokinesis and gene transcription (Rungger *et al.* 1984, Scheer *et al.* 1984, Voet and Voet 1990).

We also found topoisomerase II to be readily cross-linked by DHMO and DHSN. Topoisomerase II is a protein crucial to the formation of DNA by catalyzing negative supercoiling and when inhibited by drugs such as novobiocin, DNA replication cannot proceed (Voet and Voet 1990). Topoisomerase II has also been found to be involved in modulating the superhelical structure of DNA in transcriptionally active regions that may be necessary for drug action (Wang 1985). Transcribed regions of DNA are the preferred target for several DNA-reactive molecules such as aflatoxin B_1 (Irvine and Wogen 1983) and bleomycin and neocarzinostatin (Beckman *et al.* 1987). If bifunctional alkylating agents such as DHMO and DHSN can bind actin or topoisomerase II in the cell, this could explain in part the ability of PAs to impede DNA replication and alter normal DNA behavior.

To explain the observed anticarcinogenic effects of PAs, we tested glutathione and metallothionein in our system and showed both to cross-link readily with DHMO and DHSN. Glutathione, a tripeptide that binds electrophilic species and allows them to be excreted from the body, is the most predominant intracellular thiol (Meister and Anderson

1983) and contains approximately 40% cysteine by weight. Glutathione has been shown to be involved in cross-links induced by standard cross-linkers *in vitro* such as chromium (Borges and Wetterhahn 1989) and dehydrosenecionine (Reed *et al.* 1992), and the cysteine residues of glutathione are crucial to formation of GSH cross-links (Salnikow *et al.* 1992). Metallothionein, found mainly in the cytoplasm and but also in the nucleus, is known to be associated with binding and transport of heavy metals such as cadmium and cooper and contains 28% cysteine residues (Nordberg *et al.* 1972, West *et al.* 1995). It has been suggested that metallothioneins are free radical scavengers and protect the body somewhat like GSH against reactive electrophilic species. Elevated levels of intracellular glutathione (Hamilton *et al.* 1985) and metallothionein (Kelly *et al.* 1988) are known to be associated with resistance to drugs presumably because of the ability to bind the drug and make it excretable. We have shown glutathione and metallothionein to be molecular targets for formation of pyrrole cross-links in our system and formation of these complexes in the cell could deter the DNA cross-linking effect of pyrroles.

In summary, our *in vitro* system has provided insight into some characteristics that make a protein an attractive target for PA-induced cross-linking. We hypothesized that the number of free cysteine residues and therefore the number of sulfhydryl groups that would be available for binding are key elements to formation of cross-links. The mode of action of PAs is the ability to form DNA-DNA or DNA-protein cross-links with cellular nucleophiles and thus alter the normal cell processes. The cross-links induced by PAs could have negative effects to the cell as in the case of binding actin or topoisomerase II to alter normal DNA processes and replication or beneficial effects such as binding to electrophilic scavengers like GSH or metallothionine as a detoxifying mechanism. The nucleophiles we tested *in vitro* and found to form cross-links with DHMO and DHSN help explain the antimitotic, carcinogenic, and anticarcinogenic effects of PAs.

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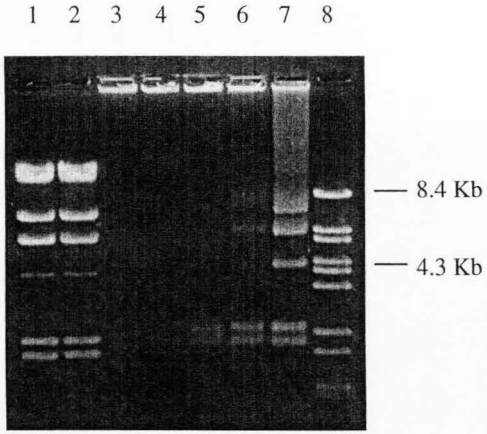

