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Molecular Toxicology of Pyrrolizidine Alkaloids

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MOLECULAR TOXICOLOGY OF PYRROLIZIDINE ALKALOIDS

by

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ABSTRACT

Molecular Toxicology of Pyrrolizidine Alkaloids

by

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Major Professor: Dr. Roger A. Coulombe, Jr.
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Pyrrolizidine alkaloids are cytotoxic, carcinogenic, and anti-carcinogenic *in vivo* and *in vitro,* and they produce many hazardous effects in humans and animals. Pyrrolizidine alkaloids (PAs) also cross-link with DNA and/or protein. However, whether such cross-linking is important to the toxic action of PAs is not known. In addition, the exact mechanism underlying these DNA cross-links or cytotoxicity is also not clear.

In three separate studies, I characterized the nature of PA-induced DNA cross-links and the relationships between PA structures and cross-linking potency. In the first study (Chapter II), I found that cross-linking potency of PA congeners coincided with their abilities to cause cytopathologic effects. Macrocyclic α,β-unsaturated diesters PAs and their pyrrolic metabolites were the most potent inhibitors of colony formation, and inducers of cytopathologic changes and megalocyte formation. The macrocyclic α,β-saturated diester PA and open diesters PAs slightly inhibited colony formation, and slightly changed cell morphology. Retronecine and indicine N-oxide were completely inactive. In the next study (Chapter III), I found that pyrrolic macrocyclic metabolites were

more potent DNA cross-linkers than their parent compounds as determined by alkaline elution. The pyrroles of the macrocyclic diester PAs were potent DNA-DNA (inter- and/or intra) cross-linkers in *Bst* EII-digested λ-phage DNA or pBR322 plasmid DNA but dehydroretronecine and indicine N-oxide were not. I also examined which DNA sequences were more susceptible to PA-induced cross-links by using a series of restriction endonucleases to determine sequence specificity. The most favorable cross-linking site for PAs appeared to be 5'-d(GG) and 5'-d(GA) although other sites, 5'-d(CC) or 5'-d(CG), might be also preferable cross-linking targets. In the next study (Chapter IV), I characterized the nature of DNA-protein interactions induced by PAs, because I found in previous studies that PA-induced cross-links are largely protein associated. In PA or pyrrolic PA exposed cells, cross-linked proteins with molecular weights 40 - 60 kD were detected. Two-dimensional electrophoretic analysis revealed that these proteins were probably acidic in nature. In an *in vitro* system utilizing pBR322 or *Bst* EII-digested λ-phage DNA, dehydrosenecionine induced DNA-protein cross-links with BSA, indicating that such interactions might be related to amino acid composition of protein.

These results confirmed that PA-induced DNA cross-links (DNA-DNA, DNA-protein cross-links) are influenced by three structural features: the C1,2 unsaturation of pyrrolizidine ring, α,β-unsaturation, and size of the macrocyclic diester ring. The ability to form cross-links was closely related to the known toxic potencies of these PAs. From this research, I also conclude that DNA cross-linking is the most critical event leading to PA-related diseases and that cross-linking is due to pyrrolic metabolites of PAs, not via a common metabolite as was once thought.

(114 pages)

CHAPTER I

INTRODUCTION

Genotoxicity

DNA is a well-known intracellular target for genotoxic agents and some anti-neoplastic drugs. Some of these agents induce cross-links in DNA which may involve nuclear or non-nuclear proteins that have important roles in gene regulation (Banjar *et al.*, 1984; Lemaire *et al.*, 1991). The interaction of DNA and genotoxics can cause permanent or semi-permanent genomic changes. The damaged DNA can be fully or partially repaired, although incorrectly repaired DNA can induce genomic changes such as transition or transversion mutations.

Pyrrolizidine alkaloids

Pyrrolizidine alkaloids (PAs) are common natural plant alkaloids produced by genera such as *Senecio*, *Crotalaria*, *Heliotropium*, and *Symphytum* (Bull *et al.*, 1968; Smith and Culvenor, 1981). Pyrrolizidine alkaloids are cytotoxic, carcinogenic, and anti-carcinogenic (Culvenor, 1968; Svoboda and Reddy, 1972; Newberne and Rogers, 1973; Schoental, 1975; Kuhara *et al.*, 1980; King *et al.*, 1987). Humans and other animals may be exposed to PAs in food and feed. PAs or their toxic metabolites are secreted in the milk of lactating dairy cattle and rats, and especially young animals are susceptible to PA toxicity (Schoental and Magee, 1959; Dickinson *et al.*, 1976). Pyrrolizidine alkaloids are toxic to most animal species. Horses, rats, and chickens are very susceptible, while guinea pigs, sheep, and goats are resistant to PA toxicity (Schoental, 1963; Bull *et al.*, 1968; White *et al.*, 1973; Chesney and Allen, 1973; Shull *et al.*, 1976; Goeger *et al.*, 1982; Peterson and Jago, 1984). These species differences

of PA toxicity have been explained by the differences in the rate of metabolic conversion of PAs to toxic metabolites (pyrroles) in the different animal species.

Pyrrolizidine alkaloid poisoning in humans is endemic in some geographical locations. In Afghanistan and West India, contamination of food crops or the ingestion of herbs for the treatment of certain ailments is associated with PA-related liver toxicities such as veno-occlusive disease (Tandon and Tandon, 1975; Mohabbat *et al.*, 1976; Tandon *et al.*, 1976a; Tandon *et al.*, 1976b; Tandon *et al.*, 1977; Tandon *et al.*, 1978). Pyrrolizidine alkaloid-related hepatotoxicity may be a high risk factor for liver disease and a cause of mortality in a segment of the population, especially in developing countries where ingestion of PAs in food or herbal medicines is common (Tandon *et al.*, 1975; Tandon *et al.*, 1978; Stillman *et al.*, 1979; Huxtable, 1980).

Other PA-related effects include gastrointestinal lesions, pulmonary hypertension, right ventricular hypertrophy, central nervous system disorders, and veno-occlusive disease of the liver in both animals and humans (Bras and Hill, 1956; Hill, 1960; McLean, 1970; Bras, 1973). In animal experiments, a single exposure to PAs progresses relentlessly to advanced chronic liver disease and cirrhosis (Schoental and Magee, 1957, 1959; Nolan *et al.*, 1966). Pyrrolizidine alkaloids have also been reported to be synergistic with aflatoxins in causing liver cirrhosis, hepatoma, or lung toxicity in primates and rats (Cook *et al.*, 1950; Lin *et al.*, 1974; Culvenor, 1983).

The structures of some PAs and their metabolites are shown in Figure 1. Pyrrolizidine alkaloids can be divided into four major structural groups: the macrocyclic α,β-unsaturated diesters (senecionine, seneciphylline, riddelliine, retrorsine), the macrocyclic saturated diester (monocrotaline), open diesters (heliosupine, latifoline), and necine base (retronecine). McLean (1970) reported

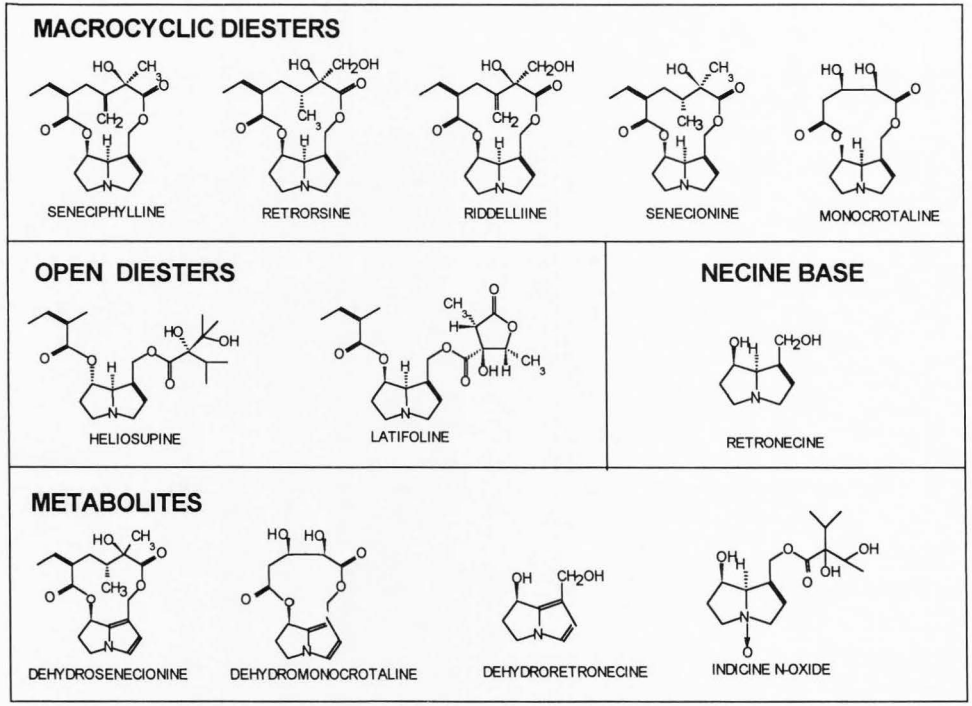


Fig. I-1. Chemical structures of pyrrolizidine alkaloids and their metabolites.

that essential structures for the biological toxicities of PAs include a C1,2-double bond in the pyrrolizidine ring and branched chain acids, and esterification with a 9-hydroxyl and 7-hydroxyl substituent.

Pyrrolizidine alkaloids are not biologically toxic *per se* but must first be metabolized primarily by cytochrome P-450 mixed-function oxidase to either the dehydro form (pyrrole), which is highly toxic, or the N-oxide form, which is less toxic. The pyrrole is formed by allylic oxidation in pyrrolizidine ring followed by cytochrome P-450 mixed-function oxidase mediated dehydration (Mattocks, 1968; Mattocks and White, 1971). The major metabolic routes of PAs are shown in Figure 2. The major metabolic routes of the macrocyclic diester PAs in animals are hydrolysis of the ester groups, N-oxidation, and dehydrogenation to the pyrrole (Mattocks, 1986). The pyrrole alkylates cellular nucleophiles at either or both ester linkages (Fig. 3). The relative animal toxicity of various PAs is highly correlated to its metabolic fate (hydrolysis, dehydrogenation, N-oxidation) which determines whether a larger or smaller proportion of the dose is converted into toxic metabolites (Mattocks, 1970). For example, horses can convert PAs to a larger proportion of pyrroles compared with sheep or goats, which produced a larger proportion of N-oxides (White *et al.*, 1973; Goeger *et al.*, 1982). The hydrolysis rate of various PAs is inversely related to the presence of steric hindrance around the ester groups (Mattocks, 1982; Bull *et al.*, 1968).

Interaction of PAs with DNA

Various PAs are mutagenic, carcinogenic, or anti-carcinogenic. In animals and mammalian cells exposed to PAs, megalocyte formation is a common cytopathologic sequelae which is postulated to be a result of the anti-mitotic action of PAs (Bull and Dick, 1959; Tandon *et al.*, 1978; McLean and Mattocks,

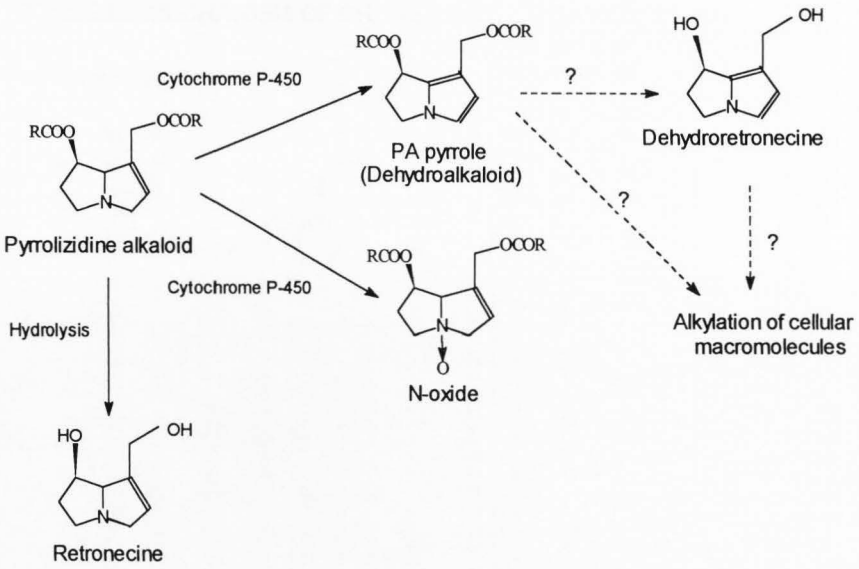
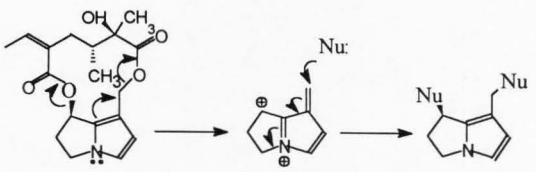


Fig. I-2. Known and postulated metabolic pathways of pyrrolizidine alkaloids.

POSSIBLE MECHANISM OF CROSS-LINK FORMATION BY PAs

via S_N1 Solvolysis



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

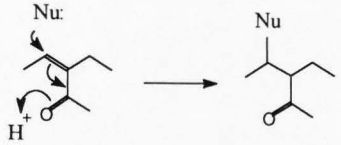


Fig. I-3. Possible reaction mechanisms between macrocyclic unsaturated diester PAs with a nucleophilic atom (Nu).

1980; Kim *et al.*, 1993). Lasiocarpine and its pyrrolic metabolite, dehydroheliotridine, have been shown to specifically inhibit the late S or early G2 phase of the cell cycle (Svoboda and Reddy, 1972; Samuel and Jago, 1975). Results from our laboratory showed that the macrocyclic α,β-unsaturated diester PAs (seneciphylline, riddelliine, retrorsine, and senecionine) induced the most potent cross-linking activity, and also the most potent inhibition of colony formation, suggesting that the DNA cross-links have a direct effect on mitosis (Hincks *et al.*, 1991; Kim *et al.*, 1993).

Several PAs have also been reported to be genotoxic in the *Salmonella*/mammalian microsomal (Ames) assay (Yamanaka *et al.*, 1979) or in the unscheduled DNA synthesis assay (Green *et al.*, 1981; Mirsalis *et al.*, 1993). Mori *et al.* (1985) found that 13 of 15 PAs were inducers of unscheduled DNA synthesis in primary hepatocyte cultures. Petry *et al.* (1984) reported that monocrotaline and jacobine induced DNA-DNA cross-links but not single-strand breaks in freshly isolated rat hepatocytes. Seneciphylline and senecionine covalently bind to DNA and have a bifunctional alkylating capability in rat liver, lung, and kidney *in vivo* (Candrian *et al.*, 1985).

Robertson (1982) showed that dehydromonocrotaline formed an adduct at the N^2 position of deoxyguanosine in synthetic oligonucleotides. Similar PA-induced cross-linking site specificity was also reported in synthetic DNA duplexes *in vitro* (Woo *et al.*, 1993). Their results strongly indicated N^2 of deoxyguanosine residues at the sequence 5'-d(CG) are the most common binding site for pyrrole-derived bifunctional electrophiles. However, the exact mechanisms of toxic, carcinogenic, or anti-carcinogenic actions of these compounds are not yet clear.

Pyrrolizidine alkaloid-induced cross-links

Our laboratory investigated how PA structural features influenced DNA cross-links using the alkaline elution assay (Hincks *et al.*, 1991). With 8 PAs of differing stereochemistry and functional groups, potent DNA-DNA and DNA-protein cross-linking abilities were found in cultured bovine kidney epithelial cells with an external metabolizing system. The ranking of the PA-induced DNA-DNA cross-links and DNA-protein cross-links was the macrocyclic α,β-unsaturated diesters (seneciphylline > riddelliine > retrorsine > senecionine), open diester (heliosupine > latifoline), the macrocyclic saturated diester (monocrotaline), and the necine base (retronecine) (Reed *et al.*, 1988; Hincks *et al.*, 1991). Two critical structural determinants for cross-linking activity were the presence of a macrocyclic necic acid ester, and α,β-unsaturation in the macrocyclic diester.

Experimental methods for the investigation of DNA cross-links

There are several strategies for studying of DNA-DNA and/or DNA-protein cross-links. One of these is alkaline elution, which was developed by Kohn and Grimek-Ewig (1973). Alkaline elution is based on the discrimination of various lengths of [^{3}H]-thymine labeled DNA by filter retention. Different filter systems can be used for detection of different types of DNA damage. Polycarbonate filters are used for detecting single strand breaks, and polyvinylcarbonate filters are used for detecting DNA-DNA and/or DNA-protein cross-links.

Recently, more specific molecular biological approaches have been developed to characterize the sequence-specific interaction with DNA and genotoxics. The most important of these is the DNA mobility shift or "band-shift" assay (O'Connor and Fox, 1989; Banjar *et al.*, 1984; Oleinick *et al.*, 1987), which

is based on the retarded mobility of DNA-DNA or DNA-protein complexes compared to untreated, non-cross-linked DNA during polyacrylamide or agarose gel electrophoresis. This method is very useful to study DNA-DNA or DNA-protein cross-links, and DNA cross-linked sites and sequence specificity in plasmid DNA using restriction endonucleases. Cellular DNA cross-linked proteins can also be characterized by 2-D gel electrophoresis in which proteins can be separated on the basis of isoelectric point in the first dimension, followed by separation on the basis of molecular weight using SDS-PAGE (O'Farrell, 1975; Ames and Nikaido, 1976; O'Farrell *et al.*, 1977).

