FINAL

Report on Carcinogens Background Document for

Riddelliine

August 11, 2008

U.S. Department of Health and Human Services
Public Health Services
National Toxicology Program
Research Triangle Park, NC 27709

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FOREWORD

The Report on Carcinogens (RoC) is prepared in response to Section 301 of the Public Health Service Act as amended. The RoC contains a list of identified substances (i) that either are known to be human carcinogens or are reasonably be anticipated to be human carcinogens and (ii) to which a significant number of persons residing in the United States are exposed. The Secretary, Department of Health and Human Services (HHS), has delegated responsibility for preparation of the RoC to the National Toxicology Program (NTP), which prepares the report with assistance from other Federal health and regulatory agencies and nongovernmental institutions.

Nominations for (1) listing a new substance, (2) reclassifying the listing status for a substance already listed, or (3) removing a substance already listed in the RoC are reviewed in a multi-step, scientific review process with multiple opportunities for public comment. The scientific peer-review groups evaluate and make independent recommendations for each nomination according to specific RoC listing criteria. This background document was prepared to assist in the review of riddelliine. The scientific information used to prepare Sections 3 through 5 of this document must come from publicly available, peer-reviewed sources. Information in Sections 1 and 2, including chemical and physical properties, analytical methods, production, use, and occurrence may come from published and/or unpublished sources. For each study cited in the background document from the peer-reviewed literature, information on funding sources (if available) and the authors' affiliations are provided in the reference section. The draft background document was peer reviewed in a public forum by an *ad hoc* expert panel of scientists from the public and private sectors with relevant expertise and knowledge selected by the NTP in accordance with the Federal Advisory Committee Act and HHS guidelines and regulations. This document has been finalized based on the peer-review recommendations of the expert panel and public comments received on the draft document. Any interpretive conclusions, comments, or statistical calculations made by the authors or peer reviewers of this document that are not contained in the original citation are identified in brackets [ ].

A detailed description of the RoC nomination review process and a list of all substances under consideration for listing in or delisting from the RoC can be obtained by accessing the 12th RoC at http://ntp.niehs.nih.gov/go/9732. The most recent RoC, the 11th Edition (2004), is available at http://ntp.niehs.nih.gov/go/19914.

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PEER-REVIEW

The draft background document on Riddelliine was peer reviewed by the Report on Carcinogens (RoC) expert panel for Riddelliine and Aristolochic Acid. The panel met in a public forum at the Sheraton Chapel Hill Hotel, Chapel Hill, NC on January 24 – 25, 2008. Members of the expert panel are as follows:

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Criteria for Listing Agents, Substances or Mixtures in the Report on Carcinogens

U.S. Department of Health and Human Services
National Toxicology Program

The criteria for listing an agent, substance, mixture, or exposure circumstance in the RoC are as follows:

Known To Be Human Carcinogen:

There is sufficient evidence of carcinogenicity from studies in humans*, which indicates a causal relationship between exposure to the agent, substance, or mixture, and human cancer.

Reasonably Anticipated To Be Human Carcinogen:

There is limited evidence of carcinogenicity from studies in humans*, which indicates that causal interpretation is credible, but that alternative explanations, such as chance, bias, or confounding factors, could not adequately be excluded,

or

there is sufficient evidence of carcinogenicity from studies in experimental animals, which indicates there is an increased incidence of malignant and/or a combination of malignant and benign tumors (1) in multiple species or at multiple tissue sites, or (2) by multiple routes of exposure, or (3) to an unusual degree with regard to incidence, site, or type of tumor, or age at onset,

or

there is less than sufficient evidence of carcinogenicity in humans or laboratory animals; however, the agent, substance, or mixture belongs to a well-defined, structurally related class of substances whose members are listed in a previous Report on Carcinogens as either known to be a human carcinogen or reasonably anticipated to be a human carcinogen, or there is convincing relevant information that the agent acts through mechanisms indicating it would likely cause cancer in humans.

Conclusions regarding carcinogenicity in humans or experimental animals are based on scientific judgment, with consideration given to all relevant information. Relevant information includes, but is not limited to, dose response, route of exposure, chemical structure, metabolism, pharmacokinetics, sensitive sub-populations, genetic effects, or other data relating to mechanism of action or factors that may be unique to a given substance. For example, there may be substances for which there is evidence of carcinogenicity in laboratory animals, but there are compelling data indicating that the agent acts through mechanisms which do not operate in humans and would therefore not reasonably be anticipated to cause cancer in humans.

*This evidence can include traditional cancer epidemiology studies, data from clinical studies, and/or data derived from the study of tissues or cells from humans exposed to the substance in question that can be useful for evaluating whether a relevant cancer mechanism is operating in people.

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Executive Summary

Introduction

Riddelliine is a pyrrolizidine alkaloid (PA) of the macrocyclic diester class. PAs are esters of unsaturated basic alcohols (necine bases) and necic acids and have been estimated to be present in approximately 6,000 plant species in 12 families distributed throughout the temperate and tropical regions of the world. Riddelliine was nominated by the National Institute of Environmental Health Sciences for possible listing in the Report on Carcinogens based on the results of a National Toxicology Program bioassay that reported clear evidence of carcinogenic activity in rats and mice.

Human Exposure

Riddelliine and riddelliine *N*-oxide (a metabolite of riddelliine that can be converted back to riddelliine) occur in plants of the genus *Senecio* that are found in sandy desert areas of the western United States and other parts of the world. At least 15 *Senecio* species have been identified that are used in herbal medicines or possibly as food worldwide. Herbal products containing PAs, including several herbal teas, have been extensively documented as causing toxicity in humans. Two cases of accidental poisoning of infants were reported from the southwestern United States in which *Senecio longilobus*, a species known to contain riddelliine as well as seneciphylline, senecionine, and retrorsine, was accidentally used to prepare an herbal tea known as gordolobo yerba. *Senecio* species containing riddelliine are not generally used as food plants in the United States, but ingestion by humans could result from direct contamination of foodstuffs by parts of *Senecio* plants or from indirect introduction of the alkaloid through products derived from animals that have fed on the plants. Evidence for ingestion of these products comes from reports of toxicity in animals and humans. Cases have been reported from outside the United States of accidental human poisoning from grains and flours contaminated with *Senecio* plant parts. PAs have also been detected in eggs, and honey has been shown to contain either PAs or pollen from PA-containing plants. Experimental studies of cows fed *Senecio* plants have demonstrated that PAs can be transmitted in milk.

Human Cancer Studies

No studies on the relationship between human cancer and exposure to riddelliine were identified.

Studies in Experimental Animals

When administered by gavage, riddelliine caused significantly increased incidences of malignant and benign tumors at multiple tissue sites in B6C3F_1 mice and F344/N rats. In B6C3F_1 mice, exposure to riddelliine caused hemangiosarcoma in the liver in males and alveolar/bronchiolar tumors in females. In F344/N rats, exposure to riddelliine caused hemangiosarcomas in the liver in both sexes. Hepatocellular adenoma and mononuclear-cell leukemia in both sexes of rats were also considered to be treatment related. Liver nodules were observed in a small study in Wistar rats exposed to riddelliine via drinking water followed by intraperitoneal injection(s) of riddelliine. The riddelliine metabolites dehydroretronecine (*R*-DHP) and dehydroheliotridine (*S*-DHP) caused tumors in rodents exposed by dermal application, subcutaneous injection, or intraperitoneal injection. In addition, ingestion of dried plant materials or extracts containing riddelliine caused liver tumors in rats and chickens.

Absorption, Distribution, Metabolism, and Excretion

Riddelliine and other PAs are absorbed primarily via ingestion (though dermal absorption can occur), distributed to the liver, and excreted in the urine and feces. Riddelliine has three primary metabolic pathways: (1) hydrolysis of the ester group(s) to form the necine base, (2) oxidation of the necine base (of riddelliine) to the corresponding *N*-oxide (which may be reduced to riddelliine), and (3) hydroxylation of the necine base (of riddelliine), followed by dehydration to form the corresponding dehydroriddelliine (pyrrolic) derivative. This pyrrolic derivative is then hydrolyzed to form the racemic (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine (DHP), which is a 50/50 mixture of the optically pure *R*-DHP and *S*-DHP enantiomers. Metabolism of PAs to the reactive pyrrolic ester metabolites in humans and rodents is mainly catalyzed by CYP3A and CPY2B6 isozymes of cytochrome P450. Metabolism of PAs to the corresponding *N*-oxides is catalyzed by both cytochrome P450- and flavin-containing monooxygenase.

Mechanisms of Genotoxicity and Tumorigenicity

DHP can bind DNA, which may be a key step leading to its genotoxicity and tumorigenicity. A set of eight DHP-derived adduct peaks has been detected in DNA reacted with riddelliine in the presence of rat microsomes. Dose-dependent DHP adduct formation has also been detected in livers of rats and mice exposed to riddelliine. Adduct levels were higher in endothelial cells than in parenchymal cells in rats and were more persistent in endothelial cells than in parenchymal cells in both rats and mice suggesting that tumor specificity was due to higher levels of DNA damage in the cells that form liver hemangiosarcomas. The kinetic parameters (V_{max} and K_m) for formation of DHP are comparable in human and rat microsomes, and the same profile of DHP-adduct peaks is also detected. In addition, other PAs have been shown to be metabolized to DHP and to cause liver tumors and, to a lesser extent, tumors of other organs, including the CNS, lung, pancreas, bladder, skin, testes, pituitary, and adrenal gland, in rats.

DNA-adduct formation may play a role in the genotoxicity of riddelliine. Riddelliine induced a higher frequency of mutations in non-neoplastic endothelial cells (but not in parenchymal cells) in the cII gene mutation assay in transgenic Big Blue rats. The predominant mutations observed were G·C to T·A transversions, which are consistent with riddelliine-induced formation of DNA adducts involving G·C base pairs. Riddelliine also induced mutations in a *S. typhimurium* strain (TA100) that detects base-pair substitutions (in the presence of metabolic activation) but not in three other *S. typhimurium* strains that detect frameshift mutations (with or without metabolic activation). In addition to mutations, riddelliine also induced other types of genetic damage in mammalian experimental studies. *In vitro*, riddelliine increased the frequency of sister chromatid exchange and chromosomal aberrations in Chinese hamster ovary cells, cell transformation in BALB/c-3T3 fibroblast cells, and DNA cross-linking, but not DNA strand breaks in bovine kidney epithelial cells. In rats exposed *in vivo*, riddelliine induced S-phase synthesis in hepatocytes and endothelial cells and increased p53 expression in endothelial cells but did not induce micronucleus formation in polychromatic erythrocytes. In mice, riddelliine caused unscheduled hepatocyte DNA synthesis (in females only), but did not induce micronucleus formation.

Mutations in the k-*ras* gene and increased p53 gene expression were detected in hemangiosarcomas from mice treated with riddelliine.

In addition to the formation of exogenous (DHP-DNA adducts), the formation of endogenous DNA adducts and formation of DNA-DNA and DNA-protein cross-links have also been proposed as mechanisms of tumorigenicity. Riddelliine metabolites appear to cause damage to endothelial cells, as shown by karyomegaly and cytomegaly and accumulation of intravascular macrophages in many organs. Short-term exposure to riddelliine in rats increased apoptosis and S-phase nuclei in endothelial cells and hepatocytes. Increased levels of p53 protein were detected in endothelial cells, and vascular endothelial growth factor (VEGF), an endothelial cell-specific mitogen, was increased in hepatocytes. Development of hemangiosarcoma in the liver may have resulted from endothelial cell DNA-adduct formation, apoptosis, proliferation of endothelial cells, and mutations. Increased expression of VEGF protein also could have contributed by stimulating endothelial cell proliferation.

Metabolites and analogues of riddelliine have shown carcinogenic and genotoxic properties in experimental animals. Since many of the PAs share a common metabolic activation pathway, the genotoxic and carcinogenic effects are similar to those observed with riddelliine. DHP-DNA adducts, mutations, clastogenic effects, liver tumors in rats and, to a lesser extent, tumors of other organs, including the CNS, lung, bladder, pancreas, skin, testes, pituitary, and adrenal gland, have been observed in studies with other PAs or plant extracts containing PAs.

The genotoxicity, tumorigenicity, and toxicity of PAs vary, but the structure-activity relationships are not well defined. In general, the macrocyclic diester types are the most genotoxic and the monoesters types the least. While the ability of PAs to form cross-links has been proposed to affect their toxicity, only limited data are available for this potential relationship.

Toxicity

The liver is the primary target organ in humans, experimental animals, and livestock. Veno-occlusive disease is a characteristic lesion in humans poisoned by PAs. Other

common effects in humans include ascites, splenomegaly, hepatomegaly, centrilobular hepatic necrosis, and cirrhosis. Young children appear to be particularly susceptible since many of the case reports involve infants and young children. Livestock poisoned by ingesting PA-containing plants often develop fatal liver disease. [The available data indicate interspecies differences in susceptibility with sheep, guinea-pigs, gerbils, hamsters, and rabbits showing resistance, while rats, cattle, horses, and chickens are highly susceptible.] The lungs are the second most common site of PA toxicity, but not all PAs affect the lungs. The primary site of damage is the pulmonary vasculature. The 11-membered macrocyclic diesters such as monocrotaline are particularly active in the lung but only at doses that were equal to or greater than doses causing liver toxicity.