FIG. III-1. Effect of cysteine on dehydrosenecionine (DHSN)-induced DNA cross-links in Hind III-digested λ-phage DNA at w:w:w ratios (DNA:DHSN:cysteine) of 1:2.5:0.5 (lane 4), 1:2.5:1 (lane 5), 1:2.5:2 (lane 6), and 1:2.5:4 (lane 7). Controls with vehicle (DMSO) are in lane 1 (1:2.5:0) and lane 2 (1:2.5:4), while lane 3 is DNA with DHSN only (1:2.5:0) and lane 8 is BST E II-digested λ-phage DNA as a marker. Increasing amounts of cysteine in the reaction partially overcome the DHSN cross-linking effect on the DNA and can be seen as increased electrophoretic mobility of the DNA bands as compared to the control in lane 3.

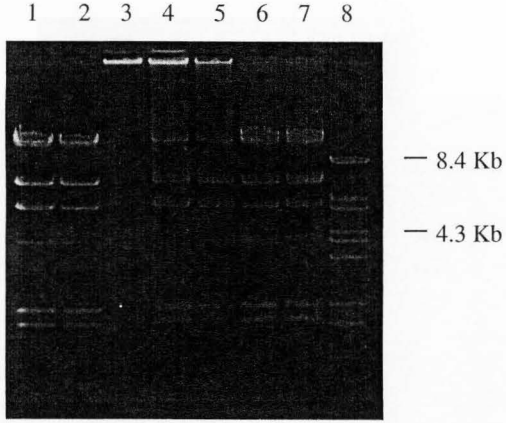

FIG. III-2. Effect of cysteine on dehydromonocrotaline (DHMO)-induced DNA cross-links in Hind III-digested λ-phage DNA at w:w:w ratios (DNA:DHMO:cysteine) of 1:2.5:0.5 (lane 4), 1:2.5:1 (lane 5), 1:2.5:2 (lane 6), and 1:2.5:4 (lane 7). Controls with vehicle (DMSO) are in lane 1 (1:2.5:0) and lane 2 (1:2.5:4), while lane 3 is DNA with DHMO only (1:2.5:0) and lane 8 is BST E II-digested λ-phage DNA as a marker. Increasing amounts of cysteine in the reaction partially overcome the DHMO cross-linking effect on the DNA and can be seen as increased electrophoretic mobility of the DNA bands as compared to the control in lane 3.

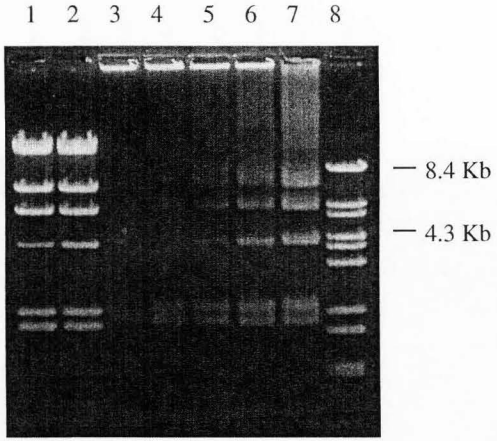

FIG. III-3. Effect of glutathione on dehydrosenecionine (DHSN)-induced DNA cross-links in Hind III-digested λ-phage DNA at w:w:w ratios (DNA:DHSN:glutathione) of 1:2.5:0.5 (lane 4), 1:2.5:1 (lane 5), 1:2.5:2 (lane 6), and 1:2.5:4 (lane 7). Controls with vehicle (DMSO) are in lane 1 (1:2.5:0) and lane 2 (1:2.5:4), while lane 3 is DNA with DHSN only (1:2.5:0) and lane 8 is BST E II-digested λ-phage DNA as a marker. Increasing amounts of glutathione in the reaction partially overcome the DHSN cross-linking effect on the DNA and can be seen as increased electrophoretic mobility of the DNA bands as compared to the control in lane 3.