The purpose of this study was to determine the molecular mechanisms of PA toxicity and examine which PA structures are critical for their molecular and cytopathological effects using mammalian cells or target DNA systems. This study will contribute to a better understanding of how PAs exert their acute and chronic toxicity, carcinogenesis, and potential anti-carcinogenesis.

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CHAPTER II

STRUCTURAL INFLUENCES ON PYRROLIZIDINE ALKALOID-INDUCED CYTOPATHOLOGY

ABSTRACT

Pyrrolizidine alkaloids (PAs), which are common constituents of hundreds of plant species around the world, have been reported to have cytotoxic, carcinogenic, anti-neoplastic, or genotoxic activity *in vivo* and *in vitro*. The mechanisms of these biological toxicities are not yet clear. The ability of eight PA congeners to inhibit mitosis and induce megalocyte formation in cultured bovine kidney epithelial (MDBK) cells was studied to examine possible structural influences on these endpoints. Representatives of the three PA structural groups, the macrocycles (seneciphylline, senecionine, riddelliine, retrorsine, monocrotaline), open diesters (heliosupine, latifoline), and a necine base (retronecine), were co-cultured for 2 hr with NADPH generating system using the rat liver S9. Macrocylic PAs with α,β-unsaturation (seneciphylline, senecionine, riddelliine, retrorsine) showed a dose-dependent inhibition of colony formation at 50, 100, and 300 μM and induction of megalocytosis at 500 μM. Colony growth resumed 3 weeks after removal of PAs at 50 and 100 μM, and normal cellular morphology returned 5 or 6 weeks after removal of PAs at 500 μM. The saturated macrocyclic (monocrotaline) and open diesters (heliosupine and latifoline) elicited only a slight inhibition of colony formation and had no effect on cellular morphology at 500 μM. The necine base (retronecine) had no effect on either colony formation or cell morphology. Pyrrolic PAs (dehydrosenecionine, dehydromonocrotaline, dehydroretronecine) were more active in inhibition of colony formation than their parent compounds and were potent inducers of

abnormal cellular morphology at 500 μM. An N-oxide metabolite, indicine-N-oxide, was completely inactive. The results support previous studies showing that there are structural influences on PA-induced cytopathological effects.

INTRODUCTION

Pyrrolizidine alkaloids (PAs), which are common plant constituents, have been reported to have potent anti-mitotic, hepatotoxic, and genotoxic activity *in vivo* and *in vitro* (Petry *et al.*, 1984, 1986: Kuhara *et al.*, 1980; Mattocks, 1968; Mori *et al.*, 1985; Schoental, 1975). Pyrrolizidine alkaloids induce severe acute or chronic liver and lung toxicities (Bull and Dick, 1959; McLean, 1970). Mattocks (1968) suggested that pyrroles are also potent anti-mitotic agents.

Pyrrolizidine alkaloids require metabolic activation to exert their biological action. Activated PAs then interact with cellular constituents (Green *et al.*, 1981; Segall *et al.*, 1985; Griffin and Segall, 1986). Pyrrolizidine alkaloids are metabolically converted by cytochrome P-450 mixed-function oxidase to two major products: highly reactive and toxic pyrrole and the less toxic N-oxide (Mattocks and White, 1971). Pyrrolizidine alkaloids are classified into four major groups: macrocyclic diesters, open diesters, monoester, and necine base. The biological toxicities of PAs are thought to be related to α,β-unsaturation in the structure of the necic acid esters and C1-C2 unsaturation of the necine base (Bull *et al.*, 1968; Mattocks, 1986; McLean, 1970; Hincks *et al.*, 1991) and size of the macrocyclic ring could also be important. We demonstrated (Hincks *et al.*, 1991) that a group of 12-membered ring macrocyclic PAs bearing an α,β-unsaturated ester function in the macrocycle were more potent DNA cross-linkers than was monocrotaline, which contains an 11-membered macrocycle

and lacks the α,β-unsaturation. These PAs were also more potent than open diesters and a necine base, retronecine.

Some PAs induce DNA-protein and/or DNA-DNA interstrand cross-links in several *in vivo* or *in vitro* systems (Petry *et al.*, 1984, 1986; Candrian *et al.*, 1985; Black and Jago, 1970; Hincks *et al.*, 1991; Niwa *et al.,* 1991; Reed *et al.*, 1988). Pyrrolizidine alkaloids have been studied as chemotherapeutic agents (Letendre *et al.*, 1981; King *et al.*, 1987). For example, indicine N-oxide, derived from *Heliotropium indicum*, has been found to have anti-tumor activity against leukemia and solid tumors. Many PAs are also mutagenic in the *Salmonella I* mammalian microsomal assay (Yamanaka *et al.*, 1979) and are genotoxic in the unscheduled DNA synthesis assay (Green *et al.*, 1981; Mori *et al.*, 1985).

In PAs-exposed animals, enlarged cells or "megalocytes" are a common pathologic sequela and are postulated to be a result of the anti-mitotic action of PAs (Bull and Dick, 1959; Tandon *et al.*, 1978; McLean and Mattocks, 1980). Lasiocarpine (Svoboda and Reddy, 1972) and its pyrrolic metabolite, dehydroheliotridine, have been shown to specifically inhibit late S or early G2 phase of the cell cycle (Samuel and Jago, 1975).

We have recently identified structural features that appear to be important in the DNA cross-linking capabilities of PAs (Hincks *et al.*, 1991). The purpose of the present study was to determine whether there are similar structure-activity relationships with respect to the cytotoxic and anti-proliferative action of a similar group of PAs *in vitro*.

MATERIALS AND METHODS

Chemicals. Seneciphylline and senecionine were isolates from *Senecio vulgaris* (common groundsel) and *Senecio triangularis* and furnished by Segall

(1979 a; b). Retronecine was made by hydrolysis of riddelliine. Riddelliine was an isolate from *Senecio riddellii* furnished by Molyneux *et al.* (1979). Monocrotaline was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Heliosupine was an isolate from *Cynoglossum officinale* (hounds-tongue) furnished by Knight *et al.* (1984). Latifoline was isolated and identified from *H. floribunda* by Hagglund *et al.* (1985). Indicine N-oxide was provided by Matthew Suffness of the National Cancer Institute. Dehydro PAs (dehydrosenecionine, dehydromonocrotaline, dehydroretronecine) were prepared from their parent compounds (senecionine, monocrotaline, retronecine) by the method of Mattocks *et al.* (1989) and the purity of products was checked by ^{1}H NMR (Culvenor *et al.*, 1970). The chemical structures of PAs used in this study are shown in Figure II-1.

Cell Culture and Treatment Conditions. The Madin Darby bovine kidney (MDBK) epithelial cell line, strain CCL 22, was obtained from American Type Culture Collection (Rockville, MD). The cells were grown in Eagle's minimum essential medium with nonessential amino acids, L-glutamine (Gibco, Grand Island, NY) and supplemented with 1 mM sodium pyruvate and 10% iron-supplemented calf serum (Hyclone laboratories, Logan, UT) at 37 ^{o}C in an atmosphere of 97% air and 3% CO_2. Cells (passage 112-130) were seeded on 60-mm culture dishes or on 10-mm cover slips 22 hr prior to treatment. The medium was replaced with fresh medium containing 50 - 500 μM of the PA with an external metabolizing system which consisted of a NADPH-generating system and rat liver S9 for the parent compounds or without external metabolizing system for the metabolites and incubated at 37 ^{o}C for 2 hr. Alkaloids were dissolved in dimethylsulfoxide (DMSO) and were added directly to the medium in a volume that did not exceed 1% of the total volume. For the parent PAs, an

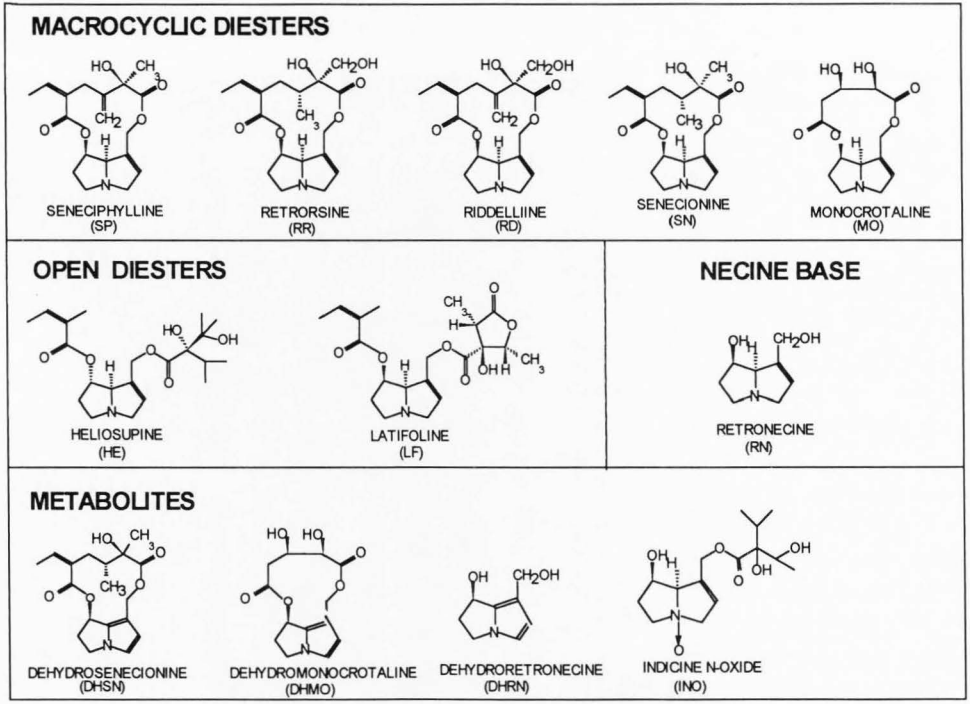

Fig. II-1. Chemical structures of pyrrolizidine alkaloids and their metabolites examined in this dissertation.

external metabolizing system was used which consisted of a NADPH-generating system and rat liver S9 as described by Madle *et al.* (1986). The S9 was prepared from Aroclor 1254-induced male Sprague-Dawley rat's liver according to Maron and Ames (1983). These fractions (protein content ca. 45 mg/ml) were pooled, stored at -80 ^{o}C, and the same batch was used in all experiments. The components of the S9 mix were 0.67 mM NADP, 0.83 mM glucose-6-phosphate, 1.33 mM $MgCl_2$, 10 mM phosphate buffer, with 2% rat liver S9 (all as final concentration in the culture medium).

Colony-Forming Efficiency and Microscopic Examination. Colony-forming efficiency was determined by seeding 100 cells on 60-mm culture dishes 22 hr prior to treatment with PAs. The examination of megalocyte formation and morphological changes was determined by seeding 500 cells on 10-mm cover slips 22 hr prior to treatment with PAs. After exposure to the PA for 2 hr, the medium was replaced with fresh medium, which contained 40 μg/ml gentamicin and 2.5 μg/ml amphotericin B, and cells were incubated for 6 weeks with medium changed every third day. At selected intervals, cells were stained with 0.2% methylene blue. Colony-forming efficiency was calculated by counting the number of growing colonies in the treated group divided by the number of colonies in the control group. All experiments were performed in duplicate and repeated at least twice. A subset of cultures was established for morphological and microscopic examination. For examination of megalocyte formation and morphological changes, the treated cells on the cover slips were fixed with methanol, stained with 0.2% methylene blue, and examined by light microscopy. Measurement of cell area was done with a Leitz microscope and computer-assisted planimetry (JAVA, Jandel Scientific) at 160X. Four fields were randomly selected and digitized for measurement, resulting in nine to twenty areas per the

selected time interval. Cell areas were measured by tracing the perimeters of digitized images of whole cells followed by computer assisted calculation of cell profile area.

Statistical Evaluations. Data were analyzed by one-way analysis of variance, and where appropriate, a *post hoc* Fischer's LSD was conducted to determine significance between groups. A level of $p < 0.05$ was chosen as significant.

RESULTS

Pyrrolizidine alkaloids were not cytocidal to the MDBK cell cultures because cells which were exposed to the highest pyrrolizidine alkaloid concentration, 500 μM, were viable as determined by trypan blue dye exclusion (data not shown). The effect of various PAs on the colony-forming efficiency of MDBK cells is shown in Figures II-2 and II-4. Of those examined, the macrocyclic α,β- unsaturated diesters (senecionine > seneciphylline > riddelliine > retrorsine) were the most potent in inhibiting colony formation. Cells resumed normal growth at 50 and 100 μM, 2 or 3 weeks after removal of these PAs (senecionine, seneciphylline, retrorsine, riddelliine) from culture, respectively. The highest concentration of these PAs (300 μM) caused a complete cessation of cell growth for 6 weeks (Fig. II-2). Cell growth was only slightly suppressed following exposure to 500 μM of monocrotaline, heliosupine, or latifoline, and normal growth resumed 2 weeks after removal of compound. The necine base retronecine and indicine N-oxide were inactive in our cell system. The pyrrolic metabolites, dehydrosenecionine, dehydromonocrotaline and dehydroretronecine, were more active in inhibiting colony formation than were their corresponding parent compounds at the same concentration (Fig. II-3, II-4).

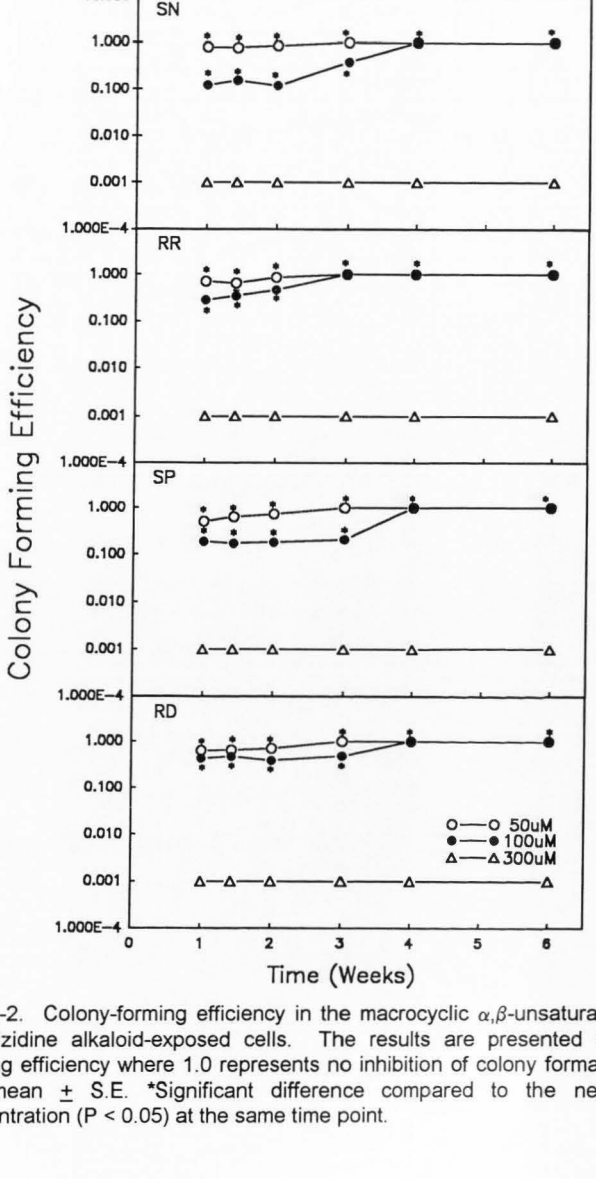

Fig. II-2. Colony-forming efficiency in the macrocyclic α,β-unsaturated diester pyrrolizidine alkaloid-exposed cells. The results are presented as colony-forming efficiency where 1.0 represents no inhibition of colony formation. Data are mean $\pm$ S.E. *Significant difference compared to the next highest concentration ($P < 0.05$) at the same time point.

The morphologic appearance of cells treated with selected PAs is presented in Figure II-5. In cells exposed to 500 μM of the macrocyclic α,β-unsaturated diester PAs, enlarged cellular morphology appeared 3 days after removal of the PA (data not shown). In these cells, the cell area was increased nearly 10-fold compared to control cells, and the cytoplasm of the megalocytes was thin and stretched, and the nucleus appeared condensed (Fig. II-5, II-6). A return to normal cellular morphology occurred after 4 weeks, but some megalocytes persisted adjacent to normal cells up to 6 weeks after treatment. In monocrotaline-treated cultures, a few megalocytes appeared, but the cell size was only sightly increased and returned to normal within 2 weeks. Treatment with heliosupine or latifoline caused slight megalocytosis and the size of the cells returned to normal within 1 or 2 weeks after treatment. Treatment with retronecine, or indicine N-oxide, did not cause any detectable cytopathological changes. Compared to their parent compounds, the pyrrolic metabolites dehydrosenecionine, dehydromonocrotaline, or dehydroretronecine were more potent inhibitors of cell division (Fig. II-4) and potent inducers of enlarged cells although persistent megalocytosis was seen only in cultures exposed to dehydromonocrotaline (Fig. II-5, II-6).