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1 Abbreviations

AUC:	area under the time-concentration curve
b.w.:	body weight
CHO:	Chinese hamster ovary
CNS:	central nervous system
dec:	decomposes (used to indicate when a substance decomposes at its boiling point or melting point)
DHH:	dehydroheliotridine, also called *S*-DHP
DHP:	racemic mixture of (+/-) 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine; see also *R*-DHP (DHR) and *S*-DHP (DHH)
DHR:	dehydroretronecine, also called *R*-DHP
DSHEA	Dietary Supplement Health and Education Act
ELISA:	enzyme-linked immunosorbent assay
GC-MS:	gas chromatography-mass spectrometry
GFHB:	German Federal Health Bureau
HPLC:	high performance liquid chromatography
i.p.:	intraperitoneal
IARC:	International Agency for Research on Cancer
LC:	liquid chromatography
LC-ES/MS:	liquid chromatography-electrospray mass spectrometry
LC-MS:	liquid chromatography-mass spectrometry
LC-MS-MS:	tandem mass spectrometry
mol wt:	molecular weight
MS:	mass spectrometry
NMR:	nuclear magnetic resonance

NTP:	National Toxicology Program
PA:	pyrrolizidine alkaloid
PCE:	polychromatic erythrocyte
ppb:	parts per billion
ppm:	parts per million
R-DHP:	dehydroretronecine, also called DHR
RTECS:	Registry of Toxic Effects of Chemical Substances
s.c.:	subcutaneous
SCE:	sister chromatid exchange
S-DHP:	dehydroheliotridine, also called DHH
s.e.m.:	standard error of the mean
SIM:	selected ion monitoring
TLC:	thin layer chromatography
UDS:	unscheduled DNA synthesis
UV:	ultraviolet
VEGF:	vascular endothelial growth factor

Table of Contents

List of Tables

List of Figures

1 Introduction

Riddelliine is a pyrrolizidine alkaloid (PA) of the macrocyclic diester class. It occurs naturally in plants (primarily of the genus *Senecio*) that are found in the western United States and other parts of the world. Cattle, horses, and sheep that consume PA-containing *Senecio* species while grazing may succumb to their toxic effects, primarily related to hepatotoxicity. The toxicity is cumulative and may occur over a period of several years. PAs are not known to be toxic *per se* but are oxidized by hepatic enzymes to pyrrolic metabolites, which are the proximate toxins. Riddelliine and other PAs exist in plants as both the free-base alkaloid and the *N*-oxide. The *N*-oxides cannot be oxidized directly to pyrroles but must first be reduced to the free base, a process that often occurs in the digestive tract. PA residues have been found in grains, milk, eggs, and honey, and the plants may contaminate human food sources or be used as dietary supplements or for medicinal purposes. Cases have been reported of accidental human poisoning from grains and flours, and herbal medicines contaminated with *Senecio* plant parts.

Riddelliine was initially nominated by the U.S. Food and Drug Administration for study by the National Toxicology Program (NTP) in its rodent bioassay program because of riddelliine's potential for human exposure and its economic impact on the livestock industry and because the toxicity of other PAs suggested that riddelliine might be carcinogenic. It was nominated by the National Institute of Environmental Health Sciences for possible listing in the Report on Carcinogens based on the results of a NTP bioassay (NTP 2003), which reported *clear evidence* of carcinogenic activity in male and female F344/N rats and B6C3F$_1$ mice.

1.1 Chemical identification

PAs are esters of unsaturated basic alcohols (necine bases) and necic acids, and have been estimated to be present in more than 6,000 plant species, i.e., approximately 3% of the world's flowering plants, in 12 families distributed throughout the temperate and tropical regions of the world (Smith and Culvenor 1981, Mattocks 1986). Necic acids are branched-chained mono- or di-carboxylic acids containing four to six carbon atoms and are typically unsaturated, hydroxylated, or epoxidized. The four most common types of necine bases found in PAs are platynecine, retronecine, heliotridine, and otonecine

(Figure 1-1). Retronecine and heliotridine are enantiomers and have been studied the most because of their abundance and toxicity.

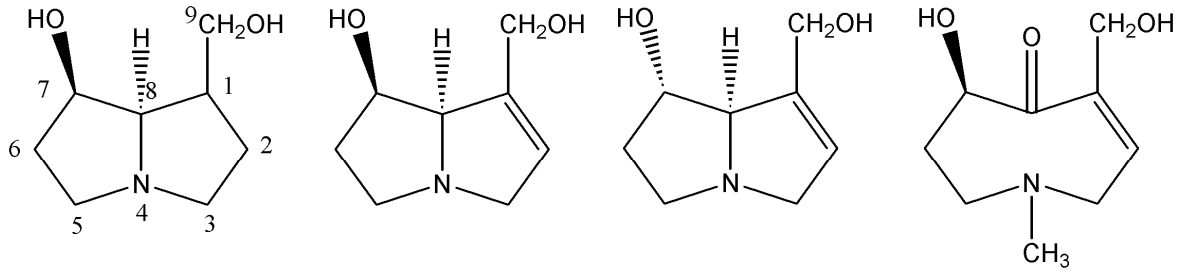


Figure 1-1. Necine bases of PAs

Note: The numbering of the atoms in the ring structure of platynecine also applies to the other bases.
Source: Fu *et al.* 2002b, used with permission.

Riddelliine consists of the necine base retronecine which is esterified with riddelliic acid, an oxygenated dicarboxylic acid (see Table 1-3). The pyrrolizidine nucleus, retronecine, consists of two fused five-membered pyrrole rings with a nitrogen atom at the bridgehead position and a 1,2-double bond. This pyrrolizidine ring system has a hydroxymethyl group at the 1-position and a hydroxyl group at the 7-position, through which the esterifying acid is attached. Riddelliine exists in plants as the free-base alkaloid and as an *N*-oxide; therefore, properties of both forms are presented below. The structures of riddelliine and riddelliine *N*-oxide are shown in Figure 1-2.

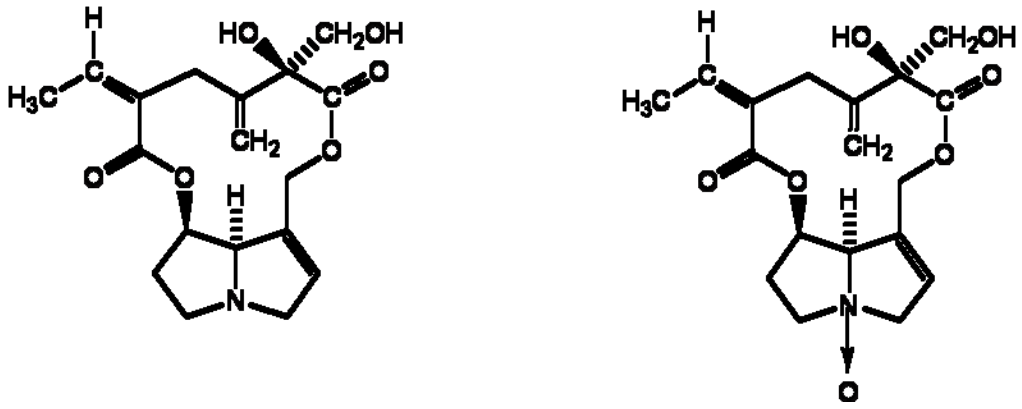


Figure 1-2. Structures of riddelliine (left) and riddelliine *N*-oxide (right)

Source: Chou *et al.* 2003a, used with permission.

Some PAs are open-chain esters (monoesters and diesters), and some form a macrocyclic diester. Riddelliine is a macrocyclic diester with a retronecine base (Figure 1-2). Structural features of PAs associated with hepatotoxicity in rats and mice include (1) a double bond in the 3-pyrroline ring, (2) one or two hydroxyl groups attached to the pyrroline ring, (3) one or two ester linkages between the base and necic acid, and (4) the presence of a branched chain on the acid moiety (Mattocks 1986, Prakash *et al.* 1999). The specific chemical or metabolic mechanisms linking these structural features with toxicity of PAs have not all been identified, but it is known that PAs with the platynecine base, which do not have the double bond between positions C-1 and C-2, are not hepatotoxic. In addition, Mattocks (1986) proposed that chain branching in the acid moiety appears to be necessary for the hepatotoxicity of the PAs because branched esters are more sterically hindered and thus are better able to resist detoxification by ester hydrolysis. Administration of an esterase inhibitor to animals increases the conversion of PAs to toxic metabolites in the liver and leads to increased hepatotoxicity. Other chemical identification information for riddelliine is provided in Table 1-1.

Table 1-1. Chemical identification of riddelliine

Characteristic	Information
Chemical Abstracts index name	13,19-didehydro-12,18-dihydroxysenecionan-11,16-dione
CAS Registry no.	23246-96-0
Molecular formula	$C_{18}H_{23}NO_6$
Synonyms	3-ethylidine-3Z,4,5,6S,9,11,13,14,14αR,14βR-decahydro-6-hydroxy-6-(hydroxymethyl)-5-methylene[1,6]di-oxacyclododecino[2,3,4-*gh*]-pyrrolizine-2,7-dione *trans*-15-ethylidine-12β-hydroxy-12α-hydroxymethyl-13-methylenesenec-1-enine

Sources: IARC 2002, NTP 2003, ChemIDplus 2007.

1.2 Physical-chemical properties

Both riddelliine and riddelliine *N*-oxide are white crystalline solids. Other physical and chemical properties of riddelliine and riddelliine *N*-oxide are summarized in Table 1-2. Riddelliine is optically active, with an optical rotation ($[\alpha]_D^{25}$) of –109.5 ($CHCl_3$). Optical rotation of the hydrochloride salt is –80.6 (H_2O). Peak ultraviolet (UV) absorption (λ_{max}) of riddelliine is < 220 nm, as is that of the *N*-oxide. The hydrochloride and methiodide salts are readily soluble in water. The solid is stable at room temperature

in diffuse light for several years (R.J. Molyneux, Western Regional Research Center, USDA, Albany, CA; email to Sanford Garner, Constella Group, LLC, Durham, NC, December 4, 2006). Alcoholic and aqueous solutions of riddelliine are stable at room temperature when protected from light. Riddelliine readily reacts with oxidizing agents to form dihydropyrrolizine and other derivatives; however, it reacts slowly with atmospheric oxygen. It is readily hydrolyzed in aqueous alkali (IARC 1976). Riddelliine *N*-oxide in solid form is stable at freezer temperature but darkens gradually over a long period when stored at room temperature in the dark.

Table 1-2. Physical and chemical properties of riddelliine and riddelliine *N*-oxide

Property[a]	Riddelliine	Riddelliine *N*-oxide
Molecular weight	349.4	365.4
Melting point (°C) HCl salt MeI salt	197–198 dec 225–226 dec 260–262 dec	156–158 dec
Boiling point (°C)	NF	NF
Density	NF	NF
Solubility: water acetone chloroform ethanol methanol	 sparingly soluble slightly soluble soluble slightly soluble soluble	 soluble insoluble insoluble slightly soluble soluble
Octanol-water partition coefficient (log K_{ow})	NF	NF
Vapor pressure	NF	NF
Vapor density	NF	NF
Critical temperature	NF	NF
Dissociation constant (pK_a)	NF	NF
Henry's law constant	NF	NF

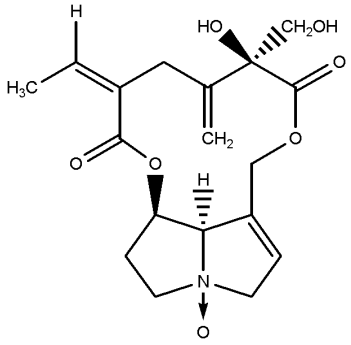
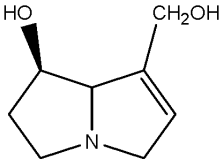
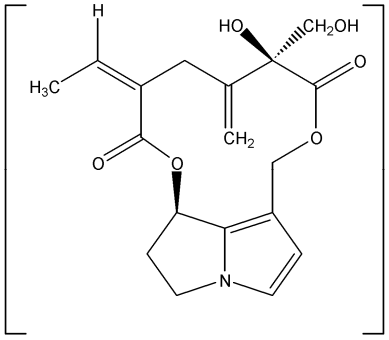
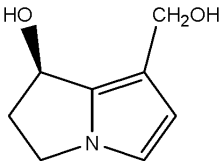
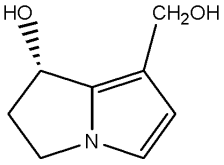
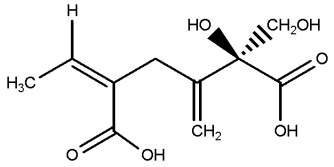
Sources: Mattocks 1986, Molyneux *et al.* 1991, Buckingham 2000; R.J. Molyneux, Western Regional Research Center, USDA, Albany, CA email to Sanford Garner, Constella Group, LLC, Durham, NC, December 4, 2006
dec = decomposes at or below its melting point; NF = not found.
[a]See Glossary for definitions of physical properties.