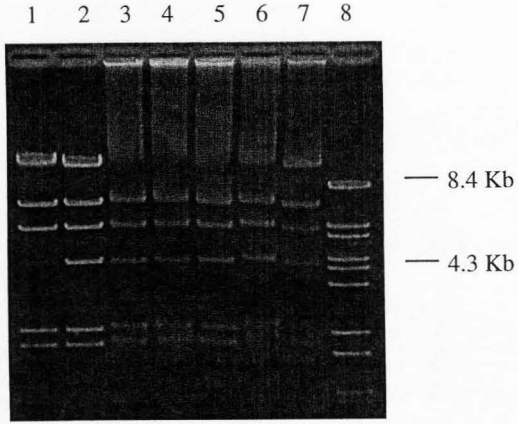

FIG. III-4. Effect of glutathione on dehydromonocrotaline (DHMO)-induced DNA cross-links in Hind III-digested λ-phage DNA at w:w:w ratios (DNA:DHMO:glutathione) of 1:2.5:0.5 (lane 4), 1:2.5:1 (lane 5), 1:2.5:2 (lane 6), and 1:2.5:4 (lane 7). Controls with vehicle (DMSO) are in lane 1 (1:2.5:0) and lane 2 (1:2.5:4), while lane 3 is DNA with DHMO only (1:2.5:0) and lane 8 is BST E II-digested λ-phage DNA as a marker. Increasing amounts of glutathione in the reaction partially overcome the DHMO cross-linking effect on the DNA and can be seen as increased electrophoretic mobility of the DNA bands as compared to the control in lane 3.

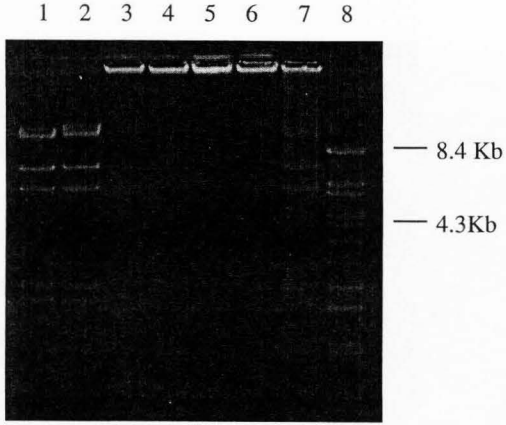

FIG. III-5. Effect of actin on dehydrosenecionine (DHSN)-induced DNA cross-links in Hind III-digested λ-phage DNA at w:w:w ratios (DNA:DHSN:actin) of 1:2.5:0.5 (lane 4), 1:2.5:1 (lane 5), 1:2.5:2 (lane 6), and 1:2.5:4 (lane 7). Controls with vehicle (DMSO) are in lane 1 (1:2.5:0) and lane 2 (1:2.5:4), while lane 3 is DNA with DHSN only (1:2.5:0) and lane 8 is BST E II-digested λ-phage DNA as a marker. Increasing amounts of actin in the reaction partially overcome the DHSN cross-linking effect on the DNA and can be seen as increased electrophoretic mobility of the DNA bands as compared to the control in lane 3.