DISCUSSION

In a previous study from our laboratory, in which we examined the relative DNA cross-linking abilities of pyrrolizidine alkaloids in the same cell system, we found that PAs with the 11-membered macrocyclic α,β-unsaturated ester group of PAs (senecionine, seneciphylline, retrorsine, riddelliine) were the most potent DNA cross-linkers (Hincks *et al.*, 1991). Several workers have also reported PA-induced DNA-protein and/or DNA-DNA cross-links, and PA-induced cytotoxicity

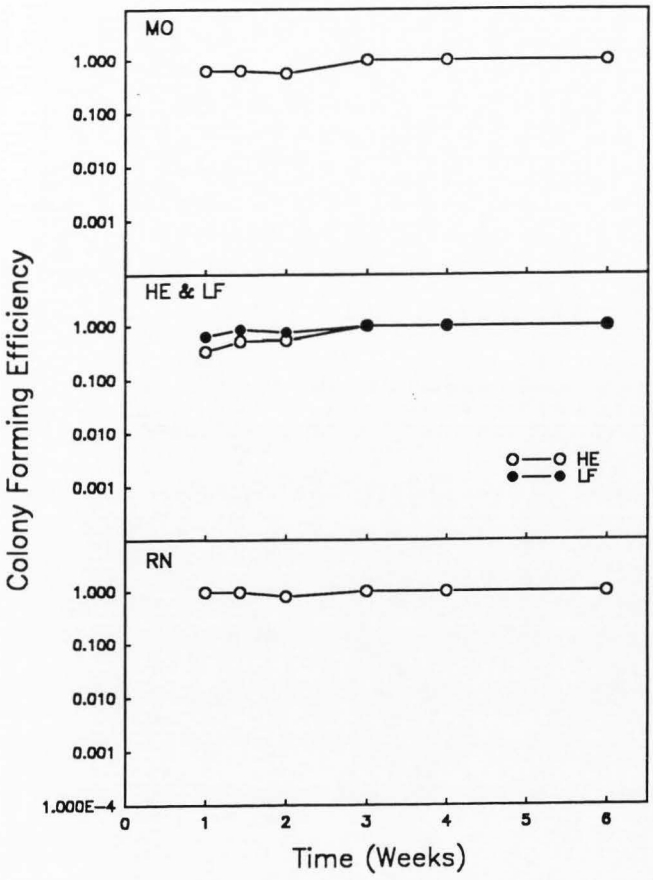

Fig. II-3. Colony-forming efficiency of cell cultures exposed to 500 μM of the macrocyclic α,β-saturated diester, open diester, and necine base pyrrolizidine alkaloid-exposed cells.

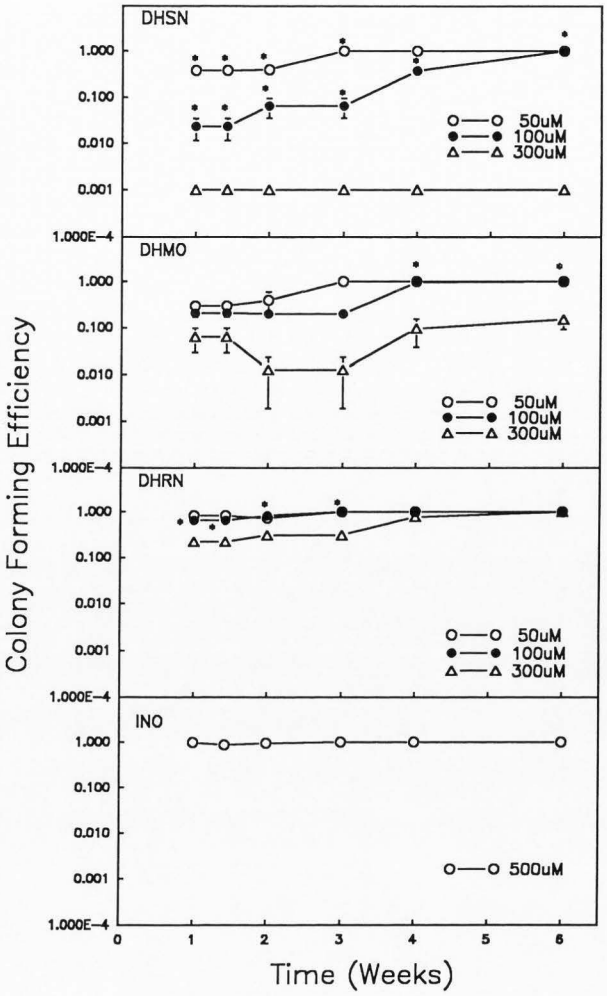

Fig. II-4. Colony-forming efficiency of cell cultures exposed to 500 μM of the pyrrolic metabolites (DHSN, DHMO, DHRN) and indicine N-oxide (INO). *Significant difference compared to the next highest concentration at the same time point ($P < 0.05$).

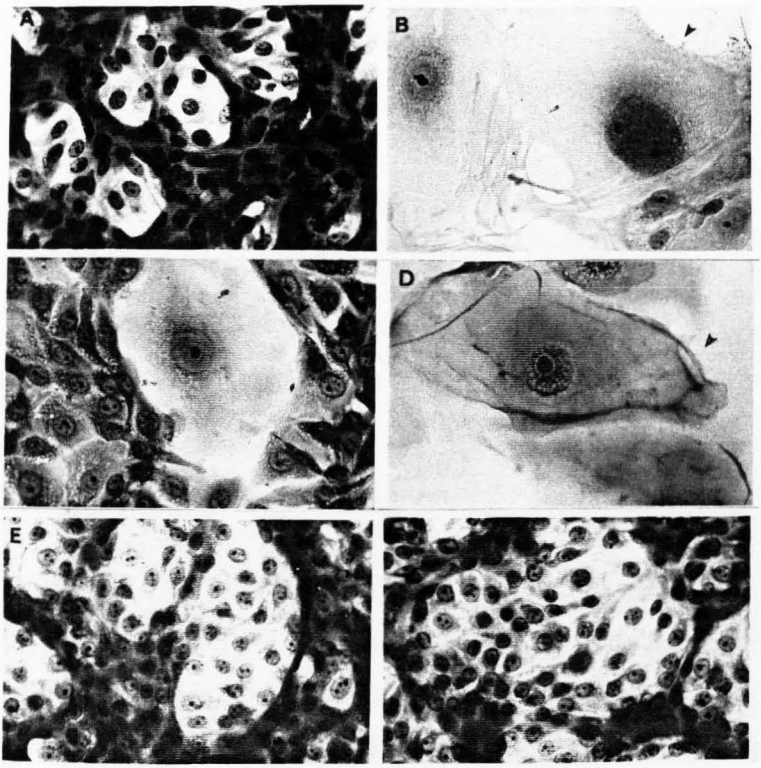

Fig. II-5. Phase-contrast photomicrographs of pyrrolizidine alkaloid-exposed cells. Cells were exposed to 500 μM of pyrrolizidine alkaloids, washed, supplemented with fresh medium, and then cultured for 6 weeks. Cells were exposed to (a) DMSO, (b) seneciphylline (SP), (c) retrorsine (RR), (d) riddelliine (RD), (e) monocrotaline (MO), and (f) retronecine (RN). X400.

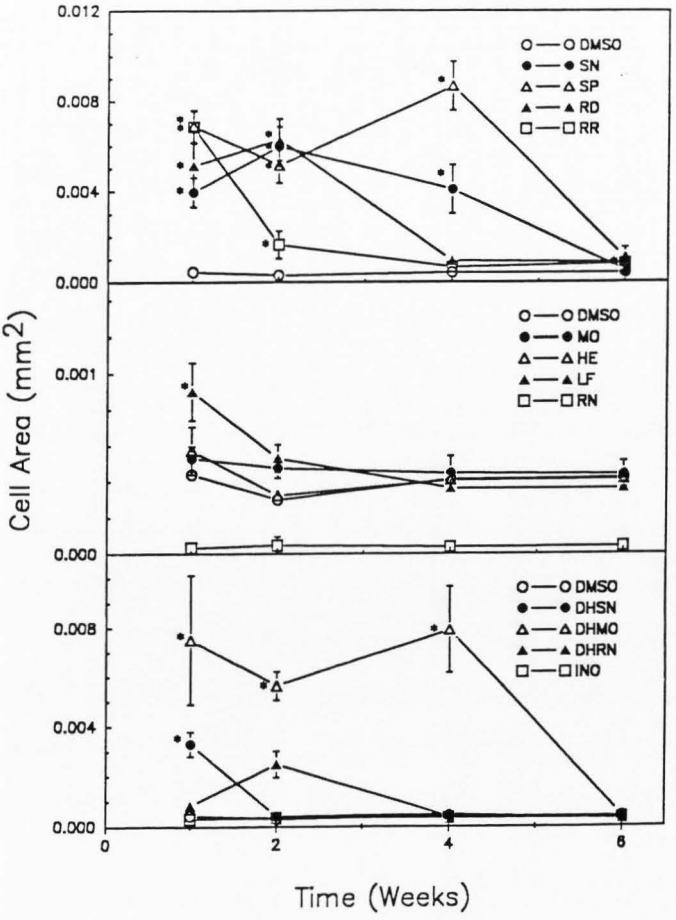

Fig. II-6. Changes in cell area due to 500 μM of pyrrolizidine alkaloid treatment. The exposed cells were washed, supplemented with fresh medium, and cultured for 6 weeks. The results are represented as cell areas (mm^2) by computer-assisted calculation of cell profile area. *Significant difference ($P < 0.05$) compared to control cells.

in vivo or *in vitro* systems (Black and Jago, 1970; Cardrian *et al.*, 1985; Griffin and Segall, 1986; Hayes *et al.*, 1984, Lieber *et al.*, 1981; Moore *et al.*, 1989; Petry *et al.*, 1984, 1986; Powis *et al.*, 1979). The present data indicate that those PAs, which were shown in previous studies to be potent DNA cross-linkers, are also potent inducers of cytopathologic changes. Therefore, taken together with previous work, the present data support the hypothesis that DNA cross-linking activity is an important mechanism underlying PA bioactivity. Pyrrolizidine alkaloids with the macrocyclic α,β-unsaturated diester were by far the most potent inducers of megalocytosis. Monocrotaline, the 12-membered macrocyclic that does not have an α,β-unsaturated ester group, showed only weak effects as did the open diesters, heliosupine and latifoline. The necine base, retronecine, which is not a potent DNA cross-linker, was also inactive in the present study as was indicine N-oxide.

A key to the structure-activity relationships was the discovery that pyrrolic PAs were more potent inhibitors of colony formation than their parent compounds. Recently, Müller *et al.* (1992) demonstrated the importance of metabolic activation in the genotoxic activity of PAs. Particularly striking was the fact that dehydromonocrotaline was highly active, while the parent compound, which was activated by the external metabolizing system, had very low activity. This suggests that the structural features which distinguish monocrotaline from the other macrocyclic pyrrolizidine alkaloids are not important determinants of biological activity itself, but are related to proper formation of the pyrrolic metabolite. It is likely that the external metabolizing system converts monocrotaline more rapidly to the N-oxide than to the dehydro form, while the opposite is the case for seneciphylline, retrorsine, riddelliine, and senecionine. This is in line with the relative *in vitro* conversion by rat liver microsomal

enzymes of monocrotaline to N-oxide or pyrrole compared to retrorsine and senecionine (Mattocks and Bird, 1983). It is also possible that pyrrolic PAs are hydrolyzed in our system to dehydroretronecine (Mattocks, 1986).

Those PAs which were most active in suppressing colony formation were also most active in inducing megalocytosis. Pyrrolizidine alkaloid-induced mitotic inhibition and formation of megalocytosis have been observed *in vivo* or *in vitro* (Armstrong *et al.*, 1972; Bull and Dick, 1959; Mattocks, 1986; Mattocks and Legg, 1980; Schoental and Magee, 1957; 1959). A series of PAs were shown to induce megalocytosis that persisted for up to 2 weeks in cultured liver cells and that the time frame of cell enlargement coincided with that of mitotic inhibition (Mattocks and Legg, 1980). Megalocytes with large and abnormally shaped nuclei are frequently seen in livers of PA-exposed animals, which is consistent with an inhibition at the late S or G2 phase of the cell cycle, a condition in which synthesis occurs but not cell division (Samuel and Jago, 1975).

The mechanism of PA-induced megalocytosis has long been presumed to involve mitotic inhibition. Jago (1969) suggested that megalocyte formation by pyrrolizidine alkaloids is due to the persistent inhibition of mitosis (Jago, 1969; Peterson, 1965). However, Armstrong *et al.* (1972) found that lasiocarpine induced a partial inhibition of DNA synthesis in cultured human fetal liver and lung cells but the phenomenon was not directly related to mitotic inhibition. Megalocytes induced by lasiocarpine were able to synthesize DNA but failed to divide (Armstrong *et al.*, 1972).

In my system, cells exposed to the most potent PAs regenerated approximately 6 weeks after treatment. Similarly, Schoental and Magee (1959) reported that megalocytes that persisted for up to 2 years appeared in the livers of PA-treated rats. Megalocytosis is also stimulated either by partial

hepatectomy, or following administration of thioacetamide or a necrogenic agent such as dimethylnitrosamine (Downing and Peterson, 1968; Samuel and Jago, 1975; Mattocks, 1978). Culvenor *et al.* (1976) reported that chronic treatment with dehydromonocrotaline or dehydrosenecionine caused severe liver damage in the rat, but there was no evidence of megalocyte formation. My work demonstrated that dehydromonocrotaline was a more potent inducer of megalocytes compared to monocrotaline that was activated with an external metabolic system. However, dehydrosenecionine or dehydroretronecine were only mildly active in inducing megalocytes. The explanation for this observation is not clear, but it is possible that these reactive pyrrolic (dehydro) PAs bind with nonnuclear constituents in the cell.

One PA examined here, indicine N-oxide, has been investigated for its anti-tumor activity. Poster *et al.* (1981) suggested that the anti-tumor activity of indicine N-oxide is mediated via an anti-mitotic effect. Letendre *et al.* (1984) also studied anti-tumor activity of indicine N-oxide in human acute leukemia and reported myelo-suppression, hepatotoxicity, and anti-leukemic activity. My results would indicate, at least in the cell system employed, that indicine N-oxide is only modestly active in mitotic inhibition compared to other PAs. This would be expected because indicine N-oxide must first be reduced to indicine before it can be activated to the pyrrolic form of metabolite, which is responsible for toxicity (Powis *et al.*, 1979). It is also possible that in this cell system, indicine N-oxide does not enter the cell efficiently.

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CHAPTER III

CHARACTERIZATION OF DNA-DNA CROSS-LINKS BY PYRROLIZIDINE ALKALOIDS

ABSTRACT

Pyrrolizidine alkaloids (PAs), which are common constituents of hundreds of plant species around the world, are potent cross-linkers with DNA and/or protein, and are also cytotoxic, carcinogenic, and anti-carcinogenic. In this study, I investigated PA-induced DNA-DNA cross-links induced by PAs as assessed by alkaline elution, electrophoretic band shifting, primer extension using PCR, and differential restriction endonuclease digestion. At 300 and 500 μM, chemically activated PAs were potent DNA cross-linkers as assessed by alkaline elution with a potency order of dehydrosenecionine > dehydromonocrotaline > dehydroseneciphylline > dehydroriddelliine. At 500 μM, the DNA cross-linking activity of these metabolites exceeded that of their parent compounds. Dehydroretronecine and indicine N-oxide did not induce detectable DNA cross-links. Macrocyclic diester pyrroles also induced DNA-DNA cross-links in *Bst* EII-digested λ-phage DNA or *Bam* HI-digested pBR322 DNA in a dose-dependent manner. Dehydrosenecionine and dehydromonocrotaline were potent DNA-DNA interstrand cross-linkers but dehydroretronecine and indicine N-oxide were not. Because dehydrosenecionine showed a preference for cross-linking at the *Ava* II, *Fok* I, or *Taq* I recognition site in pBR322 plasmid DNA, this appeared to cross-link 5'-d(GG) or 5'-d(GA) sequences most frequently. The macrocyclic diester pyrroles such as dehydrosenecionine, dehydroseneciphylline, dehydroriddelliine, and dehydromonocrotaline were the most potent inducers of DNA-DNA cross-links, indicating that the cross-linking

potency is inherent to the PA and that the cross-link formation does not proceed through a common reactive intermediate.

INTRODUCTION

Pyrrolizidine alkaloids, which are found in a variety of plants including *Senecio, Crotalaria, and Heliotropium,* are hepatotoxic, carcinogenic, mutagenic, or anti-carcinogenic *in vivo* and *in vitro* (1-7). Humans and other animals are susceptible to the toxic effects of PAs. Monocrotaline and jacobine induce DNA-protein and DNA interstrand cross-links but not DNA strand breaks in rat liver *in vivo* (8, 9). Monocrotaline pyrrole induces DNA cross-links and inhibits cell proliferation as well as morphologic, hemodynamic, and biochemical changes in porcine pulmonary artery endothelial cells and rat lung (10, 11).