1.3 Metabolites

This section identifies the primary metabolites of riddelliine. A more detailed discussion of the metabolism of riddelliine is provided in Section 5.1.3.

Riddelliine is absorbed from the digestive tract and metabolized in the liver (Williams *et al.* 2002). Metabolites resulting from chemical modification of the pyrrolizidine nucleus are referred to generally as pyrrolic metabolites. *In vitro* metabolism of riddelliine by rat or mouse liver microsomes generates riddelliine *N*-oxide and 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine (DHP) as major metabolites and retronecine as a minor metabolite (Yang *et al.* 2001a, Fu *et al.* 2002b, Chou *et al.* 2003c, 2004). The two enantiomers of DHP are dehydroretronecine (*R*-DHP or DHR) and dehydroheliotridine (*S*-DHP or DHH). Dehydroriddelliine was presumed by Chou *et al.* to be formed as an intermediate that then was hydrolyzed to DHP. Activated pyrroles of PAs (dehydro-PAs), including dehydroriddelliine, are reactive and unstable in solution, polymerizing in the presence of moisture and acid (Mattocks *et al.* 1989). Riddelliine metabolites and the riddelliic acid side chain are shown in Table 1-3.

Table 1-3. Riddelliine metabolites

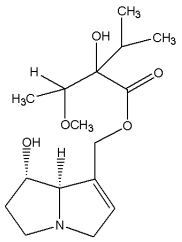
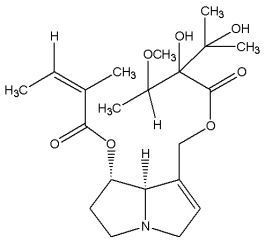
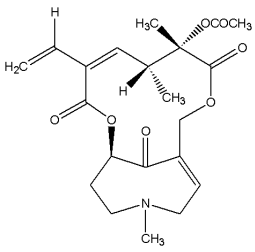
Metabolite	Molecular weight	Structure
Riddelliine *N*-oxide	365	
Retronecine	155	
Dehydroriddelliine	347	
Dehydroretronecine (*R*-DHP, or DHR)	153	
Dehydroheliotridine (*S*-DHP, or DHH)	153	
Riddelliic acid	232	

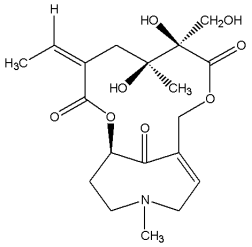
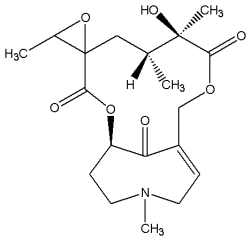
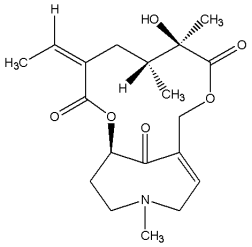
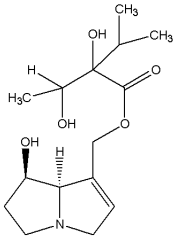
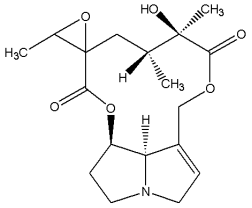
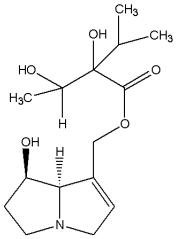
Sources: Fu *et al.* 2002b, Chou *et al.* 2003c, 2004, Yang *et al.* 2001a.

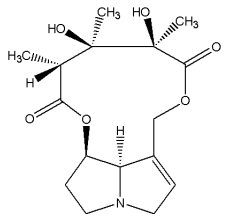
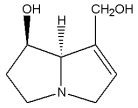
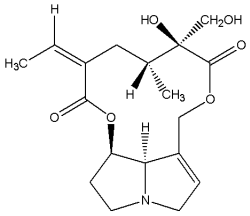
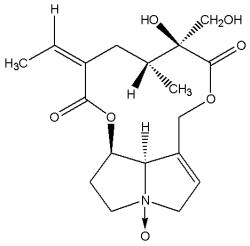
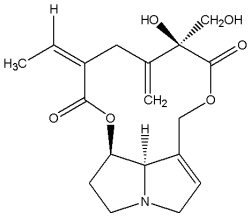
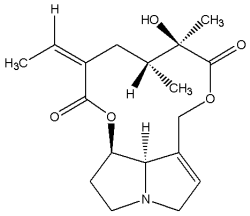
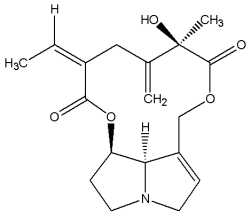
1.4 Riddelliine analogues

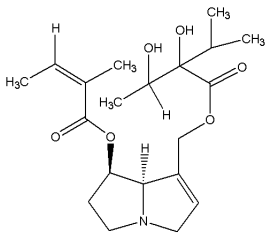
PAs number approximately 400, not including the corresponding *N*-oxides. They may be divided into three major categories: monoesters, diesters, and macrocyclic diesters. Riddelliine is a macrocyclic diester. Of the 148 macrocyclic diester alkaloids, the majority have 12-membered rings (Hartmann and Witte 1995). Riddelliine has a structure similar to that of senecionine, seneciphylline, and retrorsine, with which it frequently co-occurs in *Senecio* species. The closely related structures of these alkaloids are shown in Table 1-4. Riddelliine has hundreds of analogues; only those that have induced tumors in rats are listed here. In addition to riddelliine and the retronecine base, these include 14 PAs and one *N*-oxide form, from three plant families. The names of these compounds, their chemical structures, plant families, and species are shown in Table 1-4. (See Section 5.5 and Table 5-9 for additional information about the carcinogenicity of riddelliine analogues in experimental animals.)

Table 1-4. PAs that have caused tumors in rats

Base type	Compound	Chemical structure	Plant family	Species
Heliotridine	heliotrine		Boraginaceae	*Heliotropium* spp.
Heliotridine	lasiocarpine		Boraginaceae	*Heliotropium* spp.
Otonecine	clivorine		Compositae (Asteraceae)	*Lingularia dentata*

Base type	Compound	Chemical structure	Plant family	Species
Otonecine	hydroxysenkirkine		Compositae (Asteraceae)	*Senecio* spp.
Otonecine	petasitenine		Compositae (Asteraceae)	*Senecio* spp.
Otonecine	senkirkine		Compositae (Asteraceae)	*Senecio* spp. *Petasites* spp.
Retronecine	intermedine		Boraginaceae	*Amsinckia* spp.
Retronecine	jacobine		Compositae (Asteraceae)	*Senecio* spp.
Retronecine	lycopsamine		Boraginaceae	*Amsinckia* spp.

Base type	Compound	Chemical structure	Plant family	Species
Retronecine	monocrotaline		Leguminosae (Fabaceae)	*Crotalaria* spp.
Retronecine	retronecine		Leguminosae (Fabaceae)	*Crotalaria* spp.
Retronecine	retrorsine		Compositae (Asteraceae)	*Senecio* spp.
Retronecine	retrorsine *N*-oxide (also known as isatidine)		Compositae (Asteraceae) Leguminosae (Fabaceae)	*Senecio* spp. *Crotalaria* spp.
Retronecine	riddelliine		Compositae (Asteraceae) Leguminosae	*Senecio* spp. *Crotalaria juncea*[a]
Retronecine	Senecionine[b]		Compositae (Asteraceae)	*Senecio* spp.
Retronecine	seneciphylline		Compositae (Asteraceae)	*Senecio* spp.

Base type	Compound	Chemical structure	Plant family	Species
Retronecine	symphytine		Boraginaceae	*Symphytum officinale*

Adapted from Fu *et al.* 2002b.

[a]Based on a single seed sample; see Section 2.3.1.

[b]Based on testing of plant extracts that contained senecionine.

2 Human Exposure

This section discusses use, production, environmental occurrence, environmental fate, general population exposure, occupational exposure, analytical methods, biological indices of exposure, and regulations and guidelines for riddelliine. Information on other PAs is also included because of the similarities in the chemistry and botanical distribution of riddelliine and other PAs. Thus, evidence for exposure to other PAs illustrates potential routes of exposure that could also occur with plants containing riddelliine.

Riddelliine and riddelliine *N*-oxide are naturally occurring PAs found in plants (primarily of the genus *Senecio*) that grow in sandy desert areas of the western United States and other parts of the world. The available information on human exposure to riddelliine and other PAs is based primarily on case reports of liver toxicity associated with ingestion of herbal products and contaminated grains and flours. The diagnosis of PA toxicity is difficult to establish, and additional cases of poisoning by PAs have probably occurred (Huxtable 1980a).

Riddelliine *N*-oxide also is discussed in this section and throughout the document because it can be converted back to riddelliine after ingestion (see Section 5.1 and Figure 5-2). The quantities of PA *N*-oxides present in plants are highly variable (Fu *et al.* 2002a) but often can be nearly equal to or even greatly exceed the quantities of parent PAs; in some cases, plants may contain only the *N*-oxide form (Mattocks 1986). Of particular concern is that PA *N*-oxides are much more water soluble than the corresponding PAs. When plants containing PAs and PA *N*-oxides are used as herbal tea or herbal medicine (e.g., in Chinese herbal medicine), much more PA *N*-oxide than PA will be extracted and ingested. Consequently, it is important to assess the risk to humans posed by drinking herbal teas (including bush teas, comfrey teas, or herb-derived decoctions) that contain PAs and/or PA *N*-oxides.

2.1 Use

Riddelliine and riddelliine *N*-oxide have no known commercial uses, and no vendors for these products were identified. However, riddelliine-containing plants have occurred in folk medicines and herbal teas in the United States and other parts of the world (Section

2.3.2). The riddelliine-containing plant *Senecio longilobus* has been used in medicinal herbal preparations in the United States and *S. jacobaea* and *S. vulgaris*, both of which have been shown to contain riddelliine (Table 2-1), have been reported to be used in medicinal preparations in other parts of the world (Mattocks 1986).

Although riddelliine-containing plants are not used for food in the United States, it has been reported that two plants of the *Senecio* genus (*S. burchellii* and *S. inaequidens*) have been used as "spinach" in South Africa. Although riddelliine has been found primarily in plants of the *Senecio* genus, it has not, however, been confirmed that the plants used as "spinach" contain riddelliine (see Table 2-1).

2.2 Production

Riddelliine for experimental purposes has been isolated from *S. riddellii*, and riddelliine *N*-oxide for large animal feeding experiments has been synthesized from riddelliine by oxidation with hydrogen peroxide in ethanol (Molyneux *et al.* 1991).

No data on U.S. production volume, sales, or imports of riddelliine or riddelliine-containing plants were identified. However, after a case of PA poisoning in Arizona in which *S. longilobus* was identified as an ingredient in an herbal tea that was consumed by the patient prior to onset of symptoms (Stillman *et al.* 1977), the distribution of the herb was traced to a major U.S. importer who also was a major supplier of herbs in the western United States (Huxtable 1980b). *Senecio*-containing products have been inadvertently distributed by pharmacies and herb stores and also could be consumed by people who gather herbs for private use (Fox *et al.* 1978). (See Section 2.3.2 for further discussion on PA poisonings from herbal products.)

2.3 Occurrence and exposure

This section presents information on the environmental fate and transport and the occurrence of riddelliine and other PAs in plants, herbal products, food, dust, and insects and the potential for human exposure to these substances. The general population may be exposed to riddelliine or other PAs by contacting or ingesting plants, herbal products, or animal products that either naturally contain or have been contaminated with these chemicals. Information on other PAs is also included because of the similarities in the

chemistry and botanical distribution of riddelliine and other PAs and because of the potential for similar routes of exposure.

The available information on exposure to riddelliine and other PAs is based primarily on case reports of liver toxicity (mostly veno-occlusive disease, which is a blockage of the small veins in the liver resulting in liver damage [see also Glossary]) associated with ingestion of herbal products and contaminated foods. Specific information on riddelliine or other individual PAs is often not available because PA exposure assessments of case studies were performed on total PAs, and specific PAs were not assessed (Huxtable 1980b). The assessment of the exposures leading to the PA toxicity is one of the major obstacles in confirming that poisoning with PAs has occurred. Diagnosis of PA poisoning has usually been based on liver symptoms or pathology and analysis of PAs in ingested herbs or foods. Diagnosis can be complicated by the time interval between exposure and disease onset and similarities of clinical symptoms with other diseases. Hence, it is likely that cases of PA poisoning in the United States might have been unreported or misdiagnosed. Numerous pathways for potential exposure exist, and these are discussed in the remainder of this section.