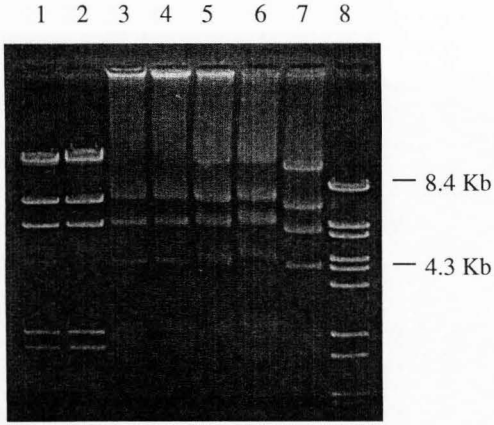

FIG. III-6. Effect of actin on dehydromonocrotaline (DHMO)-induced DNA cross-links in Hind III-digested λ-phage DNA at w:w:w ratios (DNA:DHMO:actin) of 1:2.5:0.5 (lane 4), 1:2.5:1 (lane 5), 1:2.5:2 (lane 6), and 1:2.5:4 (lane 7). Controls with vehicle (DMSO) are in lane 1 (1:2.5:0) and lane 2 (1:2.5:4), while lane 3 is DNA with DHMO only (1:2.5:0) and lane 8 is BST E II-digested λ-phage DNA as a marker. Increasing amounts of actin in the reaction partially overcome the DHMO cross-linking effect on the DNA and can be seen as increased electrophoretic mobility of the DNA bands as compared to the control in lane 3.

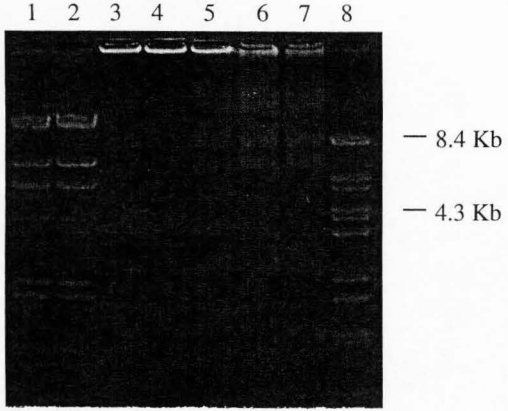

FIG. III-7. Effect of metallothionein on dehydrosenecionine (DHSN)-induced DNA cross-links in Hind III-digested λ-phage DNA at w:w:w ratios (DNA:DHSN: metallothionein) of 1:2.5:0.5 (lane 4), 1:2.5:1 (lane 5), 1:2.5:2 (lane 6), and 1:2.5:4 (lane 7). Controls with vehicle (DMSO) are in lane 1 (1:2.5:0) and lane 2 (1:2.5:4), while lane 3 is DNA with DHSN only (1:2.5:0) and lane 8 is BST E II-digested λ-phage DNA as a marker. Increasing amounts of metallothionein in the reaction partially overcome the DHSN cross-linking effect on the DNA and can be seen as increased electrophoretic mobility of the DNA bands as compared to the control in lane 3.

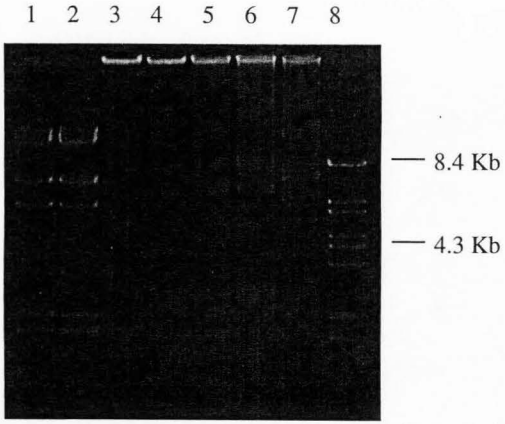

FIG. III-8. Effect of metallothionein on dehydromonocrotaline (DHMO)-induced DNA cross-links in Hind III-digested λ-phage DNA at w:w:w ratios (DNA:DHMO:metallothionein) of 1:2.5:0.5 (lane 4), 1:2.5:1 (lane 5), 1:2.5:2 (lane 6), and 1:2.5:4 (lane 7). Controls with vehicle (DMSO) are in lane 1 (1:2.5:0) and lane 2 (1:2.5:4), while lane 3 is DNA with DHMO only (1:2.5:0) and lane 8 is BST E II-digested λ-phage DNA as a marker. Increasing amounts of metallothionein in the reaction partially overcome the DHMO cross-linking effect on the DNA and can be seen as increased electrophoretic mobility of the DNA bands as compared to the control in lane 3.