Pyrrolizidine alkaloids are metabolized by the cytochrome P-450 mixed-function oxidase, resulting in a highly reactive and toxic pyrrole, and N-oxide, which is less toxic (12-15). The PA-induced cytopathologic changes *in vivo* and *in vitro* are due to the anti-mitotic effects of PAs (16-19), which inhibit the late S or early G2 phase of the cell cycle (20, 21).

Dehydroretronecine reacts with purine and pyrimidine nucleotides. The N-2 site of guanosine, the N-6 site of adenosine, and the O-2 site of uridine and deoxythymidine have been identified as targets (22-24). Dehydroretronecine also cross-links pBR322 plasmid DNA and M13 viral DNA (25). Our laboratory has shown that the macrocyclic α,β-unsaturated diesters such as seneciphylline, riddelliine, retrorsine, and senecionine are potent DNA cross-linkers in cultured cells (26). These PAs were also potent inhibitors of colony formation and inducers of cytopathologic changes, an indication that PA-induced DNA cross-linking activity is closely related to the inhibition of cell proliferation (19, 26).

The purpose of this study was to characterize the nature of PA-induced DNA cross-links and to determine if there are structural features that influence DNA-DNA cross-link formation by PAs. Several representative parent PAs and chemically activated pyrrolic PAs were used to determine possible structure-activity relationships.

MATERIALS AND METHODS

Materials

Chemicals. Senecionine, seneciphylline, retrorsine, and retronecine were isolates from *Senecio vulgaris* and *Senecio triangularis* and furnished by Segall (27, 28). Retronecine was made by hydrolysis of riddelliine. Riddelliine was an isolate from *Senecio riddellii* furnished by Molyneux et al. (29) and monocrotaline was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Indicine N-oxide was provided by Matthew Suffness of the National Cancer Institute. Pyrrolic metabolites (dehydrosenecionine, dehydroseneciphylline, dehydroriddelliine, dehydromonocrotaline, and dehydroretronecine) were prepared from their parent compounds (senecionine, seneciphylline, riddelliine, monocrotaline, and retronecine, respectively) by the method of Mattocks *et al.* (30), and purity was confirmed by ^{1}H NMR (31). Pyrrolic metabolites were dissolved in DMSO, and stored at -80 ^{o}C in capped amber vials filled with inert gas. The chemical structures of PAs used in this study are shown in Figure 1.

Cis-dichlorodiammine platinum (II), mitomycin C, lysozyme (EC 3.2.1.17), RNAse I (EC 3.1.2.75), proteinase K (EC 3.4.21.14), ampicillin, tetracycline, and ethidium bromide were obtained from Sigma Corporation (St. Louis, MO). Eagle's Minimum Essential Medium was purchased from Sigma Corporation (St.

Louis, MO) and iron supplemented calf serum was purchased from Hyclone Laboratories, Inc. (Logan, UT). Methyl-[^{3}H]-thymidine was purchased from New England Nuclear (Boston, MA). *Bst* EII-digested λ-phage DNA was obtained from New England BioLabs (Beverly, MA). Bacto-tryptone and bacto-yeast were purchased from Difco (Detroit, MI). Restriction endonucleases (*Ava* II, *Eco* RII, *Fok* I, *Hinf* I, *Mbo* II, *Taq* I) were obtained from Promega Corporation (Madison, WI). *Taq* polymerase, dATP, dTTP, dCTP and dGTP were obtained from Perkin Elmer Cetus (Norwalk, CT). The primers used for the polymerase chain reaction (PCR) were synthesized by the Biotechnology Center (Utah State University, Logan, UT) via an oligonucleotide synthesizer (Applied Biosystem 380B DNA synthesizer). Swinex filter holders and polyvinylchloride filters were obtained from Millipore Corporation (San Francisco, CA).

Methods

Cell Culture and Treatment Conditions. Madin Darby bovine kidney (MDBK) epithelial cells, strain CCL 22 (American Type Culture Collection, Rockville, MD), were grown in Eagle's Minimum Essential Medium with nonessential amino acids, L-glutamine, 1 mM sodium pyruvate, and 10% iron supplemented calf serum at 37 ^{o}C in an atmosphere of 97% air and 3% CO_2. Cells (passage 112-130) were seeded in 60-mm diameter culture dishes for alkaline elution assay 24 hr prior to labeling with methyl-[^{3}H]-thymidine. The medium was then replaced with fresh medium, and treated with 300 or 500 μM of PAs or pyrroles. Parent PAs were activated with an external metabolizing system consisting of a NADPH-generating system and rat liver S9 as described by Hincks *et al.* (26). Pyrrolizidine alkaloids were added directly into the medium in a volume that did not exceed 1% of total volume. Cell viability for all treatment

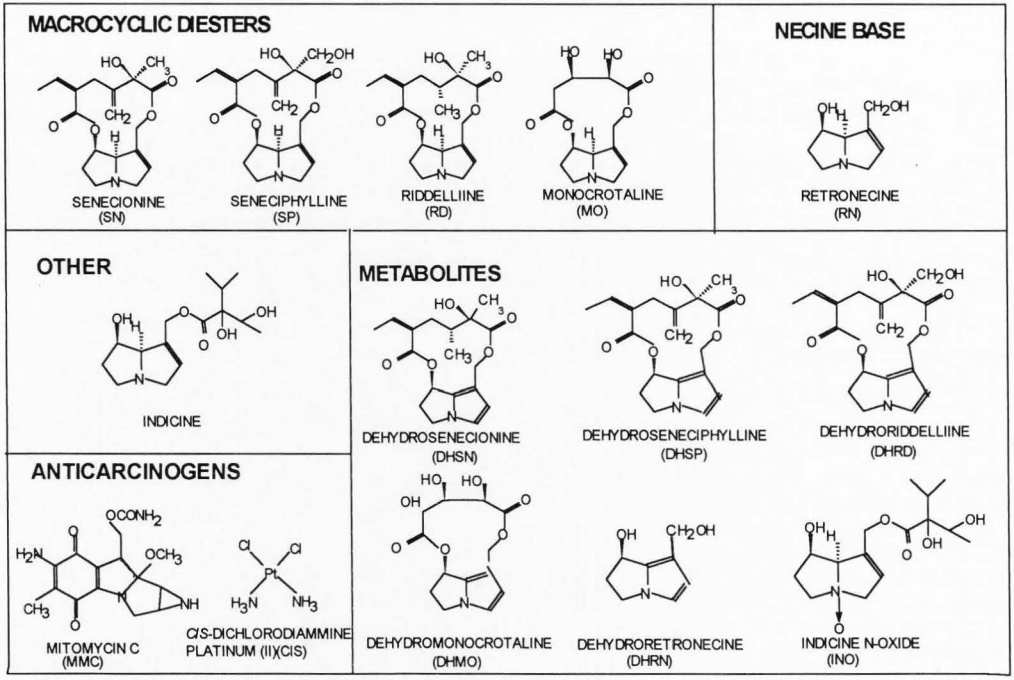


Fig.III-1. Chemical structures of PA, pyrrolic PAs, and anti-carcinogens examined in this dissertation.

groups exceeded 90% as determined by trypan blue dye exclusion (data not shown).

Cellular DNA Cross-links by Alkaline Elution. The PA-treated cells were washed and the medium was replaced with cold PBS (0.12 M NaCl, 4 mM KCl, 0.5 mM Na_2HPO_4, 0.1 mM KH_2PO_4, pH 7.4). The cells were then exposed to 1000 rad of γ-irradiation at 0 ^{o}C using a ^{137}Cs irradiator at a dose rate of 171 rad/min. The source was calibrated using the Fricke's ferrous sulfate dosimetry method (32). Alkaline elution was conducted as described previously (26).

DNA-DNA Cross-links in λ-phage or pBR322 DNA. pBR322 plasmid DNA was amplified in bacterial *E. coli host (*strain RR1) by overnight culture at 37 ^{o}C in LB medium (1% bacto-tryptone, 0.5% bacto-yeast, and 0.89 M NaCl) containing 15 μg/ml tetracycline and 50 μg/ml ampicillin. pBR322 plasmid DNA was purified by the rapid alkaline method (33). Briefly, *E. coli* cells were harvested by centrifugation at 5000 rpm for 10 min at 4 ^{o}C and the pellet was lysed (25 mM NaCl, 10 mM EDTA, 15% sucrose, 2 mg/ml lysozyme) for 20 min at 0 ^{o}C. The lysate was incubated in 0.04 M NaOH with 0.04% SDS for 10 min, and 0.075 M $NaC_2H_3O_2$, pH 4.6 was added. The mixture was incubated for 20 min at 0 ^{o}C followed by centrifugation at 10,000 rpm for 15 min. RNAse (1 mg/ml) was added to the supernatant and the mixture was incubated for 20 min at 37 ^{o}C. The sample was purified by phenol : chloroform (1:1, v/v) extraction and precipitated with ethanol. The DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4), and repurified by adding 0.4 M NaCl and 6.5% (v/v) polyethylene glycol for 1 hr at 0 ^{o}C, then centrifuged at 10,000 rpm for 10 min. The plasmid was dissolved in TE buffer, pH 7.4 (10 mM Tris-HCl, 1 mM EDTA). *Bam* HI-digested (1 U/μg DNA for 1 hr at 37 ^{o}C) pBR322 plasmid DNA or *Bst* EII-digested λ-phage DNA (NEB) was treated with the pyrrolic

(dehydrosenecionine, dehydromonocrotaline, dehydroretronecine) or N-oxide metabolite (indicine N-oxide) at DNA : PA (w:w) ratios of 1:0.5, 1.0, 2.0 for 2 hr at 0 ^{o}C. A similar treatment ratio of *cis*-dichlorodiammine platinum(II) or mitomycin C was used as a positive cross-link control. After treatment, DNA samples were precipitated with ethanol to remove excess cross-linkers. The DNA-DNA cross-links were analyzed on 1% agarose gels stained with ethidium bromide (34). The observed DNA band shifting was photographed by a Polaroid MP-4 camera system using Polaroid Type 667 film.

Pyrrolizidine Alkaloid-induced Interruption of Primer Extension by PCR. pBR322 plasmid DNA was incubated with pyrrolic metabolites (dehydrosenecionine, dehydroseneciphylline, dehydroriddelliine, dehydroretronecine), indicine N-oxide or known DNA cross-linkers (*cis*-dichlorodiammine platinum(II), mitomycin C) for 2 hr at 0 ^{o}C and precipitated with ethanol. The treatment ratios (DNA:PA, w:w) were 1 : 0.001, 0.01, 0.1, 0.25, 0.5, 1.0, and 2.0.

The treated pBR322 plasmid DNA was then used as a template for PCR. Two primers, one complementary to the forward sequences from the *Pst* I recognition site (5' GCT AGA GTA AGT AGT TCG CC 3'), and one complementary to the reverse sequences from the *Bam* HI recognition site (5' CAC GAT GCG TCC GGC GTA GA 3') were used. The reaction mixture (50 μl), which consisted of 4 ng of template DNA, 100 μM of each primer, 1 mM of each dNTP (dATP, dCTP, dGTP, dTTP), *Taq* polymerase buffer, and 1.25 U of *Taq* polymerase, was cycled for 1 min 10 sec at 55 ^{o}C, 1 min 20 sec at 70 ^{o}C, and 1 min 10 sec at 93 ^{o}C for 40 cycles in a DNA thermal cycler (EricComp, San Diego, CA). The PCR products were separated on 1% agarose gels and then stained with ethidium bromide.

Pyrrolizidine Alkaloid-induced Interruption of Restriction Endonuclease Digestion in pBR322 DNA. Circular or supercoiled double-stranded pBR322 plasmid DNA and *Bst* EII-digested λ-phage DNA, which contained 13 fragments of linear double-stranded DNA, were treated with PA for 2 hr at 0 °C, and precipitated with ethanol. The DNA : PA ratios used were 1:0.5 and 1:1 (w:w). The DNA was then digested by 3 U/μg DNA for 3 hr at 37 °C with *Ava* II, *Fok* I, *Hinf* I or *Mbo* II or for 3 hr at 63 °C with *Eco* RII or *Taq* I. The digests were separated on 1% agarose gels stained with ethidium bromide (34). The recognition sites, size of fragments, number of cuts, and recognition sequences of restriction enzymes are shown in Table III-1. *Bst* EII-digested λ-phage DNA was treated with 1:1 ratio (w:w) of PA for 2 hr at 0 °C, precipitated with ethanol, and then digested by 3 U/μg DNA for 3 hr at 37 °C with *Eco* RI.

Statistical Evaluation. Data were analyzed by one-way analysis of variance, and where appropriate, a *post-hoc* Fisher's LSD was conducted to determine significance between groups. The significance level was $P < 0.05$.

RESULTS

Cellular DNA Cross-links by Alkaline Elution

DNA cross-links induced by pyrrolic PAs and their parent PAs are shown in Tables III-2 and III-3. The pyrrolic metabolites such as dehydrosenecionine, dehydroseneciphylline, dehydroriddelliine, and dehydromonocrotaline potently induced potent DNA cross-links at 300 μM and 500 μM. The pyrroles were generally more potent DNA cross-linkers than their parent compounds. The potency of DNA cross-linking activities induced by dehydroretronecine and indicine N-oxide were similar to their parent compounds, retronecine and indicine, respectively. The potency of DNA cross-linking activity induced by

Table III-1. Recognition sites of restriction enzymes and fragment sizes of pBR 322 plasmid.

ENZYME	RECOGITION SITE	NO. OF CUTS	LOCATION OF RECOGNITION SITES	FRAGMENT SIZE (BP)
Ava II	5'G↓GWCC3'	8	799 887 1136 1439 1481 1760 3504 3726	1744 1434 303 279 249 222 88 42
Eco RII	5'CC↓WGG3'	6	130 1059 1442 2500 2621 2634	1857 1058 929 383 121 13
Fok I	5'GGATG(N)$_8$↓3'	12	112 133 987 1032 1681 1770 1848 2007 2148 3346 3527 3814	1175 854 659 649 287 182 181 141 78 66 45 44
Hinf I	5'G↓ANTC3'	10	632 852 1006 1304 1525 2029 2373 2448 2844 3361	1632 517 504 396 344 298 221 220 154 75
Mbo II	5'GAAGA(N)$_8$↓3'	11	464 738 1009 1601 2352 3123 3214 3969 4047 4156 4352	792 755 751 592 494 271 253 196 109 78 70
Taq I	5'T↓CGA3'	7	24 339 652 1127 1268 2573 4017	1444 1305 475 368 315 313 141

W : A or T, and N : any base
Arrows represent the cutting site

dehydromonocrotaline was similar to that of dehydroseneciphylline. Monocrotaline was only as potent as retronecine. About half of the total cross-links induced by pyrrolic PAs were proteinase K-resistant or DNA-DNA cross-links (Table III-3).

DNA-DNA Cross-links in λ-phage or pBR322 DNA

Band shifts induced by pyrrolic PAs in *Bst* EII-digested λ-phage DNA are presented in Figures III-2 and III-3. In this experiment, DNA-DNA cross-links caused an apparent increase in molecular weight of the target DNA evident as a band shifting toward the top of the gel. In this system, dehydrosenecionine induced the potent DNA-DNA cross-links, and did so in a dose-dependent manner (Fig. III-2). Dehydromonocrotaline also cross-linked, but it was less potent than dehydrosenecionine which is evident as less band shifting toward the top of the gel. Dehydroretronecine and indicine N-oxide did not cause band shifting (Fig. III-2). *Cis*-dichlorodiammine platinum (II), used as a positive control, induced band shifting at a potency similar to dehydromonocrotaline. Mitomycin C, which was also included as a standard, did not induce band shifting in this system (Fig. III-2). Compared to pyrrolic metabolites, parent PA itself did not induce any DNA-DNA cross-linking at 500 μM (Fig. III-3). Similarly, in *Bam* HI-digested pBR322 DNA, dehydrosenecionine and dehydroseneciphylline were the most potent inducers of DNA-DNA cross-links (Fig. III-4), especially at the high dose ratio. Dehydromonocrotaline also induced potent DNA-DNA cross-links, but the potency was less than macrocyclic α,β-unsaturated diester pyrroles (dehydrosenecionine, dehydroseneciphylline) and similar potency of dehydroriddelliine at a 1:2 (DNA:PA, w:w) dose ratio. Dehydroretronecine or

Table III-2. Pyrrolizidine alkaloid-induced total DNA cross-links[1,2,3,4]

ALKALOIDS	METABOLITES		PARENT PAs
	300 μ M	500 μ M	500 μ M
Dehydrosenecionine	1.46 $\pm$ 0.14[a]	2.13 $\pm$ 0.24[a]	1.16 $\pm$ 0.18[a]
Dehydroseneciphylline	0.74 $\pm$ 0.17[b,c]	1.59 $\pm$ 0.18[b]	1.05 $\pm$ 0.09[a]
Dehydroriddelliine	0.68 $\pm$ 0.08[b]	1.35 $\pm$ 0.24[b]	1.00 $\pm$ 0.07[a]
Dehydromonocrotaline	1.11 $\pm$ 0.18[c]	1.49 $\pm$ 0.22[b]	0.09 $\pm$ 0.05[b]
Dehydroretronecine	0.18 $\pm$ 0.01[d]	0.18 $\pm$ 0.02[c]	0.12 $\pm$ 0.03[b]
Indicine N-oxide	ND[4]	0.03 $\pm$ 0.01[c]	0.05 $\pm$ 0.01[b]

[1]Treatments were for 2 hr at 37 °C.
[2]Data presented as mean total DNA cross-link indices $\pm$ S.E. from at least three independent experiments.
[3]Cross-link indices with different superscripts are significantly different ($P < 0.05$) by one-way ANOVA and F-test within same concentration.
[4]ND, not detectable.