2.3.1 Occurrence in plants

Riddelliine has been identified in at least 13 species of the genus *Senecio* (Table 2-1) (Mattocks 1986, Hartmann and Witte 1995) and has been reported to occur in very low yield (< 0.003%) in a single sample of seeds of the legume *Crotalaria juncea* (Adams and Gianturco 1956). However, it was not detected in a second seed sample examined, and other investigators have not reported its presence in *C. juncea* or any other *Crotalaria* species. [PAs in *Crotalaria* generally are of the 11-membered macrocyclic type, in contrast to the 12-membered-ring structure of most *Senecio* alkaloids, and the occurrence of riddelliine in *Crotalaria* therefore appears to be chemotaxonomically unlikely. Furthermore, the fact that riddelliine was isolated in large quantities from *S. riddellii* by Adams *et al.* (1942) and structurally identified during the same time period (Adams and Van Duuren 1953) as the *C. juncea* report suggests that intralaboratory contamination could have occurred. Prakash *et al.* (1985) also reported trace amounts of riddelliine in *C. juncea*, but the experimental procedures described were not consistent

with isolation of macrocyclic diester class of alkaloids, and the structure was not rigorously confirmed by spectroscopic methods. Further research is needed to establish that riddelliine is an authentic constituent of *C. juncea*, and in the absence of confirmatory evidence, its presence in *C. juncea* should be regarded with suspicion.] Riddelliine co-occurs in most *Senecio* species with its *N*-oxide, the quantity of the latter often exceeding that of the free base.

Table 2-1. Plant species identified as containing riddelliine

Species	Synonym	Common name
Senecio aegypticus		
Senecio ambrosioides	*Senecio brasiliensis*	
Senecio cruentus		
Senecio cymbalarioides		
Senecio desfontanei	*Senecio coronopifolius*	
Senecio douglasii var. longilobus[a]	*Senecio longilobus*	woody or threadleaf groundsel
Senecio eremophilus		
Senecio jacobaea (erucifoline chemotype)[a]		tansy ragwort, stinking willie
Senecio riddellii[a]		Riddell's ragwort, Riddell's groundsel
Senecio spartioides[a]		broom groundsel
Senecio vulgaris[a]		common groundsel
Senecio pseudo-orientalis		
Senecio vernalis		
Crotalaria juncea		

Sources: Adams and Govindachari 1949, Bull *et al.* 1968, Huxtable 1980b, Mattocks 1986, Sener *et al.* 1986a, Sener *et al.* 1986b, Molyneux *et al.* 1991, Knight and Walter 2003.
[a]North American species.

The prototypical riddelliine-containing *Senecio*, Riddell's groundsel (*S. riddellii*), generally grows in desert areas of western North America, especially in sandy soils. It is a low, shrubby plant with bright green, thread-like leaves and intensely yellow composite flowers. The plant sprouts in the early spring and dies back to a woody crown in the early fall, although sufficient moisture from summer rains may initiate regrowth on the stems. The early-season growth and regrowth at periods when little other green leafy material is available may make it attractive to grazing animals. This plant was one of the earliest *Senecio* species to be identified as poisonous to animals, causing "walking disease" in

horses in Nebraska and adjacent areas of Colorado and Wyoming (see Sections 4 and 5.6). The syndrome was characterized by aimless wandering and cirrhosis of the liver.

Riddelliine and riddelliine *N*-oxide are the predominant alkaloids in *S. riddellii*, occurring in yields of up to 18% of the dry weight of the plant (Molyneux and Johnson 1984); however, alkaloid content may be highly variable, depending on growth stage, environmental conditions, and location (Johnson *et al.* 1985a). It has been calculated that at 18% total PA, as little as 33 g of dry or 176 g of fresh *S. riddellii* consumed per day would be toxic to a 300-kg cow. In other *Senecio* species, riddelliine is frequently accompanied by structurally related alkaloids, such as senecionine, seneciphylline, and retrorsine, and their corresponding *N*-oxides (Molyneux *et al.* 1979), which differ from riddelliine only in the structure of the esterifying moieties (senecic, seneciphyllic, and isatinecic acids, respectively).

PAs and their *N*-oxides have been estimated to be present in approximately 6,000 plant species, i.e., about 3% of all flowering plant species, belonging to disparate genera (Smith and Culvenor 1981). The impetus for their isolation and identification has been primarily the association of specific plants with livestock poisoning. A general review of PA occurrence, metabolism, and toxicity in relation to effects on livestock has been published (Stegelmeier *et al.* 1999). Many plants not occurring in major livestock production areas have not been analyzed for the presence of PAs, so it is likely that riddelliine will be found in additional species, especially in previously unexamined *Senecio* species.

The environmental fate of PAs is not well established. In *Senecio* species, the alkaloids are biosynthesized in the roots and, as the *N*-oxides, translocated in the phloem to the flower structure, where they are preferentially stored (Hartmann *et al.* 1989). After flowering, the PA content of the remaining plant is drastically reduced, presumably because the majority of the alkaloid is dispersed in seeds and flower fragments. Nevertheless, the alkaloid content in the remaining leaves can be appreciable. For example, in *S. riddellii* collected in Oklahoma over a five-year period, the total alkaloid content in the leaves immediately before senescence ranged from 3% to 6% on a dry-

weight basis (Johnson *et al.* 1985a). Hartmann and Witte (1995) concluded that there is no evidence for PA turnover or degradation in living vegetative plant parts. However, in germinating seeds of *Crotalaria,* the alkaloids are rapidly *N*-oxidized and catabolized as a source of nitrogen (Toppel *et al.* 1988).

Plants that do not biosynthesize PAs can acquire them through root parasitism. *Castilleja* species have been shown to assimilate PAs from *Liatris punctata*, *Senecio atratus,* and *S. triangularis* (Stermitz and Harris 1987, Mead *et al.* 1992), and transfer from *S. triangularis* to *Pedicularis* species also has been documented (Schneider and Stermitz 1990). *Castilleja rhexifolia* has been used as a traditional remedy, and PAs may therefore be ingested indirectly via this route.

2.3.2 Herbal products

Herbal products containing PAs, some from plants of the genus *Senecio*, have been extensively documented as causing toxicity in humans (Huxtable 1989a). These materials are consumed in many forms, including capsules of ground plant material, tinctures produced by solvent (usually alcohol) extraction, and teas brewed from the dried plant. Herbal products are consumed for a variety of reasons, among them to treat digestive disorders, as a cough suppressant and nasal decongestant, as a sore throat remedy, as general "cure-alls" for everyday aches and pains, and to promote longevity. The inherent variability in alkaloid content of plants, even within a species, due to plant part, maturity, and location, compounded by the different preparation methods, makes alkaloid intake highly variable and estimates problematic. In the United States, prior to 2001, these products were essentially unregulated, having been classified as natural food products under the Dietary Supplement Health and Education Act (DSHEA) of 1994, and no safety standards were imposed. In 2001, the FDA issued an advisory to dietary supplement manufacturers to remove comfrey products from the market. The advisory states that any product containing PAs is considered adulterated under DSHEA. The German Federal Health Bureau (GFHB 1992) also has established regulations restricting levels of PAs in orally consumed herbal products with proven health benefits. Other European countries have imposed similar limits, and it is likely that consistent regulations will be applied throughout the continent in the future (van Engelen *et al.* 1997).

In the United States, two cases of accidental PA poisoning involving ingestion of herbal tea containing *Senecio longilobus* have been reported (Stillman *et al.* 1977, Fox *et al.* 1978) (see Section 5.6). Both cases involved infants who were given a tea known locally in the southwestern United States as "gordolobo yerba." This tea normally is made from *Gnaphalium macounii* (common names include clammy cudweed and western cudweed) and used as a folk remedy, particularly as a cough suppressant for childhood ailments. However, in these cases, *S. longilobus* was mistaken for *G. macounii* in the collection of the tea ingredients, as the plants resemble one another. *S. longilobus* contains high levels (up to 8.7%) of a mixture of macrocyclic diester alkaloids (Johnson *et al.* 1985a), of which a significant proportion (ca. 20%) is riddelliine (seneciphylline constituted ca. 50% of the PA content in both young and mature whole plants, while senecionine and retrorsine were present in slightly lower proportions [10% to 15%] than riddelliiine) (Molyneux *et al.* 1979). One case involved a six-month-old female infant who regularly had been given a hot-water infusion of *S. longilobus* and who subsequently developed veno-occlusive disease which progressed to hepatic fibrosis and cirrhosis (Stillman *et al.* 1977). It was calculated that the child received 70 to 147 mg of total PAs in the two weeks before admission to the hospital (Huxtable 1980b). Based on the proportion measured in other, whole-plant samples of *S. longilobus*, the riddelliine content of this dose would have been 14 to 28 mg, although senecionine, retrorsine, and in particular, seneciphylline, also were consumed. The other case involved a two-month-old boy who, over a four-day period, had been given gordolobo yerba, which mistakenly contained *S. longilobus*. The herb was found to contain 1.5% by weight of hepatatoxic PAs (specific PAs not reported, but *S. longilobus* has been shown to contain riddelliine, as well as seneciphylline, senecionine, and retrorsine) and it was estimated that the infant probably consumed 66 mg of mixed alkaloids over the four-day period. The infant was initially diagnosed with Stage II Reye's syndrome. However, based on autopsy results, the cause of death was ruled to be PA intoxication.

After the first case of PA poisoning in the United States reported by Stillman (1977) noted above, the distribution for the herbal product that had been linked to the poisoning was traced. Huxtable (1980b) reported that the *S. longilobus*, which had been used in the herbal product, had been collected in Mexico and imported into the United States by a

major wholesaler. The importer was also a major supplier of herbs in the western United States. Huxtable noted that the importer stated that *S. longilobus* had been imported and sold by this company for two generations. Other cases of suspected PA poisoning have been reported among Mexican-Americans in Arizona who had ingested herbal teas, including gordolobo yerba, prior to disease onset; however, there was no documentation of whether PAs had been ingested (Huxtable 1980b, 1992).

Another closely related species with similar medicinal usage by Hispanic communities in the southwestern United States and northern Mexico is *Packera candidissima* (sometimes called *Senecio candidissimus*), which contains 0.76% senecionine-type alkaloids in the roots and 0.36% in the aerial parts (Bah *et al.* 1994).

One of the most conspicuous examples of PA poisoning by herbal remedies outside of the United States is that of "bush teas" in the West Indies and Jamaica. These infusions have been prepared from various plants, including *Crotalaria fulva*, which contains the 11-membered macrocyclic diester PA fulvine. These folk remedies have been most commonly administered for treatment of colds, digestive upsets, and teething pain. In Jamaica in the 1950s, an epidemic of veno-occlusive disease occurred in children from ingestion of bush teas (Bras *et al.* 1954). (See Section 5.6 for a discussion of the toxicity of the teas.) The bush teas were made from leaves of *Crotalaria* or *Senecio* and contained PAs. A subsequent educational campaign has largely eliminated use of such remedies and the consequent occurrence of liver disease in children.

Another example of an herbal remedy with widespread usage is comfrey (*Symphytum officinale*), which contains monoester PAs. This plant is used primarily in teas, but capsules containing ground plant material have been marketed, and Russian comfrey (*S. uplandicum*) has been used in a similar manner. Comfrey teas have been used as a remedy for abdominal pain (Bach *et al.* 1989) and to treat Crohn's Disease (Weston *et al.* 1987). The overall PA content is considerably lower than generally found in *Senecio* species, ranging up to 0.2% in leaves and 0.4% in roots (Roitman 1981), and the monoester-type alkaloids are less acutely toxic than the macrocyclic diester class (Culvenor *et al.* 1980). Despite the relatively low concentration of PAs, comfrey

preparations have consistently been documented as being responsible for classic veno-occlusive disease (Ridker *et al.* 1985, Weston *et al.* 1987, Bach *et al.* 1989, McDermott and Ridker 1990), and comfrey even was found to have killed a young man who had consumed the leaves as a vegetable (Yeong *et al.* 1990). In some of these cases, it was possible to calculate an approximate PA intake. For example, a woman diagnosed with veno-occlusive disease and centrilobular necrosis was found to have ingested an estimated 15 μg/kg body weight (b.w.) of PAs daily from comfrey tea and comfrey-pepsin capsules over the preceding four months, for a minimum total PA dose of 85 mg (Ridker *et al.* 1985). The quantity of total PA (free base plus *N*-oxide) in comfrey preparations was determined to be 270 μg/g in samples of leaf capsules and 2,900 μg/g in root capsules (Huxtable 1989a), and a cup of comfrey-root tea, brewed according to package specifications, contained 8.5 mg of total alkaloids (Roitman 1981). In a study analyzing the PA content of comfrey teas, Research Triangle Institute (RTI 2001) identified the PAs symphytine (1.6 to 8.4 μg/L) and echimidine (1.5 to 14.5 μg/L) in teas prepared from the leaves of comfrey.

Two studies of poisoning in children in South Africa with hepatic veno-occlusive disease reported the presence of PAs in either the urine of the cases or in the herbal remedies to which they were exposed. Steenkamp *et al.* (2000) confirmed the presence of PAs in the urine of four cases of veno-occlusive disease in children for whom an on-admission urine specimen was available. These 4 cases were part of a total of 20 children identified with veno-occlusive disease thought to be caused by exposure to traditional remedies; however, no on-admission urine samples were available for the other 16 cases. Steenkamp *et al.* noted that the most common genera containing PAs in South Africa are *Senecio* species and *Crotalaria* species. The presence of the PA retrorsine in the traditional herbal remedies administered to two sets of twin infants (a boy and a girl in each set) admitted to a Johannesberg hospital with veno-occlusive liver disease was determined by GC-MS (concentrations not provided) (Conradie *et al.* 2005).