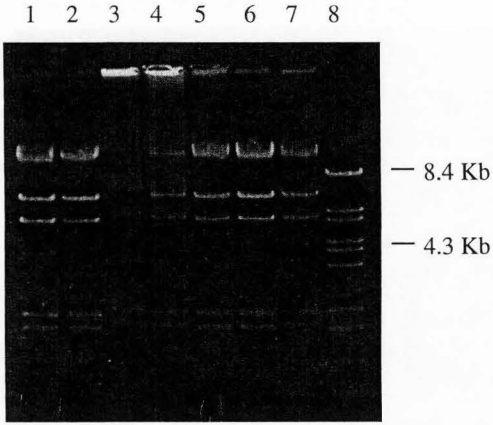

FIG. III-9. Effect of topoisomerase on dehydrosenecionine (DHSN)-induced DNA cross-links in Hind III-digested λ-phage DNA at w:w:w ratios (DNA:DHSN:topoisomerase) of 1:2.5:0.5 (lane 4), 1:2.5:1 (lane 5), 1:2.5:2 (lane 6), and 1:2.5:4 (lane 7). Controls with vehicle (DMSO) are in lane 1 (1:2.5:0) and lane 2 (1:2.5:4), while lane 3 is DNA with DHSN only (1:2.5:0) and lane 8 is BST E II-digested λ-phage DNA as a marker. Increasing amounts of topoisomerase in the reaction partially overcome the DHSN cross-linking effect on the DNA and can be seen as increased electrophoretic mobility of the DNA bands as compared to the control in lane 3.

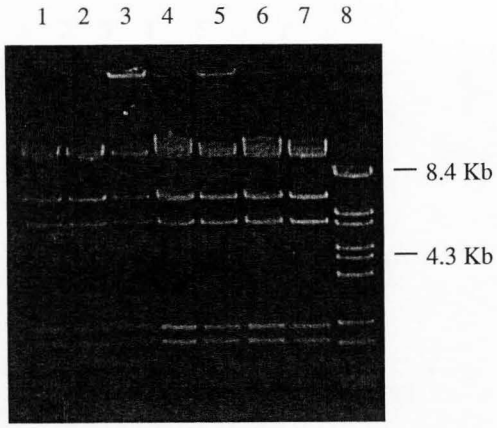

FIG. III-10. Effect of topoisomerase on dehydromonocrotaline (DHMO)-induced DNA cross-links in Hind III-digested λ-phage DNA at w:w:w ratios (DNA:DHMO:topoisomerase) of 1:2.5:0.5 (lane 4), 1:2.5:1 (lane 5), 1:2.5:2 (lane 6), and 1:2.5:4 (lane 7). Controls with vehicle (DMSO) are in lane 1 (1:2.5:0) and lane 2 (1:2.5:4), while lane 3 is DNA with DHMO only (1:2.5:0) and lane 8 is BST E II-digested λ-phage DNA as a marker. Increasing amounts of topoisomerase in the reaction partially overcome the DHMO cross-linking effect on the DNA and can be seen as increased electrophoretic mobility of the DNA bands as compared to the control in lane 3.