Table III-3. Pyrrolizidine alkaloid-induced proteinase K-resistant (DNA-DNA) cross-links[1]

ALKALOIDS	300 μM[2,3]	%[4]
Dehydrosenecionine	0.67 ± 0.09[a]	0.46 ± 0.05
Dehydroseneciphylline	0.25 ± 0.04[b]	0.49 ± 0.09
Dehydroriddelliine	0.36 ± 0.05[a,b]	0.55 ± 0.12
Dehydromonocrotaline	0.53 ± 0.04[a,b]	0.51 ± 0.10
Dehydroretronecine	0.08 ± 0.04[c]	0.46 ± 0.26
Indicine N-oxide	ND[5]	ND

[1]As measured by alkaline elution.
[2]Data are presented as mean DNA proteinase K-resistant cross-link indices ± S.E. which we define as DNA-DNA cross-links. Data are from at least three independent experiments.
[3]Values with different superscripts are significantly different ($P < 0.05$) by one-way ANOVA and F-test.
[4]The fraction of DNA-DNA cross-links ± S.E. of total DNA cross-links.
[5]ND, not detectable.

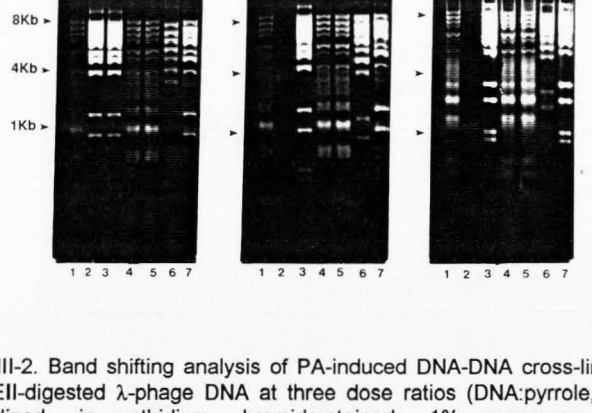


Fig. III-2. Band shifting analysis of PA-induced DNA-DNA cross-links in *Bst* EII-digested λ-phage DNA at three dose ratios (DNA:pyrrole, w:w) visualized in ethidium bromide-stained 1% agarose gels. Dehydrosenecionine (lane 2) was the most potent inducer of DNA-DNA cross-links. **Lane 1**, DMSO. **Lane 2**, dehydrosenecionine. **Lane 3**, dehydromonocrotaline. **Lane 4**, dehydroretronecine. **Lane 5**, indicine N-oxide. **Lane 6**, *cis*-dichlorodiammine platinum (II). **Lane 7**, mitomycin C. **M**, molecular weight markers (➢)

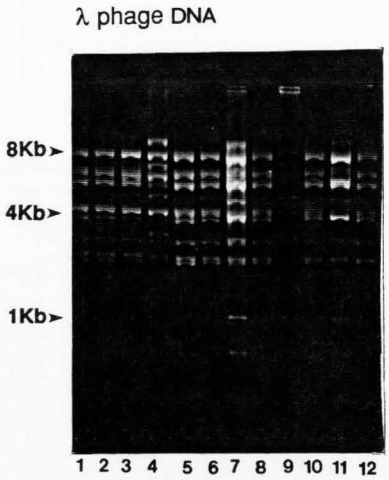


Fig. III-3. Band shifting analysis of DNA cross-links in *Bst* EII-digested λ-phage DNA by PA alone or pyrroles at 500 μM in ethidium bromide-stained 1% agarose gel. Monocrotaline (lane 8) alone did not induce any DNA cross-links compare to dehydromonocrotaline (lane 9). **Lane 1-2**, λ-phage *Bst* EII-digested DNA alone. **Lane 3**, 4 mM of mitomycin C. **Lane 4**, 1 mM of cis-dichlorodiammine platinum (II). **Lane 5**, DMSO. **Lane 6,** senecionine. **Lane 7**, dehydrosenecionine. **Lane 8**, monocrotaline. **Lane 9**, dehydromonocrotaline. **Lane 10**, retronecine. **Lane 11**, dehydroretronecine. **Lane 12**, indicine N-oxide.

indicine N-oxide did not induce DNA-DNA cross-links. *Cis*-dichlorodiammine platinum (II) induced mild DNA cross-links, and mitomycin C did not induce DNA-DNA cross-links at the same dose.

Pyrrolizidine Alkaloid-induced Interruption of Primer Extension by PCR

The ability of various pyrroles to cross-link a 1.129 kb region of pBR322 between the reverse direction of a *Bam* HI recognition site and the forward direction of a *Pst* I recognition site is presented in Figure III-5. In this experiment, the macrocyclic diesters, dehydrosenecionine and dehydromonocrotaline, interrupted the primer extension by PCR as evidenced by the disappearance of the 1.129 kb product. At the low dose ratios (1:0.001, 0.01, 0.1, 0.25; DNA:PA, w:w), these pyrroles did not induce any DNA interstrand cross-links (data not shown). However, at higher dose ratios (1:0.5, 1:1, 1:2) (Fig. III-5), dehydrosenecionine induced the most potent DNA interstrand cross-links. Dehydromonocrotaline also induced potent DNA interstrand cross-links but less than that of dehydrosenecionine. Dehydroretronecine and indicine N-oxide had no effect in this system. *Cis*-dichlorodiammine platinum(II) and dehydrosenecionine induced DNA interstrand cross-links at the same potency.

Pyrrolizidine Alkaloid-induced Interruption to Restriction Endonuclease Digestion of pBR322 DNA

The ability of pyrrolic PAs-induced DNA-DNA cross-links within recognition sites of various restriction endonucleases to inhibit the enzymatic action was then studied to determine possible sequence preferences of pyrrolic

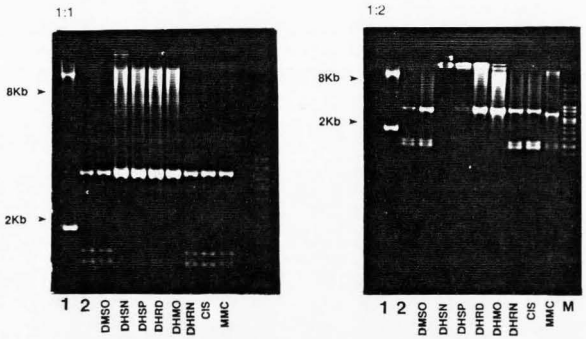


Fig. III-4. Band shifting analysis of PA-induced DNA-DNA cross-links in *Bam* HI-digested pBR322 plasmid DNA at the dose ratios 1:1, and 1:2 (DNA:pyrrole, w:w) in ethidium bromide-stained 1% agarose gels. DNA cross-linking can be seen as a shifting of DNA bands founds on top of the gel. Band shifting was most evident in DNA treated with dehydrosenecionine (DHSN) and dehydroseneciphylline (DHSP). DNA treated with *cis*-dichlorodiammine platinum (II) (CIS) and mitomycin C (MMC) are included for comparison. **Lane 1**, pBR322 alone. **Lane 2**, *Bam* HI-digested pBR322.

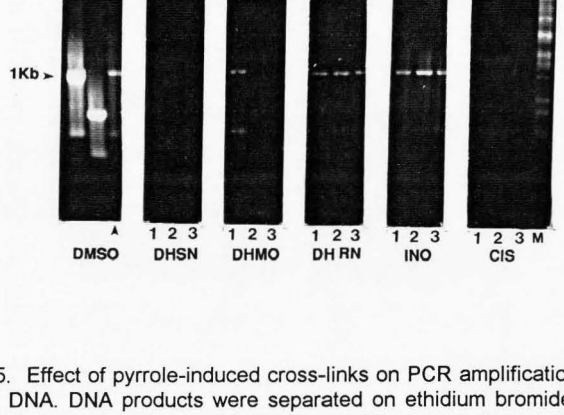


Fig. III-5. Effect of pyrrole-induced cross-links on PCR amplification of pBR322 plasmid DNA. DNA products were separated on ethidium bromide-stained 1% agarose. Primers used and amplification conditions are outlined in Materials and Methods. Dehydrosenecionine was the most potent DNA interstrand cross-linker as evident by disappearance of the amplified 1.129 kb segment. The dose ratios of DNA : pyrrole were; 1:0.5 (**lane 1**), 1:1 (**lane 2**), and 1:2 (**lane 3**) (w:w). (**Arrow head**) DMSO. (**M**) molecular weight markers.

PA-induced DNA cross-links. The enzymatic action of *Eco* RI was inhibited by treatment of *Bst* EII-digested λ-phage DNA with dehydrosenecionine as evidenced by disappearance of the 0.9 kb DNA band. Dehydromonocrotaline, dehydroretronecine, or indicine N-oxide did not inhibit the enzymatic action of *Eco* RI (Fig. III-6).

In pBR322 plasmid DNA, dehydrosenecionine significantly inhibited the endonuclease action of *Ava* II, *Fok* I, and *Taq* I at the high dose ratio (DNA:cross-linker, 1:1; w:w), moderately inhibited that of *Eco* RII (ca. 0.4 kb) and *Mbo* II (ca. 0.75, 0.6 kb), and least inhibited with *Hinf* I (ca. 0.2, 0.4, 0.75 kb) (Fig. III-7) observed as missing DNA bands. *Cis*-dichlorodiammine platinum (II) induced cross-links within the recognition sites of all of these restriction endonucleases. Dehydromonocrotaline induced few cross-linking sites (around 0.5 and 0.7 kb) with *Mbo* II. Dehydroretronecine, indicine N-oxide, or mitomycin C did not cross-link within the recognition sites of these restriction endonucleases, because the band patterns of these groups were the same as that of control.

DISCUSSION

The purpose of this study was to characterize the pyrrolic PA-induced DNA-DNA cross-links and to determine whether structural features on PAs can influence the pyrrolic PA-induced DNA-DNA cross-linking activity. From previous studies in our laboratory, macrocyclic α,β-unsaturated diester PAs such as seneciphylline, riddelliine, retrorsine, and senecionine were the most potent DNA cross-linkers as determined by alkaline elution (26), and had the most potent anti-mitotic and cytopathologic effects (19) in cell culture relative to other structural classes of PAs. In this study, I examined the types and degrees of PA-

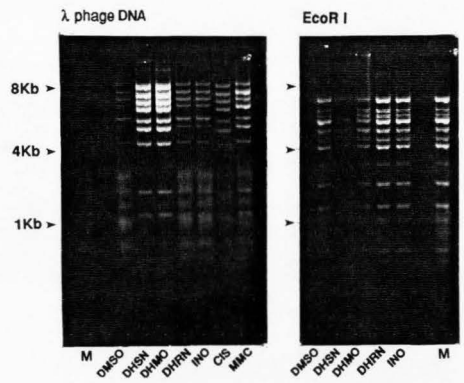


Fig. III-6. Evaluation of the ability of various pyrroles to cross-link DNA and their cross-linked sites in *Bst* EII-digested λ-phage DNA. For the detection of the cross-linked site, *Bst* EII-digested λ-phage DNA was cut with *Eco* RI after treatment with pyrroles at the dose ratio, 1:1 (DNA:pyrrole, w:w) and analyzed in ethidium bromide-stained 1% agarose gel. Dehydrosenecionine (DHSN) and dehydromonocrotaline (DHMO) induced mild DNA cross-linking at *Eco* RI recognition sites around 1 kb. DHRN, dehydroretronecine; INO, indicine N-oxide; CIS, *cis*-dicholrodiammine platinum (II); MMC, mitomycin C; M, molecular marker.

1:0.5 (DNA : cross-linker, w:w)

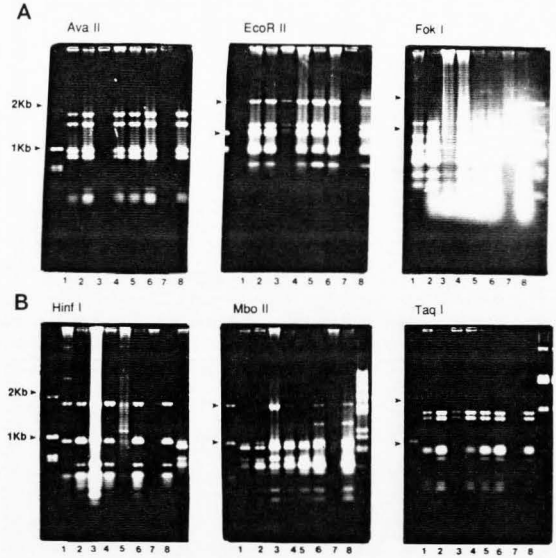
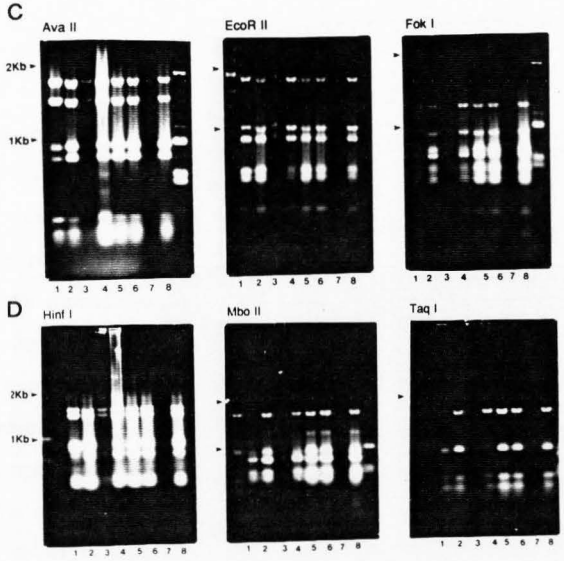


Fig. III-7. Effect of pyrrole-induced DNA cross-links on digestion pattern of pBR322 plasmid DNA by six restriction endonucleases. The DNA was treated with pyrroles or known cross-linkers as positive controls at 1:0.5 (DNA : cross-linker, w:w; panel A, B), and 1:1 (panel C, D) dose ratios using ethidium bromide-stained 1 % agarose gel. Dehydrosenecionine (lane 3) was the most potent DNA cross-linker and the cross-linked sites were at *Ava* II, *Eco* RII, *Fok* I, *Mbo* II, and *Taq* I recognition sites. **Lane 1**, pBR322 plasmid DNA alone. **Lane 2**, DMSO. **Lane 3**, dehydrosenecionine. **Lane 4**, dehydromonocrotaline. Lane 5, dehydroretronecine. **Lane 6**, indicine N-oxide. **Lane 7**, *cis*-dichlorodiammine platinum (II). **Lane 8**, mitomycin C.

(continue Figure III-7)

1:1 (DNA : cross-linker, w:w)



induced DNA cross-links by alkaline elution, gel-shift analysis, PCR amplification, and interruption of restriction endonuclease digestion.

My results showed that chemically activated pyrrolic PAs were more potent inducers of DNA cross-links in cells than their parent compounds that had been activated by an external metabolizing system. Parental PAs without an external metabolizing system did not induce any DNA cross-links in *Bst* EII-digested λ-phage DNA, confirming the hypothesis that the pyrrolic metabolites are the biologically active metabolites.

As in our previous studies (19, 26), I found that the macrocyclic α,β-unsaturated diester pyrroles such as dehydrosenecionine, dehydroseneciphylline, and dehydroriddelliine, and a α,β-saturated diester pyrrole dehydromonocrotaline were potent cellular DNA cross-linkers. I also found the α,β-unsaturated macrocyclic diester pyrroles such as dehydrosenecionine and dehydroseneciphylline were more potent DNA-DNA cross-linkers than the α,β-saturated macrocyclic diester pyrrole dehydromonocrotaline in pBR322 plasmid DNA and *Bst* EII-digested λ-phage DNA. The necine base pyrrole dehydroretronecine or indicine N-oxide did not induce DNA cross-links. Thus, structural features of pyrrolic PAs were reflected in their differential DNA cross-linking activities.

My results suggested that the presence of both α,β-unsaturation and a macrocyclic ring such as in dehydrosenecionine and dehydroseneciphylline might be the most important two structural features affecting pyrrole-induced DNA cross-linking activity. Because these pyrroles were more potent than the macrocyclic α,β-saturated diester pyrrole dehydromonocrotaline, DNA cross-links might be induced via the Michael addition reaction in addition to S_N1 solvolysis, a common reaction to most of pyrrolic PAs with cellular nucleophiles

(35, 36). In addition, the α,β-unsaturated macrocyclic pyrroles (dehydrosenecionine, dehydroseneciphylline, and dehydroriddelliine) contain an 11-membered macrocycle ring, whereas dehydromonocrotaline has a 12-membered macrocycle ring which may also influence the differential cross-linking potency.