Children are uniquely susceptible to PA-containing herbal preparations (Small *et al.* 1993). A case of exposure *in utero* has been reported (Roulet *et al.* 1988) where a pregnant woman had consumed coltsfoot (*Tussilago farfara*) daily, and the newborn

infant, who died from hepatic veno-occlusive disease, was estimated to have received total PAs at a cumulative transplacental dose of 0.125 mg/kg b.w. An 18-month-old child diagnosed with veno-occlusive disease was estimated to have received total PAs (primarily seneciphylline and its *N*-oxide) at a daily dose of 60 μg/kg b.w. through consumption of a tea of *Adenostyles alliariae* daily for 15 months (Sperl *et al.* 1995). Toxicity of PAs has been reported to occur in neonatal and fetal animals with little maternal toxicity (Small *et al.* 1993, Stegelmeier *et al.* 2003).

A number of Chinese herbal therapies are made from plants containing PAs (Table 2-2). These plants are used for a variety of medicinal purposes, including treatment of infections and diseases such as bronchitis, asthma, and influenza and treatment of traumatic injuries and abscesses. Senecionine and seneciphylline are the PAs identified in these plants.

Table 2-2. Chinese herbal plants that contain analogues of riddelliine

Plant	Chinese name	Medicinal purpose	Alkaloid
Gynura segetum	ju shan qi, tu san chii	hemoptysis, peripheral blood circulation disorder	senecionine, seneciphylline
Senecio argunensis	yu yie qian li guang, zhan long cao	folk medicine, dysentery	senecionine, seneciphylline
Senecio chrysanthemoides	chien li kuang, tsang tu san chi	traumatic injury, breast abscesses	seneciphylline
Senecio nemorensis	huana wan	enteritis, hepatitis, boils	senecionine
Senecio scandens	quian li guang, chiu li ming	oral and pharyngeal infection	senecionine, seneciphylline
Tussilago farfara	kuan dong hua, chien hua	chronic bronchitis, asthma, influenza	senecionine

Source: Fu *et al.* 2001, Fu *et al.* 2002a.

2.3.3 Food

Two plants of the genus *Senecio* (*S. burchellii* and *S. inaequidens*) have been used in South Africa as a leafy vegetable similar to spinach; however, they are purportedly "not popular" (Mattocks 1986) and have not been reported to contain riddelliine. [Because it is unlikely that *Senecio* species known to contain riddelliine are used for food, ingestion by humans is most likely to result from either direct contamination of foodstuffs by parts of *Senecio* plants or from indirect introduction of the alkaloid through products derived from animals that have fed on the plants. Although no studies have specifically examined the

occurrence of riddelliine in foodstuffs, the likelihood of its occurrence can be extrapolated from more general studies and reports of PA contamination, especially with respect to *Senecio* species.] The topic has been comprehensively reviewed by Coulombe (2003), who identified 15 *Senecio* species used as either herbal medicines or food in the United States, Jamaica, Germany, Japan, and Africa. The remainder of this section discusses the occurrence of riddelliine and PAs in grains and flours, meat, milk, eggs, and honey and bee pollen.

Grains and flours

No information specific to riddelliine in grains and flours was found; however, the earliest report of human poisoning due to PAs identified *S. ilicifolius* and *S. burchelli* seeds incorporated into bread as being responsible for 80 cases of PA poisoning in South Africa, primarily in children (Willmot and Robertson 1920). The authors called the condition "senecio disease." Over 30 years later, a similar episode in South Africa was described in which 12 people were poisoned by an unidentified *Senecio* species, and 6 died (Selzer and Parker 1951).

Several large-scale episodes of human poisoning by cereal grains contaminated with seeds of PA-containing plants have been described. Particularly problematic has been contamination by *Heliotropium popovii*, which resulted in 7,800 reported cases of veno-occlusive disease in Afghanistan and 3,906 cases in Tajikistan (Tandon *et al.* 1978, Mayer and Luthy 1993). In these cases, the seeds (of which heliotrine was the preponderant PA) contaminated wheat that was consumed in bread; [baking therefore must not have destroyed the alkaloids]. Seeds of *H. popovii* are similar in size to wheat grains and therefore difficult to remove by screening. In contrast, *Senecio* seeds typically are quite small and lightweight, with a feathery pappus, which should make them easy to remove from heavier grains by winnowing.

Meat

No information specific to riddelliine in meat was found. Furthermore, the question of occurrence of PAs in meat is inherently complex. The alkaloids are oxidized in the liver to the dehydro (pyrrolic) metabolites, which are extremely reactive and rapidly bind to cellular macromolecules in the liver and red blood cells through thiol groups. It is

therefore unlikely that unreacted PAs will be sequestered, and there are no reports of their detection in meat products. However, animal experiments have indicated possible lung involvement (see Sections 4 and 5 for further discussion on lung toxicity), which is difficult to explain if the metabolites are irreversibly bound to liver tissues. Furthermore, chronic and progressive liver damage suggests that these compounds are persistent and may be recycled to cause further damage. The presence of PAs bound to liver tissue has been demonstrated by gas chromatography/mass spectrometry (GC-MS) (see Section 2.4), and [the consumption of liver from animals exposed to PAs could potentially result in exposure to humans].

Milk

No information specific to riddelliine in milk was found; however, the potential for humans to be exposed to PAs excreted in milk has been reviewed (Molyneux and James 1990). [Because the free-base alkaloids generally react rapidly and possibly irreversibly after metabolism in the liver, they are unlikely to be a source of milk contamination. The corresponding *N*-oxides, however, if not reduced in the gut to the tertiary or free-base form, are extremely water soluble; also, some of the tertiary alkaloids could be oxidized in the liver to the *N*-oxides. The *N*-oxides are rapidly excreted in the urine, but in lactating animals, an appreciable amount is sequestered in the milk.]

Lactating cows fed dried *Senecio jacobaea* with an average alkaloid level of 0.16% (through a rumen cannula) excreted only one of the plant alkaloids (jacoline, a macrocyclic diester of retronecine) in the milk, at concentrations of 0.94 to 1.67 μg/mL (Dickinson *et al.* 1976). Their suckling calves were not affected, even though the cows died of liver damage. In a similar experiment, no histopathologic changes were detected in calves consuming milk from cows fed chronic lethal doses of *S. jacobaea,* even though clinical chemistry tests suggested the presence of hepatic lesions in the calves (Johnson 1976). Johnson (1976) also reported that no gross or histopathologic effects were seen in rats following gavage daily for 30 days with milk from cows fed *S. jacobaea*. Goats fed the flowering tops of *S. jacobaea* at 1% of their body weight per day produced milk containing PAs at concentrations of 0.33 to 0.81 ppm (Deinzer *et al.* 1982). In rats fed milk from these goats at a total PA dose of 0.96 mg, swollen centrilobular hepatocytes

and biliary hyperplasia were observed, similar to effects seen in rats fed the plant at 0.001% in the diet (Goeger *et al.* 1982). [It is noteworthy that all of these experiments were performed with *S. jacobaea*, which contains lower total alkaloid levels and a lesser proportion of the *N*-oxide form than do riddelliine-containing species such as *S. longilobus* and *S. riddellii*.]

In an experiment with tritium-labeled senecionine and seneciphylline (produced biosynthetically by growing *S. vulgaris* with radiolabeled precursors), lactating rats fed these compounds excreted 0.08% of the radioactivity in the milk within 3 hours, of which 0.02% was unchanged PAs (Lüthy *et al.* 1983). [The experiment was not performed with the corresponding *N*-oxides, which would be expected to be excreted more efficiently.]

[Although no definitive information on the occurrence of riddelliine in milk is available, the general population is unlikely to be exposed to appreciable levels of riddelliine in milk, because most milk herds are not kept in the arid environments where plants containing the alkaloid are endemic. Furthermore, milk consumed by the general population usually is blended from many sources, with consequent dilution of any alkaloids present. However, individuals potentially could be exposed by consuming organic milk from a family cow or goat grazing in areas where *S. riddellii* or similar species are common, particularly in view of the exceptionally high alkaloid levels and proportion of *N*-oxides that may be present. The potential for exposure to PAs from goat's milk may be even greater as goats are relatively resistant to the toxic effects of PAs, and there is a potential for relay toxicity, especially as highly susceptible infants are likely to drink goat's milk.]

Calculation of potential excretion in milk from a cow grazing *S. riddellii* with a high alkaloid content and 10:1 ratio of *N*-oxide to free base suggests that the milk could contain riddelliine *N*-oxide at concentrations as high as 5 mg/L (Molyneux and James 1990). [Although this form of the alkaloid is not toxic *per se*, it could be reduced to the tertiary or free-base form in the gut of the consumer and thus result in hepatic damage.] Weanling pigs have been shown to be particularly susceptible to the effects of riddelliine

(Stegelmeier *et al.* 2003), [and children who are high consumers of milk from a point source might similarly be at risk.]

Eggs

No information specific to riddelliine in eggs was found; however, poisoning of poultry by contamination of feed with seeds of *Heliotropium* (Pass *et al.* 1979a) has been reported. Eggs were analyzed in one incident, involving contamination of wheat by *H. europaeum*, and shown to contain a mixture of alkaloids typical of *Heliotropium* at concentrations of 1.2 to 9.7 μg per egg (Edgar and Smith 2000). However, when Eroksuz *et al.* (2003) fed groups of 10 laying hens diets containing ground aerial parts (stems, leaves, flowers) of *S. vernalis* at 0, 0.5%, 2%, and 4% for 210 days, no free PAs were detected in the eggs.

Honey and bee pollen

No data on riddelliine levels in honey were found; however, bees gathering pollen and nectar from PA-containing plants are likely to acquire the alkaloids, especially since the highest levels have been found to occur in the flowers and seeds (see Section 2.3.1). Numerous PA-containing plants, including plants of the genus *Senecio*, in many parts of the world have been identified as sources of honey for human consumption, primarily by microscopic pollen analysis but rarely by analysis for the alkaloids (Edgar *et al.* 2002). Honey samples in Switzerland have been reported to contain PAs at 0.03 to 0.07 μg/g (Rietjens *et al.* 2005).

Bees foraging *S. jacobaea* produced honey containing PAs at concentrations of up to 3.9 μg/g (3.9 ppm) (Deinzer *et al.* 1977). All the PAs present in the plant were detected in the honey and included seneciphylline, senecionine, jacobine, jaconine, jacoline, and jacozine. [The reported amounts probably were underestimates, because no corrections were made for extraction efficiencies.] More recent analysis of honey from *S. jacobaea* by solid-phase extraction and liquid chromatography- (LC-) MS analysis showed PA levels of up to 1.48 μg/g (Crews *et al.* 1997). Reported recoveries were 57% to 70%, indicating actual levels in excess of 2 μg/g, and the profile of PAs in the honey was

characteristic of *S. jacobaea.* However, no PAs were found in samples of honey retailed in the area.

A major source of honey produced in southeastern Australia is *Echium plantagineum*, known as Paterson's Curse in Victoria and New South Wales and as Salvation Jane in South Australia (Culvenor *et al.* 1981). Analysis of four honey samples from producers in New South Wales showed PA levels from 0.27 to 0.87 μg/g, and a fifth sample purchased from an Adelaide store, labeled "*Echium* honey," had a level of 0.95 μg/g. [The extraction efficiency for GC-MS analysis was estimated by Culvenor *et al.* to be 60% to 70%, so some of the samples could have contained PAs at levels in excess of 1 μg/g.] The primary constituent was echimidine, a non-macrocyclic diester, accompanied by structurally related alkaloids. The non-macrocyclic esters are characteristic of the plant family *Boraginaceae* which includes the genera *Echium* and *Heliotropium* (Edgar *et al.* 2002).

Beales *et al.* (2004) analyzed 63 samples of Australian honey drawn from bulk containers prior to any processing at the packaging company and from 5 retail samples. The primary floral sources for the bulk samples were identified by the bee keepers as follows: 13 samples from *E. plantagineum*, 9 samples from *E. plantagineum* mix, 4 samples from *Heliotropium amplexicaule*, 2 samples from *H. europaeum*, and 35 from floral sources with no known association with PAs. The 5 retail samples included 3 samples from blended sources, 1 from *Eucryphia lucida*, and 1 from *Echium vulgare*. The concentration of total PAs in the honey attributed to known PA-producing floral sources ranged from about 0.033 to 2.2 μg/g. Concentrations of PAs in the honeys attributed to non-PA-producing plants, or in honeys from unknown sources, ranged from 0.003 to 0.8 μg/g. The only sample that did not contain detectable amounts of PAs was the retail sample from *E. lucida*.

In addition to honey, bee pollen could be a source of PA exposure. Boppré *et al.* (2005) reported the presence of PAs in 2 pollen samples from *E vulgare* collected from plants in Australia. PA concentrations in the pollen ranged from about 8,000 to 14,000 μg/g, and the authors suggested that pollen could contribute significantly to the pyrrolizidine

content of honey. Boppré *et al.* also noted that commercial bee pollen used as a food supplement could contain PAs at unsafe levels.