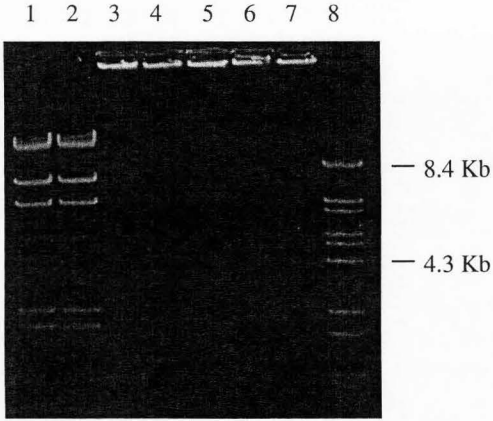

FIG. III-11. Effect of methionine on dehydrosenecionine (DHSN)-induced DNA cross-links in Hind III-digested λ-phage DNA at w:w:w ratios (DNA:DHSN: methionine) of 1:2.5:0.5 (lane 4), 1:2.5:1 (lane 5), 1:2.5:2 (lane 6), and 1:2.5:4 (lane 7). Controls with vehicle (DMSO) are in lane 1 (1:2.5:0) and lane 2 (1:2.5:4), while lane 3 is DNA with DHSN only (1:2.5:0) and lane 8 is BST E II-digested λ-phage DNA as a marker. Increasing amounts of methionine in the reaction partially overcome the DHSN cross-linking effect on the DNA and can be seen as increased electrophoretic mobility of the DNA bands as compared to the control in lane 3.

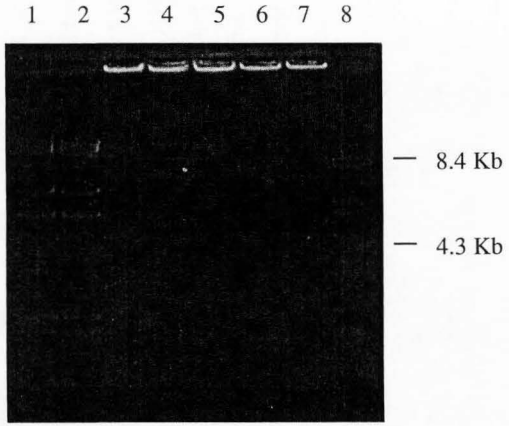

FIG. III-12. Effect of methionine on dehydromonocrotaline (DHMO)-induced DNA cross-links in Hind III-digested λ-phage DNA at w:w:w ratios (DNA:DHMO: methionine) of 1:2.5:0.5 (lane 4), 1:2.5:1 (lane 5), 1:2.5:2 (lane 6), and 1:2.5:4 (lane 7). Controls with vehicle (DMSO) are in lane 1 (1:2.5:0) and lane 2 (1:2.5:4), while lane 3 is DNA with DHMO only (1:2.5:0) and lane 8 is BST E II-digested λ-phage DNA as a marker. Increasing amounts of methionine in the reaction partially overcome the DHMO cross-linking effect on the DNA and can be seen as increased electrophoretic mobility of the DNA bands as compared to the control in lane 3.

CHAPTER IV

SUMMARY

Pyrrolizidine alkaloids (PAs) are natural plant toxins found worldwide that exhibit cytotoxic, carcinogenic, anticarcinogenic, and antimitotic properties. PAs are metabolized by the P450 (CYP) system to the relatively nontoxic N-oxide form or the highly reactive pyrroles. Pyrroles are bifunctional electrophilic alkylators that bind cellular nucleophiles such as proteins or DNA and alter normal cell processes including DNA replication and transcription. The formation of DNA-DNA or DNA-protein cross-links in the cell is a key mode of action for PAs and could help explain the varying side effects of PAs. For example, proteins, key to mitosis or gene transcription, could be targets for pyrrole binding and would explain the antimitotic and carcinogenic effects of PAs. Other protein targets may be electrophilic scavengers and the formation of PA-induced cross-links with these proteins may help explain the anticarcinogenic effects of PAs. Experimental goals were to discover some of the proteins that are good cross-linking targets for the pyrroles and what characteristics make the protein an attractive cross-linking target. Therefore, 2 studies were conducted using the potent cross-linkers dehydromonocrotaline (DHMO) and dehydro-senecionine (DHSN) to induce cross-links, first in cultured normal (MDBK) and neoplastic (MCF7) cells and then in an *in vitro* system utilizing the gel shift assay.