The differences in the relative cellular DNA cross-linking activities of parental PAs detected in this study suggested that PA structures influence their metabolites which are preferentially formed. For example, the macrocyclic α,β-unsaturated diester PAs are more readily metabolized to pyrroles than are open diesters, such as heliosupine and latifoline (37, 38). Retronecine, which is more water soluble, forms relatively little pyrrole (37-39). Thus, the DNA cross-linking activity may depend on the ratio of the biologically active form (pyrrole) to the biologically less-active form such as necine or N-oxide (38, 40, 41). In this study, macrocyclic diester pyrroles also induced more DNA cross-links than did the necine base or N-oxides.

Our laboratory (19, 26) also found that those groups of PAs differed in their DNA cross-linking potency and cytopathologic activity in cultured mammalian cells (19, 26), further indicating that DNA cross-linking formation is an important event leading to PA bioactivity. Those PAs that were the most potent cross-linkers are also more acutely toxic to animals *in vivo*. For example, senecionine was 3-6 times more acutely toxic than monocrotaline in the rats (40, 42). Another study indicated similar potency differences with respect to the induction of unscheduled DNA repair in isolated hepatocytes (43).

Moreover, the metabolic pathways of various PAs also affect the biological activities of PAs in different species (37-41, 44). Species of both farm and laboratory animals differ in their susceptibilities to PAs (45). Pyrrolizidine

alkaloids are toxic to most animal species such as chickens, horses, and rats, although some species such as guinea pigs, sheep and goats are relatively resistant (46-49). The differences in susceptibility are largely due to the metabolic pathway or conversion rate to nontoxic (necine, N-oxide) or toxic metabolites (pyrroles) in animal tissues (48).

I further characterized the nature of pyrrolic PA-induced DNA-DNA cross-links and structural influences by investigating pyrrolic PA-induced DNA-DNA cross-linking activity (inter- and/or intrastrand cross-links), and cross-linked site specificity using *in vitro* two target DNA systems: pBR322 plasmid DNA, which consists of supercoiled or circular double-stranded DNA, and *Bst* EII-digested λ-phage DNA, which consists of 13 fragments of double-stranded DNA. In both systems, the α,β-unsaturated macrocyclic diester pyrroles were the most potent inducers of DNA-DNA cross-links. Dehydromonocrotaline formed more DNA cross-links than did dehydroretronecine, and indicine N-oxide was not active. Thus, it appeared the structural features that influenced DNA cross-linking activity in cell culture hold true in target DNAs. The differences of cross-linking potency between pBR322 plasmid DNA and *Bst* EII-digested λ-phage DNA suggested that the conformation of target DNA also could influence DNA-DNA cross-linking activity induced by pyrrolic PAs.

Pyrroles that were potent inducers of DNA cross-links also effectively inhibited PCR amplification of the 1.129 kb region of the pBR322 plasmid DNA. In this experiment, dehydrosenecionine was the most potent inducer of DNA interstrand cross-links, somewhat more potent than was dehydromonocrotaline, because the onset dose ratio of dehydromonocrotaline was higher (1:1, w:w) than dehydrosenecionine (1:0.5, w:w). Dehydroretronecine or indicine N-oxide had no effect. The potency of *cis*-dichlorodiammine platinum(II) was similar to

that of dehydrosenecionine. These results suggested that pyrrolic metabolites were primarily responsible for inducing interstrand DNA cross-links and that α,β-unsaturation in or size of the macrocyclic diester ring influenced the formation of DNA interstrand cross-links by pyrrolic metabolites.

Previous work (22, 50, 51) has shown that pyrrolic metabolites alkylated synthetic oligonucleotides containing a single 5'd(CG) sequence. I investigated the sequence specificity of PA-induced DNA-DNA cross-links in pBR322 using restriction endonuclease digestion. In this study, the recognition sites of *Ava* II (5'-GG($^{A}_{T}$)CC), *Eco* RII (5'-CC($^{A}_{T}$)GG), *Fok* I (5'-GGATG), *Mbo* II (5'-GAAGA), and *Taq* I (5'-TCGA) were highly associated with cross-linking induced by dehydrosenecionine, but the recognition site of *Hinf* I (5'-GANTC) was not. These data indicate that the pyrrolic PA-induced DNA-DNA cross-linking site was preferentially related to 5'-d(GG), 5'-d(CC) or 5'-d(GA) sequences, although other sequences might also be involved. These restriction endonucleases were seldom inhibited by dehydromonocrotaline. As before, α,β-unsaturation in or size of the macrocyclic diester ring appears to be an important structural feature in pyrrole-induced DNA-DNA cross-links.

I included two bifunctional alkylating anti-cancer agents to compare the relative potency of pyrrolic PAs. The known DNA cross-linker, *cis*-dichlorodiammine platinum (II), also induced potent DNA-DNA cross-links in this study. This compound has been shown to produce potent DNA cross-links, including interstrand DNA cross-links in short duplex DNA, 5'-d$(CG)_5$, and the preferred sequences might be 5'-d(CG) or 5'-d(GC) (24). However, mitomycin C, whose DNA cross-link action is dependent upon reductive activation (52), did not induce detectable DNA-DNA cross-links in this study. Other workers have found that activated mitomycin C, which possesses structural similarities to pyrrolic

PAs, appeared to preferentially cross-link with deoxyguanosine residues at the sequence 5'-d(CG) in duplex DNA (51, 53-54). The similarity in DNA cross-linking activity or structural features of pyrroles and those anti-cancer agents suggests that the mechanism by which pyrrolic PA induces DNA cross-links might be similar to that of those anti-cancer agents.

I also investigated DNA cross-linking sequence specificity using *Eco* RI cleavage of PA-treated *Bst* EII-digested λ-phage DNA. *Eco* RI (5'-GAATTC) can cut in *Bst* EII-digested λ-phage DNA, but at low rates. The DNA cross-linking potency differences between two target DNA systems, pBR322 plasmid DNA, which consisted of circular or supercoiled double-stranded DNA, and *Bst* EII-digested λ-phage DNA, which consisted of 13 fragments of linear double-stranded DNA, suggested that DNA cross-linking activity is influenced by neighboring sequences and/or the native structure of the target DNA.

In summary, the α,β-unsaturation of the macrocyclic diester ring and possibly its size, as well as the C1-C2 double bond, are structural features that appear to affect the DNA cross-linking potency of PAs. Because the cross-linking potency among PAs roughly coincides with relative animal and cellular toxicity, PA-induced DNA cross-linking activity does not involve a common PA metabolite, dehydroretronecine. The significant portion of PA-induced cross-links that are associated with protein may also contribute to the bioactivity of these natural compounds. This will be the subject of future studies in our laboratory.

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CHAPTER IV

CHARACTERIZATION OF DNA-PROTEIN CROSS-LINKS BY PYRROLIZIDINE ALKALOIDS

ABSTRACT

Protein-associated DNA cross-links are implicated in the carcinogenic or anti-carcinogenic activity of many compounds. In this study, I investigated the DNA-protein cross-linking activity of a group of natural pyrrolizidine alkaloids (PAs) *in vitro*. The DNA cross-linked proteins induced by PAs were also isolated and characterized in mammalian cells. At 300 μM, pyrrolic metabolites such as dehydrosenecionine, dehydromonocrotaline, dehydroseneciphylline, and dehydroriddelliine induced DNA cross-links. Those that were protein-associated accounted for approximately 50% of the total cellular DNA cross-links. The simple necine base dehydroretronecine induced few DNA-protein cross-links and none were detected with indicine N-oxide. Macrocyclic PAs (senecionine, seneciphylline, riddelliine, retrorsine, monocrotaline) induced DNA-protein cross-links in cultured cells. The major proteins cross-linked to DNA from either PA-exposed cells or pyrrolic PA-exposed nuclei were in the molecular weight 40 - 60 kD range as determined by SDS-PAGE. Two-dimensional electrophoresis revealed that the proteins involved in cross-links were primarily acidic in nature. The macrocyclic pyrrole, dehydrosenecionine induced DNA cross-links in *Bst* EII-digested λ-phage DNA and pBR322 plasmid DNA with BSA as a protein target. The cross-linked proteins to DNA in patterns were similar to that induced by the anti-cancer agents, mitomycin C and *cis*-dichlorodiammine platinum(II). My data indicated that pyrrolic PAs with a macrocyclic diester such as dehydrosenecionine, dehydroseneciphylline, dehydroriddelliine, and

dehydromonocrotaline were more potent cross-linkers than the simple necine base. Furthermore, because of the potency differences between pyrrolic metabolites, it is likely that DNA-protein cross-links do not occur via a common metabolite as previously proposed. Cross-linking potency of the PAs examined here coincides with known potency differences in animal toxicity and led me to conclude that DNA-protein cross-linking activity is probably involved in PA-related diseases.

INTRODUCTION

Pyrrolizidine alkaloids are natural toxins found in a wide variety of plant species that pose significant health hazards to humans and other animals (1). Many PAs are toxic and carcinogenic (2-8) and are associated with chronic liver disease in humans and other animals (5, 9, 10). Dehydromonocrotaline and jacobine induced DNA-protein and DNA interstrand cross-links in rat liver *in vivo* (4, 5). Dehydromonocrotaline also induced DNA cross-links and caused anti-mitotic and cytopathologic effects in porcine pulmonary artery endothelial cells and rat lung (11-13). These DNA-protein cross-links are toxicologically important lesions and can also be induced by a variety of agents such as γ- and UV-irradiation, chromium, and formaldehyde (14-19). The proposed action of anti-tumor agents such as nitrogen mustard, cisplatin, mitomycin C, or nitrosourea is thought to be related to their abilities to induce DNA cross-links (18, 20-23). In our laboratory, we demonstrated that cytopathologic changes and anti-mitotic activity of PAs were related to their ability to induce DNA-DNA or DNA-protein cross-links (24, 25).

Pyrrolizidine alkaloids are bioactivated by the cytochrome P-450 mixed-function oxidase to form the bifunctional electrophilic pyrroles, which then

alkylate cellular macromolecules such as DNA and protein (4, 5, 26, 27). The toxic effects of pyrrolic metabolites are apparently related to their DNA cross-linking activity.

A previous study from our laboratory has shown that PAs cross-link DNA with protein (24). In this chapter, I investigated the characteristics of PA-induced DNA-protein cross-links. I also characterized the nuclear proteins involved in these cross-links in a cellular system, and examined the ability of a variety of pyrrolic PAs to induce DNA-protein cross-links in target DNA.

MATERIALS AND METHODS

Materials

Senecionine and seneciphylline were isolates from *Senecio vulgaris* and *Senecio triangularis,* and furnished by Segall (28, 29). Riddelliine was an isolate from *Senecio riddellii,* and furnished by Molyneux *et al.* (30). Monocrotaline was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Retronecine was prepared by hydrolysis of riddelliine isolated from *Senecio riddellii*, and indicine N-oxide was provided by Matthew Suffness of the National Cancer Institute. Pyrrolic PA metabolites (dehydrosenecionine, dehydroseneciphylline, dehydroriddelliine, dehydromonocrotaline, dehydroretronecine) were prepared from their parent compounds (senecionine, seneciphylline, riddelliine, monocrotaline, retronecine, respectively) by the method of Mattocks *et al.* (31) in our laboratory. The purity of these metabolites was checked and confirmed by ^{1}H NMR (32). The chemical structures of PAs and other compounds used in this study are shown in Figure IV-1.

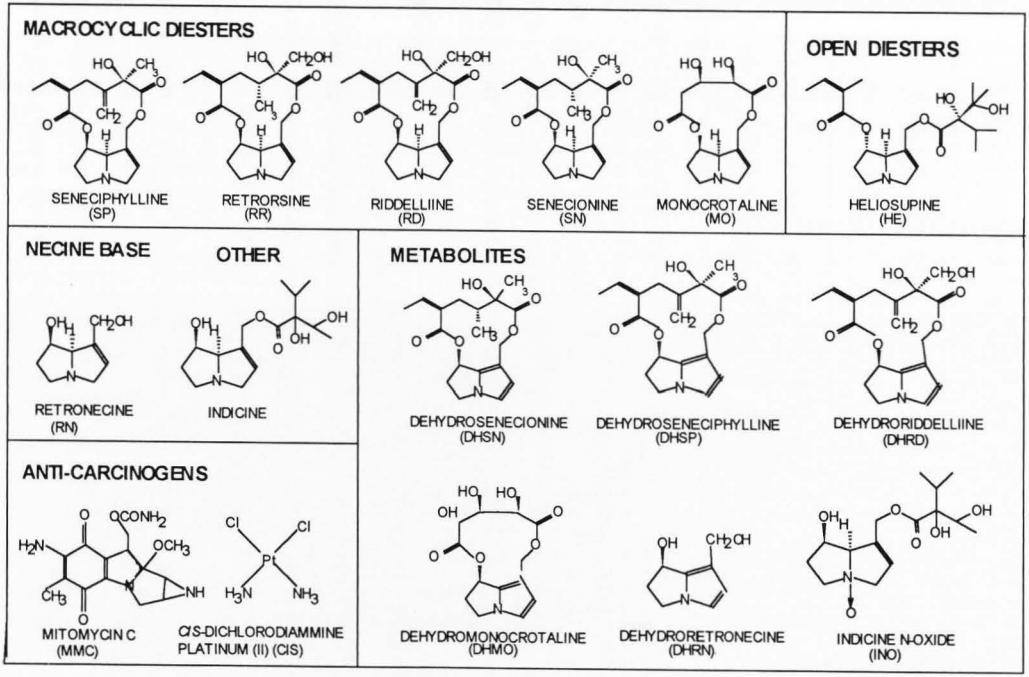

Fig. IV -1. Chemical structures of pyrrolizidine alkaloids and anti-carcinogens examined in this dissertation.

Eagle's Minimum Essential Medium was obtained from Gibco BRL (Grand Island, NY) and iron-supplemented calf serum was obtained from Hyclone Laboratories (Logan, UT). Methyl-[^{3}H]-thymidine was obtained from New England Nuclear (Boston, MA). *Cis*-dichlorodiammine platinum(II), mitomycin C, actin, myosin, lysozyme (EC 3.2.1.17), RNAse I (EC 3.1.2.75), proteinase K (EC 3.4.21.14), ampicillin, tetracycline, and ethidium bromide were obtained from Sigma Corporation (St. Louis, MO). Ampholine was purchased from Pharmacia LKB Biotechnology (Piscataway, NJ), and bacto-tryptone and bacto-yeast were obtained from Difco (Detroit, MI). Histone was purchased from Boehringer Mannheim Corporation (Indianapolis, IN). DNAse I and *Bst* EII digested λ-phage DNA were obtained from New England BioLabs (Beverly, MA). Bovine serum albumin (BSA) was obtained from Promega Corporation (Madison, WI). Swinex filter holders and polyvinylchloride filters were purchased from Millipore Corporation (San Francisco, CA).

Methods

Cell Culture and Treatment Conditions. The Madin Darby bovine kidney (MDBK) epithelial cells, strain CCL 22, obtained from American Type Culture Collection (Rockville, MD), were grown in Eagle's Minimum Essential Medium containing nonessential amino acids, L-glutamine, 1 mM sodium pyruvate and 10% iron-supplemented calf serum. Cells were cultured at 37 ^{o}C in an atmosphere of 97% air and 3% CO_2. Cells (passage 112-130) were seeded in 60-mm diameter culture dishes 22 hr prior to labeling with methyl-[^{3}H]-thymidine for alkaline elution assay or in 100-mm diameter culture dishes for the characterization of cellular DNA cross-linked proteins where parent PAs were used, and an external metabolizing system, which consisted of a NADPH-

generating system and rat liver S9 fraction, was added (24). Pyrrolizidine alkaloids and their metabolites were dissolved in DMSO and added directly to the medium in a volume that did not exceed 1% of the total volume. Cell viability for all treatment groups exceeded 90% as determined by trypan blue dye exclusion (data not shown).

Quantitation of Cellular DNA Cross-links by Alkaline Elution. Cells seeded in 60-mm diameter culture dishes were labeled with 0.1 μCi/ml of methyl-[^{3}H]-thymidine (2 Ci/mmol) for 22 hr. The medium was changed before cells were treated with PAs or their metabolites. The cells were washed and replaced with cold PBS (0.12 M NaCl, 4 mM KCl, 0.5 mM Na_2HPO_4, 0.1 mM KH_2PO_4, pH 7.4) after 2 hr incubation with PAs or metabolites. Cells were then exposed to 1000 rad of γ-irradiation at 0 ^{o}C using ^{137}Cs irradiator at the dose rate of 171 rad/min. The γ-irradiation source was calibrated using the Fricke's ferrous sulfate dosimetry method (33). Alkaline elution was performed as described in a previous paper (24).