Methods to reduce riddelliine content of foods

Riddelliine decomposes at its melting point of 197°C to 198°C. [Heating of foods above this temperature might be expected to result in the destruction of riddelliine. However, the products of thermal decomposition are not known, and in the absence of proof to the contrary, they cannot be assumed to be innocuous. Similar considerations apply to riddelliine *N*-oxide, which melts at 156°C to 158°C. Evidence from mass spectrometry suggests that the latter may initially undergo thermal deoxygenation to yield riddelliine. Nevertheless, the episodes of veno-occlusive disease resulting from consumption of bread made from wheat contaminated with PA-containing seeds (see "Grains and flours" above) suggest that heating is not effective as a means of destroying the alkaloids.]

2.3.4 Dust

[Detection of PAs in dried, ground plant material indicates that the alkaloids are likely to be present in flower and leaf fragments or dusts from senescent plant material. Individuals conducting harvesting operations in fields highly infested with PA-containing weed species might inhale them directly into the lungs, a target organ (see Table 4-3 and Section 5.6).]

2.3.5 Insects

As discussed above, bees can assimilate PAs and incorporate them into honey (Edgar *et al.* 2002). Phloem-feeding insects also can sequester them and excrete PAs in honeydew. The specialist aphid *Aphis jacobaeae* has been shown to sequester large amounts of PAs from its host, *Senecio jacobaea*, as well as from *S. pellucidus* and *S. silvaticus*, at levels of up to 3.5 mg/g; these PAs were then transferred from the aphid to predatory ladybird beetles at a level of 4.9 mg/g (Witte *et al.* 1990). Honeydew extracted from green peach aphids feeding on *S. vulgaris* flower buds contained senecionine, its *N*-oxide, and hydrolytic products including retronecine (Molyneux *et al.* 1990). Some species of *Lepidoptera* acquire PAs from plants and in some cases incorporate the PAs into their eggs, presumably for protection against insect predators (Dussourd *et al.* 1988). PAs that are not known to occur in plants have been identified in the pupae of *Lepidoptera* and are

believed to result from re-esterification of retronecine of plant origin; these PAs include callimorphine from *Tyria jacobaeae* and creatonotine from *Creatonotos transiens*.

2.3.6 Occupational exposure

[Individuals that may have an increased risk of occupational exposure to PAs include ranchers, farmers, and herbalists. Ranchers or farmers tending livestock, or harvesting hay or crops that are infested with PA-containing plants might contact or inhale dust that contains portions of these plants. In addition, individuals who harvest herbs and prepare herbal remedies have an increased risk of exposure through direct contact and inhalation of dust from the dried preparations. The lungs have been shown to be vulnerable to damage by PAs (Mattocks 1986) (see Table 4-3 and Section 5.6), and direct exposure, rather than secondary exposure following hepatic metabolism, should be a matter of concern.]

2.4 Analytical methods

The large number of known, structurally diverse PAs has complicated the development of appropriate techniques, but numerous methods have been reported for their quantitative and qualitative analysis (Roeder 1999). The primary application has been for analysis of plant samples in which the alkaloids are known or suspected to occur.

2.4.1 Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) spectroscopy has been used to determine PA content of *Senecio* species and in some cases may provide information regarding relative composition of individual alkaloids (Molyneux *et al.* 1979, Pieters *et al.* 1989). [Such techniques should be directly applicable to foods such as cereal grains or herbal remedies, but their use for analysis of samples with large amounts of potentially interfering substances, such as samples of meat, milk, or honey, would require considerable modification of extraction and sample preparation technique. For example, the presence of organic acids in honey may result in the formation of salts with the basic alkaloids, requiring careful pH adjustment prior to extraction, to liberate all of the alkaloids. Furthermore, most of the current methods are designed for plants in which the alkaloids are natural constituents and therefore present at relatively high levels, whereas the levels in food samples are likely to be several orders of magnitude lower.]

2.4.2 Thin-layer chromatography

Thin-layer chromatography is a rapid, low-cost technique for identification of individual PAs, with sensitivity of about 1 μg. PAs most commonly are separated on silica-coated plates, with organic solvent mixtures containing small amounts of ammonia. The *N*-oxides are much less lipophilic than the corresponding tertiary bases, and more-polar solvents are required to develop reasonable retention-factor values. The variety of applicable solvent systems was summarized by Mattocks (1986). PAs can be visualized by spraying with Dragendorff's reagent, which reacts with most classes of alkaloids. A more specific technique is to use Ehrlich's reagent, which reacts with the pyrrolic ring system of dehydropyrrolizidines. The latter can be produced by oxidation of the PAs with a pre-spray of *o*-chloranil; subsequent treatment with Ehrlich's reagent gives intense purple spots (Molyneux and Roitman 1980). The *N*-oxides cannot be converted into dehydropyrrolizidines by *o*-chloranil, because they are at the same oxidation state, but on spraying with acetic anhydride, they undergo a Polonovski rearrangement to give the corresponding pyrrole, which reacts with Ehrlich's reagent in the same way as with the tertiary bases.

2.4.3 Gas chromatography

Gas chromatography has been used for the analysis of a wide range of PA structural types, both underivatized and derivatized to improve volatility (Culvenor *et al.* 1981). Via GC-MS, specific individual alkaloids can be identified without the need for specific individual standards. This technique has been used to characterize the PA composition of *Senecio* species (Stelljes *et al.* 1991). Selected-ion monitoring should provide unequivocal identification. Witte *et al.* (1993) established that about 100 underivatized PAs, encompassing diverse structural types, could be identified by retention indices on two different capillary columns in combination with the molecular ion and group-specific fragmentation patterns. An interlaboratory collaboration showed that such data were sufficient to unequivocally identify the individual alkaloids, without the need for a standard for each individual alkaloid. [However, *N*-oxides, because of their extremely polar nature and tendency to undergo on-column thermal deoxygenation, cannot be analyzed by GC without time-consuming prior reduction to the free bases.]

Rats exposed to PAs form pyrrolic metabolites that can alkylate both soluble and tissue-bound thiol groups resulting in relatively stable pyrrolic thioethers that can persist for long periods in tissues (see Section 2.5). The sulfur-bound pyrrolic metabolites can be liberated from tissue samples by cleavage with silver nitrate and reaction *in situ* with ethanol to form an ethoxy derivative that can be identified by GC-MS (Mattocks and Jukes 1990). When this technique was used with rats fed monocrotaline continuously in drinking water at 20 mg/L, pyrroles were detected in the blood after 12 days and in liver after 25 days (Mattocks and Jukes 1992). This technique has also been used to establish exposure of horses and yaks to PAs, by showing the pyrroles to be bound to circulating hemoglobin and to be present in preserved liver tissue (Seawright *et al.* 1991, Winter *et al.* 1992, Winter *et al.* 1993). GC-MS is able to demonstrate unequivocally that an animal has previously been exposed to PAs, and since it is effective on dried blood and preserved liver samples, the samples can be transported or stored for further testing (Winter *et al.* 1992). [Although this method is useful as a qualitative indicator of exposure, quantitation of metabolites has been problematic.]

2.4.4 High-performance liquid chromatography

The desirability of analyzing for both free-base and *N*-oxide PAs, preferably simultaneously, presents a difficult problem, because of their vastly different physical properties. High-performance liquid chromatography (HPLC) offers the greatest potential to achieve this, even though the two alkaloid forms represent extremes of lipophilicity and hydrophilicity (Brown *et al.* 1994). An ion-pairing technique, which converts all of the alkaloids into ionized forms, has been used for HPLC separation of a number of macrocyclic PA free bases and their corresponding *N*-oxides. [Nevertheless, conventional HPLC methods are severely limited by the alkaloids' lack of a significant chromophore in the UV spectrum, with consequent reduction in sensitivity.] This limit has recently been circumvented by the use of evaporative light scattering detection, which is applicable to both tertiary bases and *N*-oxides, although the limit of detection in plant material (ca. 40 μg) was somewhat higher than desirable (Schaneberg *et al.* 2004). The development of LC-MS systems may provide the solution to such detection problems and, in association with tandem mass spectrometry (LC-MS-MS), should provide high-sensitivity analysis of the alkaloids within a complex matrix without prior clean-up.

Preliminary results of HPLC-MS analysis of extracts of honey produced from *Heliotropium europaeum*, *H. amplexicaule*, and *Echium plantagineum* have shown excellent resolution between structurally similar PAs, with unequivocal identification of most of the alkaloids present (Edgar *et al.* 2002).

2.4.5 Immunoassay

Immunoassays should be particularly suited to analysis of PAs in foodstuffs, because they are extremely sensitive, capable of detecting natural compounds in the parts-per-billion range, and less subject to matrix interference than chromatographic methods. A class-specific enzyme-linked immunosorbent assay (ELISA) of one of the most common necine bases, retronecine, has been described (Roseman *et al.* 1992), and other immunoassays have been reported that are specific for a particular alkaloid or show cross-reactivity to a small group of alkaloids having similar structure, such as the macrocyclic diester type (Bober *et al.* 1989, Roeder and Pflueger 1995, Langer *et al.* 1996, Roseman *et al.* 1996, Zündorf *et al.* 1998). More recently, it has been demonstrated that the problem of detection of both free base and *N*-oxide forms of the same alkaloid can be overcome, specifically for the case of riddelliine by the generation of polyclonal antibodies to a riddelliine-protein conjugate, and the potential for use of ELISA to detect and estimate PAs in plants and feeds has been reviewed (Lee *et al.* 2001, 2003).

2.5 Biological indices of exposure

Potential biomarkers of exposure include DHP-derived DNA adducts (Xia *et al.* 2003, Fu *et al.* 2001, Yan *et al.* 2002, Fu *et al.* 2002b) and tissue-bound pyrroles (PA metabolites) (Mattocks and Jukes 1992, Stegelmeier *et al.* 1996, Schoch *et al.* 2000).

Metabolism of riddelliine and many other PAs *in vivo* or *in vitro* results in formation of the same eight DHP-derived DNA adduct peaks (see Section 5.2). For example, in a study of DNA adducts in the blood of F344 rats, 3 rats per sex per group were given a single dose of riddelliine by gavage at 10 mg/kg b.w. in 0.1 M phosphate buffer. DNA was extracted from whole blood, and adduct levels were measured by ^{32}P-postlabeling at 8, 24, 48, and 168 hours after dosing (Yan *et al.* 2002). After a 24-hour lag, DHP-derived DNA adducts appeared in the bloodstream, reaching a constant level within 48 to 168

hours post-dosing. During this period, adduct levels were 4-fold higher in female rats than in males.

Male rats were given a single i.p. injection of one of seven PAs (heliotrine, indicine, lasiocarpine, retrorsine, senecionine, anacrotine, or monocrotaline) at doses ranging from 9 to 500 mg/kg b.w. (based on acute toxicity) and were killed 30 hours later (Mattocks and Jukes 1992). Another group of rats was administered monocrotaline (20 mg/L) in drinking water for 12 or 25 days. Livers and heparinized blood samples were collected. Sulfur-bound metabolites were extracted and analyzed using TLC, HPLC, or GC-MS. Pyrrolic metabolites were detected in most blood and liver samples. Liver pyrroles were detected in fixed or powdered samples for up to 30 days. Pyrroles were detected in the blood of rats given monocrotaline in drinking water after 12 days and in the liver after 25 days. The authors concluded that the procedure described was applicable to the diagnosis of PA exposure in livestock.

Stegelmeier *et al.* (1996) administered (by gavage) a suspension of houndstongue containing 5 or 15 mg/kg b.w. PA for 14 days to six horses. Animals were euthanized after developing clinical illness, and the remaining animals were euthanized 252 days postexposure. Hepatic tissue-bound pyrroles were identified in all three high-dose horses but were rarely detected in the low-dose horses. The authors reported that the method was specific but lacked sensitivity.

Schoch *et al.* (2000) fed 12 sows gelatin capsules containing 0, 3, 10, or 15 mg/kg b.w. riddelliine (three per group) for about 40 days. The animals were killed the day after receiving the final dose. Pyrrolic metabolites were detected in liver and blood samples from all animals dosed with riddelliine using GC/MS-MS. However, the amount detected did not correlate with the dose, and livers fixed with formalin had greatly reduced recovery compared with liver samples that were frozen or freeze-dried.

2.6 Regulations and guidelines for the United States

No regulations or guidelines were identified for riddelliine.

In an advisory dated July 6, 2001, the FDA stated: "The agency [FDA] strongly recommends that firms marketing a product containing comfrey or another source of pyrrolizidine alkaloids remove the product from the market and alert its customers to immediately stop using the product. The agency advises that it is prepared to use its authority and resources to remove products from the market that appear to violate the Act."