In the first study, DHMO- and DHSN-induced cross-links in MDBK and MCF7 nuclei were isolated, the DNA was digested off the cross-link, and the resulting proteins associated with the cross-links were separated by SDS-PAGE and probed for the presence of actin on a Western blot. Actin is ubiquitous, abundant, and not normally bound to DNA in the nucleus. Actin was chosen because of its critical role in mitosis, abundance in the cell, and previous research documenting actin cross-links with a similar electrophilic alkylator, cisplatin. Actin was found to be present in DHMO- and DHSN-induced cross-links in both normal and neoplastic cells. In addition, actin was present in cisplatin- and

mitomycin C-induced cross-links used as positive controls. Although mitomycin C is known to cross-link DNA, and is similar in structure to PAs with its two alkylating sites nearly the same distance apart as in the pyrroles, it has not been previously documented to cross-link actin. The presence of actin in the cross-links induced by DHMO, DHSN, cisplatin, and mitomycin C in both normal and neoplastic cells indicates that: 1) all of these alkylators do cross-link proteins and 2) actin, critical to mitosis, when bound in a PA-induced cross-link could be responsible for observed antimitotic effects on the cell such as megalocytosis. Results show the actin signals seen on both the MDBK and MCF7 blots are in fact actin alone or actin still cross-linked to some DNA (with less migration in the gel than the pure actin) because the Western blots were probed with a MAP antibody sensitive to all isoforms of actin.

In the second experiment, proteins or nucleophiles were tested for their cross-linking ability, based, in part, on the consequences to the cell if the nucleophile was bound in a cross-link. The hypothesis was that abundant cellular nucleophiles with cysteine residues could provide sulfhydryl groups for the electrophilic PAs to bind and form cross-links. An *in vitro* system was developed using PAs to cross-link plasmid DNA and a chosen nucleophile. The cross-links were separated by agarose gel and when cross-linking occurred, an increased molecular weight and resulting slower migration were seen. As the ratio of nucleophile over DNA was increased, the DNA bands began to reappear, indicating that the PA was cross-linked more to the nucleophile than the DNA. The agarose gels were ethidium bromide stained, indicating the location of the DNA, but a future experiment would be to label the nucleophile to determine its location as well. Results indicate that cysteine alone cross-linked readily to both DHMO and DHSN while methionine, containing no free sulfhydryl groups, did not. Glutathione, actin, metallothinein, and topoisomerase, all containing sulfhydryl groups, were then tested and each of them was found to cross-link with DHMO and DHSN. The relative cross-linking abiltiy of the nucleophiles tested with

both DHSN and DHMO was: cysteine > GSH ≥ actin > metallothionein ≥ topoisomerase > methionine. The results also indicated that overall DHSN is a much stronger cross-linker than DHMO. PA-induced cross-linking to nucleophiles can be related to the known side effects of PAs. PA-actin cross-linking could explain the megalocytotic and antimitotic effects of PAs and verifies the results of the first experiment that actin is 1of the proteins that can form cross-links with PAs. Glutathione and metallothionine both contain cysteine residues, which are known to act as electrophilic scavengers, and therefore could be potential detoxifiers of pyrroles. Topoisomerase II is crucial to DNA negative supercoiling and if bound in a cross-link, DNA replication and transcription could be affected. In summary, the cross-links induced by PAs could have negative effects to the cell as in the case of binding actin or topoisomerase II to alter normal DNA processes and replication, or beneficial effects such as binding to electrophilic scavengers like GSH or metallothionein as a detoxifying mechanism.

More research about the nature of PA-induced DNA-protein cross-links is needed. If the proteins or nucleophiles, discovered in these experiments, such as actin, glutathione, metallothionein, and topoisomerase II, are involved in the cross-links and are associated with either mitosis, gene regulation, or a detoxification mechanism, it would help explain the antimitotic, anticarcinogenic, and the carcinogenic effects of PAs. Future research should include Western blots probed with antibodies for other nucleophiles likely to be associated with PA-induced cross-links and a determination of the specific amino acid composition that makes these nucleophiles good targets for PA cross-linking.