Isolation and Characterization of DNA Cross-linked Proteins from Cells and Nuclei. Cells (80 - 85% confluence) were treated with 1 mM of each PA for 4 hr at 37 ^{o}C. Nuclei, which were isolated from cultured MDBK cells by lysing with 0.3% Triton X-100 in 150 mM NaCl and 1 mM EDTA buffer and centrifugation at 3000 rpm, were treated with 1 mM of pyrrolic PA for 4 hr at 37 ^{o}C. Cells or nuclei were also treated with 6 mM of *cis*-dichlorodiammine platinum(II) or 400 μM of mitomycin C for 6 hr at 37 ^{o}C. The treated cells and nuclei were washed twice with ice-cold PBS containing 0.1 mM of phenylmethylsulfonyl fluoride. DNA cross-linked proteins were then isolated using a modification of the method of Banjar *et al.* (34). Briefly, treated cells or nuclei were lysed in 2% (w/v) SDS,

50 mM Tris-HCl, 0.1 mM phenylmethylsulfonyl fluoride, pH 8.0, and stirred for 7 hr on a rotatory stirrer. The cross-linked proteins were separated from non-cross-linked proteins by ultracentrifugation (100,000 x g) for 17 hr at 19 ^{o}C. The DNA cross-linked proteins were then resuspended using Down's homogenizer in 2 mM Tris-HCl, pH 7.0 including 0.1 mM phenlymethylsulfonyl fluoride, then sonicated 4 times for 20 sec at 0 ^{o}C (35). The sonicated sample was digested with DNAse I (40 μg/mg DNA) for 1 hr at 37 ^{o}C, dialyzed overnight against double distilled water, and freeze dried.

Proteins, which were cross-linked to 40 μg of DNA isolated from the treated whole cells, were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (36). The running gel consisted of 15% (samples from cells) or 11% (samples from nuclei) acrylamide, 0.4% N,N'-methylenebisacrylamide in 0.375 M Tris-HCl, 0.1% (w/v) SDS, pH 8.8. The stacking gel consisted of 4.5% acrylamide, 0.12 % N,N'-methylenebisacrylamide in 0.125 M Tris-HCl, 0.1% (w/v) SDS, pH 6.8. The freeze-dried samples were dissolved in loading buffer (1% [w/v] SDS, 5% [v/v] 2-mercaptoethanol, 10% [v/v] glycerol, 6.25 mM Tris-HCl, 0.05% [w/v] bromphenol blue, pH 6.8), and were electrophoresed at 50 V in reservoir buffer (0.192 M glycine, 25 mM Tris, 0.1 % [w/v] SDS, pH 8.3). The SDS-polyacrylamide gel was stained using a modified silver staining method (37, 38).

The proteins, which were cross-linked to 100 μg of DNA isolated from the treated nuclei, were also separated by isoelectric focusing followed by SDS-polyacrylamide gel (2-D) electrophoresis (39-41). The isoelectric focusing gel consisted of 9.5 M urea, 0.2% N,N'-methylenebisacrylamide, 3.4% acrylamide, 2% NP-40, 2% ampholines (pH 5.0 - 8.0, and 3.0 - 10.0). The protein sample was dissolved in lysis buffer (9.5 M urea, 2% NP-40, 2% ampholines, 5% β-

mercaptoethanol). The gel ran for 14 hr at 400 V followed by 1 hr at 800 V, then equilibrated with sample buffer (62.5 mM Tris, 10% glycerol, 5% β-mercaptoethanol, 2.3% SDS) for 45 - 60 min at room temperature.

The isoelectric focusing gel was then loaded on top of a SDS-polyacrylamide slab gel and connected to the stacking gel using 0.4% agarose. The separating gel consisted of 12.15% acrylamide, 0.34% N,N'-methylenebisacrylamide, 0.375 M Tris, and 0.1% SDS, pH 8.8, and the stacking gel consisted of 4.8% acrylamide, 0.13% N,N'-methylbisacrylamide, 0.12 M Tris, and 0.1 % SDS, pH 6.7. The gel was run at 40 V overnight and then stained with silver nitrate (37, 38).

DNA-protein Cross-links in pBR322 Plasmid DNA or λ-phage DNA. pBR322 plasmid DNA was amplified in bacterial *E.coli* host (strain RRI) by overnight culture at 37 ^{o}C in LB medium (1% bacto-tryptone, 0.5% bacto-yeast, 0.89 M NaCl) containing 15 μg/ml tetracycline and 50 μg/ml ampicillin. pBR322 plasmid DNA was purified by the rapid alkaline method (42). Briefly, *E.coli* cells were harvested by centrifugation at 5000 rpm for 10 min at 4 ^{o}C and the bacterial cell pellet was lysed (25 mM NaCl, 10 mM EDTA, 15 % sucrose, 2 mg/ml lysozyme) for 20 min at 4 ^{o}C. The lysate was incubated with 0.04 M NaOH and 0.04% SDS for 10 min and was then mixed with 0.075 M $NaC_2H_3O_2$, pH 4.6. The mixture was incubated for 20 min at 0 ^{o}C followed by centrifugation at 10,000 rpm for 15 min. RNAse (1 mg/ml) was added to the supernatant which was incubated for 20 min at 37 ^{o}C. The sample was purified by phenol : chloroform (1:1, v/v) extraction, and then precipitated with ethanol. The DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) and incubated with 0.4 M NaCl and 6.5% polyethylene glycol for 1 hr at 0 ^{o}C. This solution was

centrifuged at 10,000 rpm for 10 min. Plasmid DNA was dissolved in TE buffer pH 7.4 (10 mM Tris-HCl, 1 mM EDTA).

In this experiment, two target DNA systems were used: pBR322, which consists of supercoiled or circular double-stranded DNA, and *Bst* EII-digested λ-phage DNA, which consists of 13 fragments of linear double-stranded DNA. The DNA-protein cross-links were investigated in pBR322 plasmid DNA treated with dehydrosenecionine or dehydromonocrotaline, and co-incubated with BSA at the DNA : pyrrole : protein ratios of 1:1:0.5, 1:1:1 or 1:1:2 (w:w:w). The DNA-protein cross-links were also characterized in *Bst* EII-digested λ-phage DNA. The DNA was treated with dehydrosenecionine and simultaneously co-incubated with actin, BSA, histone, or myosin in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) at DNA : pyrrole : protein ratios of 1:1:0.5, 1:1:1, or 1:1:2 (w:w:w) for 2 hr at 0 ^{o}C. Dimethylsulfoxide (DMSO) was used as control. The cross-linked DNA-protein was separated on ethidium bromide stained 1% agarose gels (43). The ethidium bromide-stained DNA was photographed by a Polaroid MP-4 camera system and Polaroid Type 667 film.

Statistical Evaluations. Data were analyzed by one-way analysis of variance, and where appropriate, a *post hoc* Fisher's LSD was used to determine significance ($P < 0.05$) among groups.

RESULTS

Quantitation of DNA Cross-links by Alkaline Elution

The extent of cellular DNA cross-links by various PAs and their pyrrole or N-oxide metabolites as determined by alkaline elution is shown in Table IV-1. None of the compounds examined induced single-strand breaks (data not

shown). The pyrrolic metabolites were potent inducers of DNA-protein cross-links. The order of potency was dehydrosenecionine > dehydromonocrotaline > dehydroriddelliine > dehydroretronecine > indicine N-oxide. About 50% of DNA cross-links induced by pyrroles involved proteins, as determined by proteinase K treatment (Table IV-1).

Characterization of DNA Cross-linked Proteins from Cells and Nuclei

Because the alkaline elution experiments showed that a significant proportion at PA-induced cross-links was protein associated, I then examined the type of proteins involved in the cross-links. All of the parent PAs examined, except retronecine, induced DNA-protein cross-links in cultures of whole cells (Fig. IV-2A). The proteins most often cross-linked with cellular DNA following treatment with PAs had molecular weights in the range of ca. 40 - 60 kD. Senecionine, retrorsine, and heliosupine also induced DNA-protein cross-links (data not shown). A similar pattern of DNA cross-linked proteins was seen in isolated nuclei treated with the pyrrolic metabolites such as dehydrosenecionine, dehydroseneciphylline, dehydroriddelliine, and dehydromonocrotaline (Fig. IV-2B). Dehydroretronecine did not induce DNA-protein cross-links. *Cis*-dichlorodiammine platinum(II) (cells and nuclei) and mitomycin C (cells) also induced similar patterns of DNA cross-linked proteins, which had molecular weights in the range of ca. 40 - 70 kD with *cis*-dichlorodiammine platinum(II) and ca. 35 - 100 kD with mitomycin C.

As evaluated by 2-D gel electrophoresis, DNA cross-linked proteins induced by macrocyclic α,β-unsaturated diester pyrroles such as dehydroseneciphylline, dehydrosenecionine, and dehydroriddelliine were

Table IV-1. Pyrrolizidine alkaloid-induced cellular DNA cross-links [1,2,3]

ALKALOIDS	TOTAL DNA CROSS-LINKS[4]	DNA-DNA CROSS-LINKS[4]	DNA-PROTEIN CROSS-LINKS[4]	f[6]
Dehydrosenecionine	1.46 ± 0.13[a]	0.67 ± 0.09[a]	0.78 ± 0.11[a]	0.54 ± 0.08
Dehydroseneciphylline	0.74 ± 0.16[b]	0.25 ± 0.04[b]	0.49 ± 0.20[a]	0.51 ± 0.15
Dehydroriddelliine	0.69 ± 0.10[b]	0.36 ± 0.05[a,b]	0.31 ± 0.10[b]	0.50 ± 0.13
Dehydromonocrotaline	1.11 ± 0.17[c]	0.53 ± 0.04[a,b]	0.58 ± 0.19[a,b]	0.51 ± 0.17
Dehydroretronecine	0.18 ± 0.01[d]	0.08 ± 0.04[c]	0.09 ± 0.04[c]	0.52 ± 0.26
Indicine N-oxide	ND[5]	ND	ND	ND

[1]Treatments were 300 μM for 2 hr at 37 °C.
[2]Values with different superscripts are significantly different ($P < 0.05$) by one-way ANOVA and F-test within same type of cross-links.
[3]As measured by alkaline elution.
[4]Data presented as mean DNA cross-link indices ± S.E. from at least three independent experiments.
[5]ND, not detectable.
[6]The DNA-protein cross-linking fraction of total DNA cross-links ± S.E.

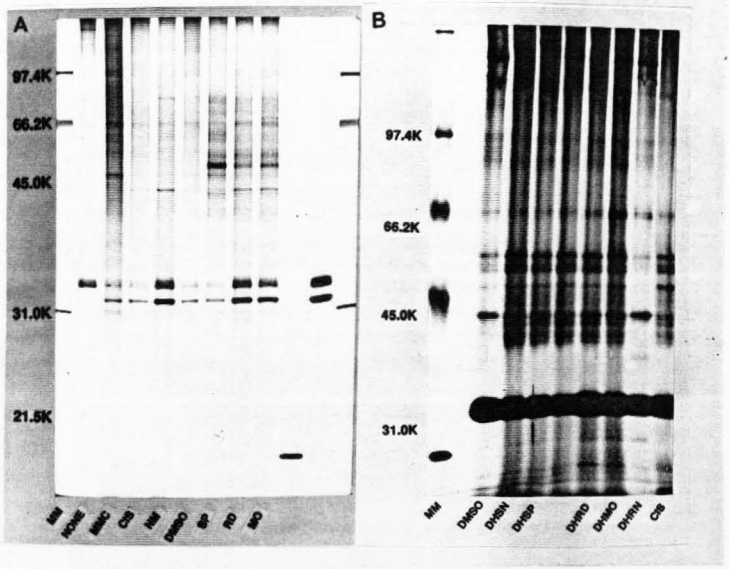

Fig. IV-2. Electrophoretic separation of DNA-protein cross-links from whole cells (A) or nuclei (B) treated with PAs or pyrrolic PAs. DNA cross-linked proteins were separated on 15% (A) or 11% (B) SDS-PAGE staining with siver nitrate. All samples were standardized to 40 μg DNA. The most prominent cross-linked proteins were around the molecular weight range 40 - 60 kD. Residual DNAse I appears around 32 kD. These data indicate that the macrocyclic diester PAs induced the most potent DNA-protein cross-links.

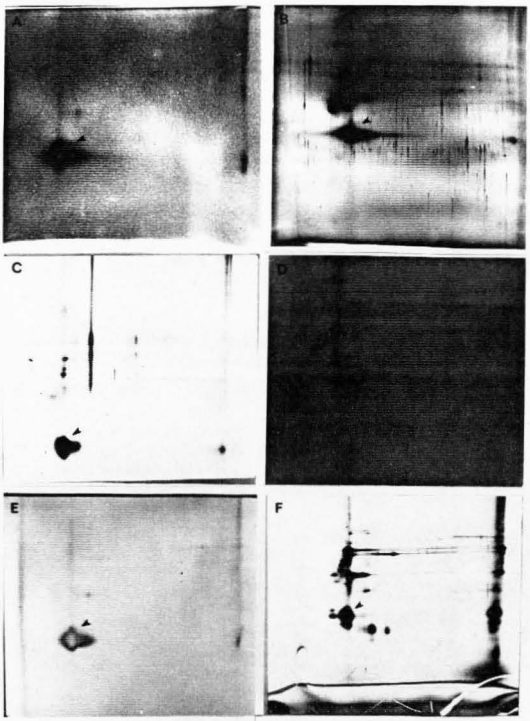

Fig. IV-3. Two-dimensional (2-D) electrophoretic separation of PA-induced DNA cross-linked proteins from treated nuclei. The DNA cross-linked proteins were separated by isoelectric focusing and then separated by 12.5% SDS-PAGE. The proteins which were cross-linked with 100 μg of DNA were used for isoelectric focusing. The most prominent proteins are at the acidic side of 2-D gel. **Panel A**, DMSO. **Panel B**, dehydroseneciphylline. **Panel C**, dehydrosenecionine. **Panel D**, dehydroriddelliine. **Panel E**, dehydroretronecine. **Panel F**, *cis*-dichlorodiammine platinum (II).

primarily in the acidic portion of the gels (Fig. IV-3A-C). The staining pattern of the DNA cross-linked proteins from dehydroretronecine-treated nuclei (Fig. IV-3E) was identical to that of the control. The pattern of DNA-protein cross-links induced by *cis*-dichlorodiammine platinum (II) was similar to that induced by the macrocyclic α,β-unsaturated diester pyrroles (Fig. IV-3F).

DNA-protein Cross-links in pBR322 Plasmid or λ-phage DNA

It was of interest to determine whether pyrrolic PA-treated DNA can cross-link with added protein such as BSA, histone, actin, or myosin *in vitro*. As can be seen in Figure IV-4, dehydrosenecionine and dehydromonocrotaline induced slight cross-links between pBR322 plasmid DNA and BSA as evidenced by the slight electrophoretic mobility change of DNA bands compared to that of the control. *Cis*-dichlorodiammine platinum(II) also induced DNA-BSA cross-links in pBR322. Dehycrosenecionine-induced DNA-protein cross-linking was also investigated in *Bst* EII-digested λ-phage DNA (Fig. IV-5). Dehydrosenecionine induced cross-links between λ-phage DNA and BSA, but did not cross-link with actin or myosin at the high dose ratio. Histone was completely cross-linked to DNA even at the lowest dose ratio (1:1:0.5; DNA : dehydehydrosenecionine : histone, w:w:w), which was evidenced by a shifting of the DNA band to the top of the gel.

DISCUSSION

We have previously shown that PAs are potent bifunctional alkylators of cellular DNA, and that a significant portion of these lesions was protein associated (24). We also found that PA structure influenced the formation of PA-induced DNA-DNA cross-links. Therefore, in this study, I characterized PA-

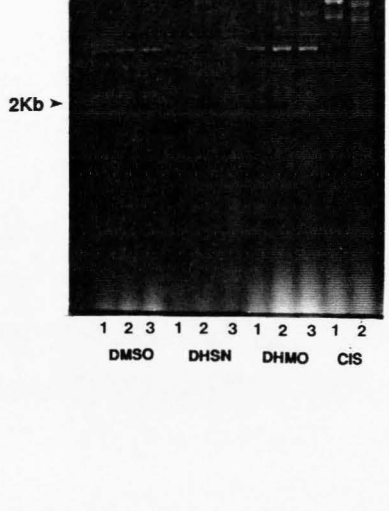

Fig. IV-4. Effect of BSA on the electrophoretic mobility of pyrrolic PAs cross-linked in pBR322 plasmid DNA. The cross-linked samples were separated by ethidium bromide-stained 1% agarose gel electrophoresis. Dehydrosenecionine induced DNA-protein cross-links dose dependently as appearing by the electrophoretic mobility changing of samples compared to DMSO. The dose ratios (DNA : cross-linker : BSA, w:w:w) were 1:1:0.5 **(lane 1)**, 1:1:1 **(lane 2)**, 1:1:2 **(lane 3)**.