2.7 Summary

Riddelliine has no known commercial uses and is not available from chemical suppliers. Riddelliine and riddelliine *N*-oxide occur in plants of the genus *Senecio* found in sandy desert areas of the western United States and other parts of the world. Environmental exposure to riddelliine and other PAs may occur through use of herbal products, ingestion of contaminated foods, or contact with plant materials. Two cases of fatal human exposure to plants containing riddelliine, in addition to seneciphylline, senecionine, and retrorsine, in an herbal tea have been reported from the southwestern United States. The potential for exposure through meat or milk from animals that have fed on PA-containing plants also has been proposed. Numerous methods have been reported for analysis of riddelliine, including NMR spectroscopy, GC, and immunoassay. DNA adducts formed from DHP and tissue-bound pyrroles (PA metabolites) (for exposure in livestock) may serve as biological indices of exposure to riddelliine. No U.S. regulations or guidelines were identified for riddelliine; however, the FDA issued an advisory dated July 6, 2001 recommendign the removal from the market of products containing comfrey or another source of PAs.

3 Human Cancer Studies

No studies or case reports on the relationship between exposure to riddelliine and cancer in humans were identified.

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4 Studies of Cancer in Experimental Animals

As discussed in Sections 1 and 2, riddelliine belongs to a class of chemicals known as PAs, which occur in a wide variety of plants found in the western United States and in temperate and tropical climates throughout the world (see Section 2.3.1 and Table 2-1). Delayed fatal liver toxicity has been reported in cattle, horses, and other livestock that ingested PA-containing plants while grazing on rangelands (Mattocks 1986) or were fed PAs under experimental conditions (Johnson *et al.* 1985b, Molyneux *et al.* 1988, Molyneux *et al.* 1991, IARC 2002) (see also Sections 2.3.1 and 5.6). Although there have been no reports of cancer in livestock exposed to PAs, no long-term, low-dose studies with these animals were identified. Several studies have investigated the carcinogenicity of riddelliine in experimental animals, and many more have examined the carcinogenicity of various PAs or of plant extracts that contain these chemicals. At least 16 PAs, including one *N*-oxide and three pyrrolic metabolites (retronecine, dehydroretronecine [*R*-DHP], and dehydroheliotridine [*S*-DHP]) have induced tumors in experimental animals (Fu *et al.* 2002b). The carcinogenicity of these other PAs is discussed in Section 5.5.1.

The carcinogenicity of riddelliine and other PAs has been reviewed (Schoental 1968a, IARC 1976, 1983, 2002 WHO 1988). Schoental and Head (1957) conducted the first carcinogenicity study of riddelliine. However, this study was reviewed by IARC (1976) and considered insufficient for evaluating the carcinogenicity of riddelliine. IARC (1976) did review other PAs and concluded that there was evidence that isatidine, lasiocarpine, monocrotaline, retrorsine, and some plant extracts known to contain PAs were carcinogenic in experimental animals (see Sections 4.5 and 5.5). More recently, IARC (2002) concluded that there was sufficient evidence for the carcinogenicity of riddelliine in experimental animals based on results of an NTP two-year bioassay (see Sections 4.1 and 4.2). It is important to note that the carcinogenic doses of PAs used in experimental animal studies are comparable with the doses in some reported instances of human poisonings, based on estimated intakes expressed as milligrams per kilogram of body weight per day (Culvenor 1983, see Section 5.6).

This section reviews the available carcinogenicity studies of riddelliine in mice (Section 4.1) and rats (Section 4.2). Non-neoplastic effects of riddelliine exposure are summarized in Section 4.3. The carcinogenicity of riddelliine metabolites (Section 4.4) and plant materials and extracts that likely contained riddelliine also are briefly reviewed (Section 4.5). The carcinogenicity data are summarized in Section 4.6.

As noted in Section 2.1, riddelliine is not available from chemical suppliers. The riddelliine used by the NTP in the subchronic (Chan *et al.* 1994) and chronic (Chan *et al.* 2003, NTP 2003) toxicity studies was from the same lot and was obtained from Dr. Russell Molyneux of the United States Department of Agriculture. The chemical was extracted and purified from *S. riddellii* plants collected from rangelands in the western United States. Its purity was 92%, with 5% retrorsine and 1.4% seneciphylline. Retrorsine and seneciphylline are both metabolized to DHP, which is the same DNA adduct-forming molecule to which riddelliine is metabolized (see Section 5.1.3 and Figure 5-2). Limited studies in animals suggest that liver tumors also may occur from exposure to retrorsine and seneciphylline (see Section 5.5.1). The only other animal study reported below was conducted by Schoental and Head (1957) using crystalline riddelliine that they noted was a gift from Professor Roger Adams who had established its structure. No other information on the source or purity of this crystalline riddelliine was reported.

4.1 Carcinogenic effects in mice

The NTP and other researchers have conducted several studies on the carcinogenicity of riddelliine in mice. Groups of 20 B6C3F$_1$ mice (6 to 8 weeks old) of each sex were administered riddelliine in 0.1 M sodium phosphate buffer by gavage five days a week for 13 weeks at doses of 0, 0.33, 1.0, 3.3, 10, or 25 mg/kg b.w. (Chan *et al.* 1994, NTP 2003). After 13 weeks, 10 mice in each group were sacrificed and examined; 5 of the remaining animals were sacrificed after a 7-week recovery period, and the other 5 were sacrificed after a 14-week recovery period. Body-weight gain was inversely related to dose level and remained depressed in the two highest-dose groups of each sex throughout the 14-week recovery period. Hepatocytomegaly was observed in the high-dose groups after 13 weeks and persisted through the recovery period in females.

Groups of 50 B6C3F$_1$ mice (5 to 6 weeks old) of each sex were included in a two-year NTP carcinogenicity study (Chan *et al.* 2003, NTP 2003). Riddelliine was administered by gavage five days per week for 105 weeks. Because the amount of riddelliine was limited, unbalanced dose groups were purposely selected to better evaluate dose-response relationships in male mice and female rats (see Section 4.2). Based on the results of the subchronic exposure studies, dose levels were 0, 0.1, 0.3, 1.0, and 3.0 mg/kg b.w. in male mice and 0 and 3.0 mg/kg b.w. in females. Survival was significantly lower ($P < 0.001$) in the high-dose groups (3 mg/kg) than in the controls due primarily to hemangiosarcoma in the liver. Mean body weights in the high-dose groups were lower than in the controls throughout most of the study, and at the end of the study were 19% lower in males and 33% lower in females. Mean body weight in males in the 1-mg/kg group was 6% lower than in controls at the end of the study. Neoplastic lesions are summarized in Table 4-1 and non-neoplastic lesions are discussed in Section 4.3. Neoplastic lesions included significantly increased ($P < 0.001$) liver hemangiosarcoma in high-dose males and significantly increased ($P < 0.001$) alveolar/bronchiolar adenoma or carcinoma combined in females. Incidences of hepatocellular neoplasia were significantly lower in some riddelliine-exposed groups than in the controls, which the NTP suggested could be due to the ability of PAs to inhibit cell division (Hincks *et al.* 1991). The NTP (2003) concluded that there was clear evidence of carcinogenic activity of riddelliine in male B6C3F$_1$ mice based on increased incidences of hemangiosarcoma in the liver and clear evidence in female B6C3F$_1$ mice based on increased incidences of alveolar/bronchiolar neoplasms.

Table 4-1. Neoplastic lesions observed in B6C3F$_1$ mice administered riddelliine by gavage for two years

Sex	Dose (mg/kg)	No. examined (no. surviving to end of study)	Tumor incidence (%)[a]						
			Liver	Liver (hepatocellular)			Lung (alveolar/bronchiolar)		
			Hemangio-sarcoma	Adenoma	Carcinoma	Combined	Adenoma	Carcinoma	Combined
Male	0	50 (39)	2 (4.4)	16 (34.2)	23 (47.7)	36 (73.4)	12 (26.3)	7 (15.2)	18 (39.1)
	0.1	50 (41)	1 (2.2)	18 (38.8)	21 (43.2)	39 (80.0)	10 (21.7)	8 (17.3)	16 (34.7)
	0.3	50 (40)	0 (0)	14 (29.0)	19 (38.4)	33 (66.0)	11 (23.1)	6 (12.4)	15 (31.1)
	1.0	50 (38)	2 (4.4)	5 (10.9)**N	20 (42.8)	23 (49.2)*N	8 (17.5)	1 (2.2)	9 (19.7)
	3.0	50 (20)***[c]	31 (66.7)***	0 (0)***N	3 (7.5)***N	3 (7.5)***N	12 (28.5)	5 (12.4)	17 (39.7)
	trend[b]		$P < 0.001$	$P < 0.001$N	$P < 0.001$N	$P < 0.001$N	$P = 0.356$	$P = 0.289$N	$P = 0.424$
Female	0	50 (34)	0 (0)	9 (20.9)	8 (19.0)	16 (36.9)	1 (2.4)	1 (2.3)	2 (4.7)
	3.0	50 (17)***[c]	1 (2.2)	0 (0)**N	0 (0)**N	0 (0)***N	9 (21.5)**	4 (9.5)	13 (30.5)***

Sources: Chan *et al.* 2003, NTP 2003.
*Significantly different ($P < 0.05$) from the control group by the Poly-3 test, which is based on an adjustment for survival to reflect the number of animals at risk of developing the tumor (see Glossary for a more complete definition of the Poly-3 test).
**Significantly different ($P < 0.01$) from the control group by the Poly-3 test.
***Significantly different ($P < 0.001$) from the control group by the Poly-3 test (tumor incidences) or life-table pairwise comparison (survival).
[a]Poly-3-estimated neoplasm incidence after adjustment for intercurrent mortality.
[b]Poly-3 test for dose-related trend.
[c]Life-table pairwise comparison (Cox method).
N = lower incidence than in controls (in the Poly-3 test) or inverse dose relationship (in the trend test).

4.2 Carcinogenic effects in rats

Groups of 20 F344/N rats (6 to 8 weeks old) of each sex were administered riddelliine in 0.1 M sodium phosphate buffer by gavage five times a week for 13 weeks at a dose of 0, 0.1, 0.33, 1.0, 3.3, or 10 mg/kg b.w. (Chan *et al.* 1994, NTP 2003). After 13 weeks, 10 rats in each group were sacrificed and examined; 5 of the remaining animals were sacrificed after a 7-week recovery period, and the other 5 were sacrificed after a 14-week recovery period. All but 1 of the male rats in the high-dose group died before the end of 13 weeks, and 5 female rats in the high-dose group died during either the first or second recovery period. Dose-related decreases in mean final body weights and body weight gains were observed at 13 weeks, but after the 14-week recovery period, body weights in all exposure groups were similar to those of the controls except for females in the two highest dose groups. Dose-related hepatopathy was observed in both sexes, and hepatocellular adenoma was observed in 2 of 10 female rats at 13 weeks and in 1 of 5 female rats after the 14-week recovery period at 1.0 mg/kg b.w.

Groups of 50 F344/N rats (5 to 6 weeks old) of each sex were administered riddelliine by gavage five days per week for 105 weeks. Based on the results of the subchronic exposure studies, dose levels were 0, 0.01, 0.033, 0.1, 0.33, and 1.0 mg/kg b.w. in females and 0 or 1.0 mg/kg b.w. in males (Chan *et al.* 2003, NTP 2003). Survival was similar to that of controls in all exposure groups except the high-dose groups. All female rats in the 1-mg/kg group died by week 97, and the study of male rats was terminated after 72 weeks, because all but 3 animals in the single dose group had died. Hemangiosarcoma in the liver was considered the cause of early death of 37/50 males and 32/50 females dosed at 1.0 mg/kg b.w. Mean body weights for both males and females also were lower in the 1.0 mg/kg b.w. dose group compared with controls throughout most of the study. Neoplastic responses included significantly increased incidences of liver hemangiosarcoma, hepatocellular adenoma and mononuclear-cell leukemia in both males and females exposed to 1 mg/kg (Table 4-2). In addition, incidences of hepatocellular adenoma or carcinoma combined were significantly increased in the high-dose female group. The adjusted incidences of tumors were calculated using the Poly-3 test, which is based on an adjustment for survival to reflect the number of animals at risk of developing the tumor. Liver hemangiosarcomas are very

rare in F344 rats and were not detected in concurrent controls or in 659 historical controls given the NTP-2000 diet. The liver hemangiosarcomas included both single and multiple neoplastic masses and metastasized to the lung, lymph nodes, pancreas, and spleen. (See Section 4.3 for a discussion of non-neoplastic lesions.) NTP (2003) concluded that there was clear evidence of carcinogenic activity of riddelliine in male and female F344/N rats based on increased incidences of hemangiosarcoma in the liver. The increased incidences of hepatocellular adenoma and mononuclear-cell leukemia in male and female rats also were considered to be treatment related.