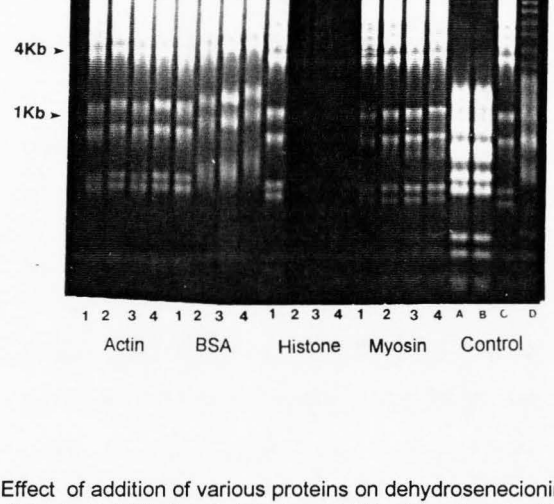

Fig. IV-5. Effect of addition of various proteins on dehydrosenecionine-induced DNA cross-links in *Bst* EII-digested λ-phage DNA at treatment ratios of 1:1:0 (**Lane 1)**, 1:1:0.5 (**lane 2**), 1:1:1 (**lane 3)**, 1:1:2 (**lane 4)** (DNA : dehydrosenecionine : protein, w:w:w) on ethidium bromide-stained 1% agarose gel. **Lane a**, λ-phage BstE II-digested DNA alone; **lane b**, DMSO; **lane c**, dehydrosenecionine; **lane d**, *cis*-dichlorodiammine platinum (II) at dose ratio 1:1 (DNA : cross-linker, w:w).

induced DNA cross-linked proteins in a mammalian cell system and in two target DNA systems, pBR322 plasmid DNA (supercoiled or circular double-stranded DNA) and *Bst* EII-digested λ-phage DNA (linear double-stranded DNA).

Similar to the formation of DNA-DNA cross-links, structural differences in the macrocyclic diester substituents of pyrrolic PAs apparently influence the formation of DNA-protein cross-links. For example, the most potent cross-linkers such as dehydrosenecionine, dehydroseneciphylline, and dehydroriddelliine all possess α,β-unsaturation in their 12-carbon macrocyclic diester moieties. Dehydromonocrotaline, which was less potent, contains an 11-atom saturated macrocyclic diester. Dehydroretronecine, a weak DNA cross-linker, has no diester substituents. Thus, the major structural features of pyrrolic metabolites which apparently influence the ability to form cross-links are the presence of both an α,β-unsaturated diester and a macrocyclic diester ring. The number of atoms in the macrocycle also appears to be an important factor of the DNA cross-links. These structural attributes also affect the DNA cross-linking potency and cytopathologic effect of the parent PAs (24, 25) and the ability to induce unscheduled DNA repair in hepatocytes (9).

In this study, chemically activated pyrroles induced a smaller proportion (50%) of DNA-protein cross-links than in a previous study from our laboratory in which PAs were metabolically activated (over 80%) (24). PAs are activated by the cytochrome P-450 mixed-function oxidase to form pyrrolic metabolites, which are highly toxic, and N-oxides, which are less toxic. The bioactivation of PAs appears to be related to an unsaturated C-1,2 bond in the necine base and the type of necic acid esters (2, 7, 44). That dehydrosenecionine induced more potent DNA cross-links than that of dehydromonocrotaline in this study affirmed the previous hypothesis that the type of side groups and number of atoms in the

macrocyclic diester pyrroles influenced their DNA cross-linking potency. Moreover, the potency of the chemically activated monocrotaline pyrrole, dehydromonocrotaline, was similar to that of the macrocyclic α,β-unsaturated diester pyrroles such as dehydrosenecionine, dehydroseneciphylline, and dehydroriddelliine, perhaps because larger proportions of other metabolites of monocrotaline such as the N-oxide form might be formed than the pyrrolic form by the cytochrome P-450 mixed-functionoxidase. The relative toxicity of various PAs in different animal species is also correlated to proportions of toxic metabolites (2, 6, 44-49).

Using SDS-PAGE and 2-D gel electrophoresis, I found that metabolically activated PAs or pyrrolic PAs cross-linked DNA with proteins in the 40 - 60 kD range and that these proteins had a net acidic charge. Because actin (molecular weight 45 kD), which is associated with gene regulation (50-52), is often cross-linked to DNA in cisplatin or chromate-treated CHO cells, as are other proteins in the molecular weight around 53 kD and 50 kD with isoelectric points of pI 5.4 - 9.0 (20), it is possible that one of the DNA cross-linked proteins might be actin. The necine base (retronecine, dehydroretronecine) and indicine N-oxide-induced DNA cross-linked proteins were not detected. The results affirmed that DNA-protein cross-links may be a critical factor in PA-induced bioactivity (5, 27), and also suggested that the majority of DNA-protein cross-links are formed by pyrrolic metabolites.

DNA cross-linked proteins in the molecular weights of 39 - 68 kD have been also identified in *cis*- or *trans*-dichlorodiammine platinum(II) or chromium compound-treated cells (19, 20). The proteins cross-linked with DNA were identified as nuclear matrix proteins (53, 54). In this study, two standard bifunctional cross-linkers, *cis*-dichlorodiammine platinum(II) and mitomycin C,

cross-linked proteins in range of 35 - 100 kD. Because activated mitomycin C has a molecular geometry similar to that of the macrocyclic pyrrolic PAs (55-57), PA-induced DNA cross-links may be similar to these induced by this standard cross-linker.

DNA-protein cross-links are thought to be involved in carcinogenic or anti-carcinogenic activity of many bifunctional alkylating agents such as nitrogen mustard and cisplatin (34, 58). *Cis*-platinum and trivalent chromium cross-link to the N^7 position of guanine (59, 60) and probably bind to cysteine (61). In this study, the interaction of pyrrolic PA and its cross-linking ability *in vitro* were also investigated using several proteins. Dehydrosenecionine and *cis*-dichlorodiammine platinum(II) induced DNA cross-links with BSA, which has a high proportion of cysteine (62). However, these compounds did not appear to form DNA cross-links with actin or myocin, each of which has a lower proportion of cysteine than BSA. Possibly I might not be able to detect these cross-links in my system. Thus, pyrrolic PA-induced DNA-protein cross-links *in vitro* appeared to be influenced by the amino acid composition of the protein. Histone was completely bound with target DNA in this study, which was probably due to the inherent affinity of this protein to DNA.

The DNA-BSA cross-linking also occurs following treatment with chromium *in vitro* (63, 64). Lin *et al.* (65) investigated metal-induced DNA cross-links to histidine and cysteine in CHO cells and Salnikow *et al.* (66) reported that more DNA complexes formed with tyrosine and cysteine in chromium-treated cells, and that histidine, methionine, or threonine were more susceptible than any other amino acid *in vitro*.

In summary, DNA-protein cross-linking is likely an important event in PA-associated toxicity, carcinogenicity, and anti-carcinogenicity. Because the major

molecular weights of PA-induced DNA cross-linked proteins were similar to those induced anti-cancer agents, the characteristics of PA-induced DNA-protein cross-links might be similar. The α,β-unsaturation of the macrocyclic ring of pyrrolic PAs is a structural feature that appears to be important in PA-induced DNA-protein cross-links. Determination of the amino acid(s) involved in PA-induced DNA-protein cross-links is the subject of studies currently under way.

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CHAPTER V

SUMMARY

Pyrrolizidine alkaloids (PAs) are natural toxins found in a wide variety of plant species that pose significant health hazards to humans and other animals. Many PAs are toxic and carcinogenic *in vivo* or *in vitro*. Pyrrolizidine alkaloids are bio-activated by the cytochrome P-450 mixed-function oxidase to form two major types of metabolites: the bifunctional electrophilic pyrroles that are highly reactive and can alkylate cellular macromolecules such as DNA and protein, and N-oxides that are least toxic. Pyrrolizidine alkaloid-induced potent DNA cross-linking activity and the structural features of PAs that influenced DNA cross-links were examined in our laboratory. However, whether such cross-links are important to the toxic action of PAs is not clear.

Thus, I examined some of the molecular mechanisms that may affect the ability of four major groups of PAs and their metabolites to induce cytopathologic effects and DNA cross-links. I conducted three studies concerning the PA-induced DNA cross-links on (1) colony formation and cytopathologic changes, (2) DNA-DNA cross-links including interstrand cross-links and the cross-linking site specificity, and (3) DNA-protein cross-links in cellular and target DNA systems.

In the first study, the ability of eight PA congeners to inhibit mitosis and to induce megalocyte formation in cultured bovine kidney epithelial (MDBK) cells was related to their structural features. The macrocyclic α,β-unsaturated diester PAs (seneciphylline, senecionine, riddelliine, retrorsine) inhibited colony formation and induced megalocytosis in a dose-dependent manner. Colony growth resumed 3 weeks after these PAs were removed, and cell morphology

reverted to normal 5 or 6 weeks after these PAs were removed. At high concentrations (500 μM), the α,β-saturated macrocyclic (monocrotaline) and open diesters (heliosupine) only slightly inhibited colony formation and did not affect cell morphology. The necine base (retronecine) did not affect either colony formation or cell morphology. The pyrrolic metabolites such as dehydrosenecionine, dehydromonocrotaline, and dehydroretronecine inhibited colony formation more than their parent compounds at 500 μM and were potent inducers of abnormal cell morphology. An N-oxide metabolite, indicine-N-oxide, was completely inactive as expected. The results clearly indicated that the structure of PAs influenced their cytopathologic effects.

In the next study, the DNA cross-linking activities of pyrrolic metabolites were compared to those of their parent PAs investigated in the cell system. Pyrrolic metabolites were more potent inducers of DNA cross-links than were their parent compounds. The most potent compounds were the macrocyclic α,β-unsaturated diester pyrroles such as dehydrosenecionine, dehydromonocrotaline, dehydroseneciphylline, and dehydroriddelliine, which induced both DNA-protein and DNA-DNA cross-links. The necine base pyrrole dehydroretronecine and the N-oxide metabolite indicine N-oxide induced relatively few DNA cross-links. Indicine N-oxide was about as potent as indicine. Thus, PA-induced DNA cross-linking activity was highly correlated with structural features and the metabolic pathways of PAs. For example, monocrotaline, which had little effect on either mitosis and megalocytosis or on the formation of cross-links, is metabolized by cytochrome P-450 mixed-function oxidase system to a relatively high proportion of N-oxide.

The comparative potency of a variety of PAs to induce DNA-DNA cross-links in *Bst* EII-digested λ-phage DNA and *Bam* HI-digested pBR322 plasmid

DNA was also studied. As in the cell system, the macrocyclic α,β-unsaturated diester pyrroles such as dehydrosenecionine, dehydroseneciphylline, or dehydroriddelliine induced significantly potent DNA-DNA cross-links in *Bst* EII-digested λ-phage DNA and pBR322 plasmid DNA, and the potency of DNA cross-links increased in a dose-dependent manner. The macrocyclic α,β-saturated diester pyrrole such as dehydromonocrotaline also induced potent DNA-DNA cross-links. However, the necine base pyrrole such as dehydroretronecine, and the N-oxide form of metabolites such as indicine N-oxide induce no DNA-DNA cross-links.

In pBR322 plasmid DNA, the macrocyclic α,β-unsaturated diester pyrrole such as dehydrosenecionine was also the most potent in inhibiting the PCR amplification of a 1.129kb region of pBR322. The macrocyclic α,β-saturated diester pyrrole such as dehydromonocrotaline was slightly less potent than was dehydrosenecionine. The necine base pyrrole, dehydroretronecine, and indicine N-oxide did not induce any interstrand DNA cross-links. The results clearly indicated that the macrocyclic α,β-unsaturated diester pyrrole (dehydrosenecionine) was the most potent inducer of DNA-DNA interstrand cross-links, and also the structure of PAs influenced their DNA-DNA interstrand cross-links.

I also determined which nucleotide sequences were likely to be involved in cross-linking in pyrrole-treated pBR322 plasmid DNA with restriction endonucleases. In DNA treated with dehydrosenecionine, which is one of the macrocyclic α,β-unsaturated diester pyrroles, the recognition site of *Ava* II, *Fok* I, and *Taq* I was mostly cross-linked, the recognition site of *Eco* RII and *Mbo* II was moderately cross-linked, and the recognition site of *Hinf* I was least cross-linked. pBR322 plasmid DNA treated with *cis*-dichlorodiammine platinum(II)

completely blocked the action of these restriction endonucleases. There was only a mild blocking effect in the macrocyclic α,β-saturated diester pyrrole, dehydromonocrotaline-treated DNA with *Mbo* II. In DNA treated with the necine base pyrrole, dehydroretronecine, or indicine N-oxide, there was no inhibition of these restriction endonucleases. From the differential inhibition of restriction endonucleases, there appeared to be no discernible rules regarding sequence specificity, but the most favorable cross-linking sites for PAs appeared to be 5'-d(GG) or 5'-d(GA) sequences, although other sequences such as 5'-d(CG) or 5'-d(CC) may be involved.

The nature of DNA-protein interactions induced by PAs was also investigated because the large portion of PA-induced cross-links was protein associated in our previous study. The cellular DNA-protein cross-linking activity of the pyrrolic metabolites was as follows: dehydrosenecionine > dehydromonocrotaline > dehydroseneciphylline > dehydroriddelliine > dehydroretronecine and those that were protein-associated accounted for approximately 50% of the total cellular DNA cross-links. However, dehydroretronecine induced few DNA-protein cross-links, and none were detected following treatment with indicine N-oxide. Thus, the macrocyclic diester pyrroles were potent cellular DNA-protein cross-links.

Because a major portion of PA-induced cellular DNA cross-links involved proteins, we characterized DNA cross-linked proteins in cell cultures by isolating and separating the cross-linked proteins using SDS-PAGE and 2-D gel electrophoresis. In cells treated with the macrocyclic α,β-unsaturated diesters (senecionine, seneciphylline, riddelliine, retrorsine), the macrocyclic α,β-saturated diester (monocrotaline) and open diester (heliosupine), the molecular weight of the proteins cross-linked with DNA ranged from 40 - 60 kD. The

chemically activated pyrroles (dehydrosenecionine, dehydroseneciphylline, dehydroretrorsine, dehydromonocrotaline) also induced a high degree of DNA cross-linked proteins in nuclei, but dehydroretronecine or indicine N-oxide did not induce DNA cross-linked proteins. Nonhistone nuclear proteins were possibly involved the PA-induced DNA-protein cross-links, and the proteins induced by macrocyclic diester pyrrole were also detected on the acidic site of 2-D gels. The cross-linked proteins to DNA in patterns were similar to that induced by the anti-cancer agents, mitomycin C, and *cis*-dichlorodiammine platinum (II). These results indicated that the macrocyclic diester PAs or pyrroles were more potent DNA-protein cross-linkers than the simple necine base.

The pyrrole-induced DNA-protein cross-links were also investigated by co-incubating actin, BSA, histone, or myosin with dehydrosenecionine in pBR322 plasmid DNA or *Bst* EII-digested λ-phage DNA. The DNA-protein cross-links were formed when dehydrosenecionine was incubated with BSA in pBR322 plasmid DNA and *Bst* EII-digested λ-phage DNA.

Taken together, these experiments indicated that DNA cross-linking activity is probably the most critical event leading to PA bio-activity. The inhibition of colony formation, cytopathologic effects, and DNA cross-linking activities of PAs are largely due to the action of pyrrolic metabolites rather than via a common pyrrolic intermediate as was once thought. These results also cinfirmed that PA-induced DNA cross-links are influenced by three structural features: the C1,2 unsaturation of the pyrrolizidine ring, α,β-unsaturation, and the size of the macrocyclic diester ring. Furthermore, because PAs cross-link in a manner similar to known anti-tumor agents, this work indicates that some PAs might have anti-carcinogenic activity.

APPENDIX

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Pharmacology and toxicology research, in vivo and in vitro : Pharmaco-kinetics, gastro-intestistinal tract related experiment, cardio-vascular experiment, psycho-somatic experiment, central nervous system experiment (surgeryt, organ perfusion, primary cell culture, tissue culture, etc.) (1974 - 1985)

Toxicology research, in vitro : Kinetics, carcinogenesis, mutagenesis, DNA damage, DNA adduct with natural toxins (aflatoxin, pyrrolizidine alkaloids, 3-MI, other plant alkaloids) in cell culture (1987 - 1994) .

Cell culture and tissue culture : Normal cell culture, cancer cell culture, pre-cancerous cell culture, transformed cell culture, primary cell culture, tissue culture, bacteria cell culture, and cell metabolism, cytopathological changes, MTT test, etc. (1987 - 1994).

Molecular biology related research : DNA related experiment (DNA-DNA or DNA-protein cross-linking), isolated DNA from bacterial system and eukaryotic system, mutagenesis, carcinogenesis, tumorigenesis, point mutation, polymerase chain reaction (PCR), DNA sequencing (Sanger method or Maxim-Gilbert method), Western or Southern blotting, probe, Ames assay, DNA adduct , etc. (1987 - 1994)

Biochemistry related research : HPLC, EC detector, chromatography, RIA (cAMP, cGMP), electrophoresis (agarose gel electrophoresis, PAGE, SDS-PAGE isoelectrofocusing, second dimensional electrophoresis, capillary gel electrophoresis), enzyme assay, etc. (1974 - 1994)

Radioisotope material (125I, 32P, 35S, 3H, 14C) and biohazard material : Including safety oriented training (1974 -1994)