Table 4-2. Neoplastic lesions observed in F344/N rats administered riddelliine by gavage for two years

Sex	Dose (mg/kg)	No. examined (no. surviving to end of study)	Tumor incidence (%)[a]				
			Liver	Liver (hepatocellular)			All organs
			Hemangiosarcoma	Adenoma	Carcinoma	Combined	Mononuclear-cell leukemia
Male	0	50 (49)	0 (0)	0 (0)	0 (0)	0 (0)	2 (4.0)
	1.0	50 (3)***[c]	43 (92.5)***	4 (13.7)*	0 (0)	4 (13.7)*	9 (28.5)**
Female	0	50 (33)	0 (0)	1 (2.3)	0 (0)	1 (2.3)	12 (27.0)
	0.01	50 (22)	0 (0)	0 (0)	0 (0)	0 (0)	8 (18.9)
	0.033	50 (28)	0 (0)	0 (0)	0 (0)	0 (0)	13 (29.9)
	0.1	50 (22)	0 (0)	0 (0)	0 (0)	0 (0)	18 (40.3)
	0.33	50 (29)	3 (7.0)	1 (2.4)	1 (NR)[d]	2 (4.8)	18 (39.0)
	1.0	50 (0)***[c]	38 (89.7)***	7 (32.3)**	1 (NR)[d]	8 (36.1)***	14 (51.6)*
	trend[b]		$P < 0.001$	$P < 0.001$	NR	$P < 0.001$	$P = 0.009$

Sources: Chan *et al.* 2003, NTP 2003.

*Significantly different ($P < 0.05$) from the control group by the Poly-3 test, which is based on an adjustment for survival to reflect the number of animals at risk of developing the tumor (see Glossary for a more complete definition of the Poly-3 test).

**Significantly different ($P < 0.01$) from the control group by the Poly-3 test.

***Significantly different ($P < 0.001$ from the control group by the Poly-3 test (tumor incidences) or life-table pairwise comparison (survival).

[a]Poly-3 estimated neoplasm incidence after adjustment for intercurrent mortality.

[b]Poly-3 test for dose-related trend.

[c]Life-table pairwise comparison (Cox method).

[d]Adjusted rate not reported, unadjusted rate = 2.0%.

NR = not reported.

Schoental and Head (1957) administered riddelliine in drinking water at a concentration of 0.02 mg/mL twice weekly for six months to 14 female and 6 male Wistar rats. During the succeeding six months, rats either continued to receive riddelliine in drinking water or were administered additional riddelliine by intraperitoneal (i.p.) injections. After one year, all surviving animals (12 females and 4 males) were injected i.p. with riddelliine at a dose of 30 mg/kg b.w. and maintained without further exposure until their deaths. Control groups consisted of 8 rats of each sex maintained on the normal diet throughout the experiment and an additional group of 3 male rats maintained on the normal diet supplemented with betaine. In the animals that survived for a year, the livers of all 4 males were grossly abnormal, with pale, solid nodules in all lobes; however, no histopathology was reported for these nodules. The surviving females were less severely affected than the males; 5 of the 12 had small liver nodules, 1 had a liver sarcoma (arising from the wall of a tapeworm cyst), and 1 had a mammary fibroadenoma. No liver nodules occurred in the controls. The authors reported that the lesions produced by riddelliine were similar to those produced by other PAs. [This early tumorigenicity study suggested a possible tumorigenic effect by riddelliine, despite its small sample size and unconventional study design.]

4.3 Non-neoplastic effects in rats and mice

In the NTP (2003) study, riddelliine exposure increased the incidences of many non-neoplastic lesions, particularly in the liver, kidney, and spleen, in both rats and mice (Table 4-3) (see Section 5.6 for a discussion of toxicity). Significantly higher incidences of non-neoplastic lesions in the bone marrow, lung, stomach, and lymph nodes also were observed in rats. Arterial inflammation was particularly severe in female mice, affecting the intestines, mesentery, ovary, and uterus, in addition to the kidney and spleen, while subcutaneous tissue edema was noted in male mice. These results demonstrated that the selected dose ranges were appropriate and that the lowest doses tested in female rats and male mice were close to the no-observed-effect levels.

Table 4-3. Incidences of selected non-neoplastic lesions in F344/N rats and B6C3F$_1$ mice exposed to riddelliine by gavage for two years

Lesions	Male rats[a]	Female rats[b]	Male mice[c]	Female mice[d]
Liver				
Hepatocyte, cytomegaly	0/50, 32/50**	0/50, 0/50, 7/50**, 23/50**, 32/50**, 29/50**	4/50, 4/50, 16/50**, 33/50**, 43/50**	0/49, 49/50**
Hepatocyte, karyomegaly			4/50, 4/50, 15/50**, 33/50**, 43/50**	0/49, 49/50**
Necrosis, focal	0/50, 23/50**	4/50, 2/50, 3/50, 4/50, 4/50, 15/50**	18/50, 9/50*, 5/50**, 6/50**, 21/50	
Eosinophilic focus	3/50, 15/50**	1/50, 2/50, 6/50, 4/50, 12/50**, 13/50**		
Mixed-cell focus	3/50, 7/50*	8/50, 10/50, 10/50,11/50, 23/50**, 5/50		
Clear-cell focus		9/50, 8/50, 9/50, 13/50, 22/50**, 2/50		
Bile duct, hyperplasia		2/50, 1/50, 4/50, 4/50, 3/50, 10/50**	2/50, 0/50, 1/50, 3/50, 6/50	0/49, 28/50**
Hemorrhage	0/50, 4/50*	0/50, 0/50, 2/50, 0/50, 1/50, 7/50**		
Hepatocyte, centrilobular necrosis		0/50, 7/50**		
Hepatocyte, centrilobular necrosis			0/50, 1/50, 3/50, 4/50, 10/50**	
Hemorrhage, focal			0/50, 2/50, 1/50, 6/50*, 21/50**	
Hyperplasia, regenerative	0/50, 49/50**	0/50, 0/50, 0/50, 0/50, 8/50**, 50/50**		
Infiltration, mixed cell				29/49, 41/50**
Kidney				
Nephropathy	0/50, 6/50**	0/50, 0/50, 0/50, 1/50, 1/50, 6/50**	46/49, 48/49, 48/50, 50/50, 50/50	18/49, 47/50**
Glomerulus, glomerulosclerosis			0/49, 1/49, 0/50, 42/50**, 41/50**	0/49, 40/50**
Renal tubule, hyaline droplet			0/49, 2/49, 1/50, 1/50, 3/50	2/49, 14/50**
Renal tubule, karyomegaly			0/49, 0/49, 0/50, 0/50, 12/50**	0/49, 1/50
Renal tubule, dilatation			16/49, 17/49, 24/50, 29/50**, 22/50	
Renal tubule, pigmentation				2/49, 27/50**
Artery inflammation				1/49, 16/50**

Lesions	Male rats[a]	Female rats[b]	Male mice[c]	Female mice[d]
Spleen				
Congestion	0/50, 24/49**	0/50, 0/50, 0/50, 1/50, 3/50, 7/50**		
Hematopoietic cell proliferation	1/50, 23/49**	24/50, 33/50*, 25/50, 26/50, 27/50, 34/50**	18/49, 16/49, 19/50, 20/50, 33/50**	32/49, 43/50*
Artery inflammation				0/49, 6/50*
Other				
Bone marrow hyperplasia	1/50, 36/49**	6/50, 3/50, 8/50, 7/50, 10/50, 32/50**		
Lung hemorrhage	1/50, 21/50**	4/50, 7/50, 1/50, 3/50, 5/50, 19/50**		
Stomach erosion	0/50, 10/50**	0/50, 0/50, 0/50, 2/49, 1/49, 9/50**		
Lymph node, mediastinal, hemorrhage	3/50, 20/50**	5/50, 8/50, 9/50, 5/50,7/50, 25/50**		

Sources: Chan *et al.* 2003, NTP 2003.
[a]For male rats, doses = control and 1.0 mg/kg b.w.
[b]For female rats, doses = control, 0.01, 0.033, 0.1, 0.33, and 1.0 mg/kg b.w.
[c]For male mice, doses = control, 0.1, 0.3, 1.0, and 3.0 mg/kg b.w.
[d]For female mice, doses = control and 3.0 mg/kg b.w.
*Significantly different from the control group ($P \leq 0.05$) by Poly-3 test, which is based on an adjustment for survival to reflect the number of animals at risk of developing the lesion (see Glossary for a more complete definition of the Poly-3 test).
**Significantly different from the control group ($P \leq 0.01$) by Poly-3 test.

4.4 Metabolites

Riddelliine and many other hepatotoxic PAs share in common the reactive metabolite DHP (see Sections 1 and 5). DHP is a racemic mixture of the enantiomers *R*-DHP and *S*-DHP. Both enantiomers have been reported to cause cancer in rats (Allen *et al.* 1975, Peterson *et al.* 1983), and *R*-DHP also caused skin tumors in mice (Shumaker *et al.* 1976, Johnson *et al.* 1978, Mattocks and Cabral 1982). [However, DHP does not appear to account for all the carcinogenic and toxic effects of riddelliine or other PAs (see Sections 5.5.1 and 5.6) because there are some differences in the tumor types induced by the various PAs. Thus, other metabolic intermediates, such as dehydroriddelliine and other PA-specific pyrroles may be important.] No carcinogenicity studies with dehydroriddelliine were identified, but there are several studies that indicate that the pyrrolic derivatives form DNA adducts (see Sections 5.2 and 5.3) and DNA cross-links, and contribute to PA-specific toxic effects (Wilson *et al.* 1992, Hoorn *et al.* 1993,

Wagner *et al.* 1993, Kim *et al.* 1999). Results from carcinogenicity studies of riddelliine metabolites are summarized below.

Retronecine is a hydrolysis product of riddelliine and can be detected in the serum of male and female rats and mice exposed to riddelliine (Williams *et al.* 2002). A tumor of the spinal cord was observed in one of ten newborn rats injected subcutaneously with retronecine (Schoental and Cavanagh 1972). However, no control group was included in this study. [This is the only study identified that has reported central nervous system (CNS) tumors after administration of riddelliine, its metabolites, or other PAs.]

4.4.1 Mice

Johnson *et al.* (1978) exposed groups of 8-week-old female Swiss mice to DHR (*R*-DHP) (20 mg/kg b.w.) by subcutaneous (s.c.) injection (8 mg/mL in 0.1 M phosphate buffer), topical application (4 mg/mL in acetone), or both. Group I (25 mice) received 0.2 mL topical applications, group II (25 mice) received 0.1 mL s.c. injections, and group III (75 mice) received both s.c. injections and topical applications. The control group (15 mice) received s.c. injections of 0.1 mL of 0.1 M phosphate buffer (pH 7) and topical applications of 0.2 mL of acetone. All mice were administered *R*-DHP once per week for the first four weeks; after six months, all animals without tumors were administered *R*-DHP weekly for two more weeks. Results were reported only for those animals in each group that were still alive at the time of appearance of the first tumor in that group. Of the 92 mice exposed to *R*-DHP that survived until the appearance of the first tumor, 68% (63 of 92) developed tumors at the application or injection site. Most were skin tumors (basal-cell or squamous-cell carcinoma). Twelve of the mice developed skin tumors that metastasized to the lung, liver, or spleen. The incidence of mice with skin tumors was 0 of 11 in the control group, 6 of 16 in group I, 13 of 21 in group II, and 28 of 55 in group III. Of 11 mice in the control group, 1 developed a pulmonary adenoma. No statistical analyses of tumor incidences between exposure groups or with controls were reported.

A solution containing *R*-DHP at a concentration of 7.65 mg/mL in acetone was applied (0.1 mL of *R*-DHP solution per mouse per application) to the backs of 21 female LACA mice weekly for up to 47 weeks (Mattocks and Cabral 1982). Controls received applications of acetone. All surviving mice were killed at 102 weeks and examined for

skin tumors. The incidence of malignant skin tumors (histological type not reported) was significantly higher ($P < 0.02$) in exposed mice (5 of 20) than in the controls (0 of 19).

4.4.2 Rats

A group of 75 male Sprague-Dawley rats received biweekly s.c. injections of DHR (*R*-DHP) at 20 mg/kg b.w. for four months, followed by biweekly s.c. injections at 10 mg/kg b.w. for another eight months (Allen *et al.* 1975, Shumaker *et al.* 1976). The control group (50 rats) received biweekly injections of 0.1 M phosphate buffer at pH 7. After four months, a partial hepatectomy was performed on 15 animals in the exposed group and 5 in the control group to investigate the effect of *R*-DHP on hepatic mitosis and to evaluate tissue changes resulting from exposure to *R*-DHP. *R*-DHP-exposed rats with partial hepatectomies had a decreased mitotic index (11.99 ± 6.6, mean ± S.D.) compared with control rats (61.7 ± 8.7), which was described by the authors as a "decided inhibition," although no statistical analysis was provided. The remaining animals were maintained for up to an additional 10 months and were sacrificed when they became moribund. Survival in the exposed and control groups was similar. After four months, body weights were lower in the *R*-DHP-exposed group, but there were no signs of illness. The dose was reduced, and by the 12th month, body weights were essentially the same in both groups. Rhabdomyosarcomas developed at the injection site in 31 of 60 *R*-DHP-exposed rats and in none of the controls, and rhabdomyosarcomas with metastases [sites not reported] were observed in 5 rats.

Four groups of 24 male hooded rats received i.p. injections of *S*-DHP and/or thioacetamide [a mitotic stimulator] over a 32-week period, beginning at 10 weeks of age, and were maintained for up to 104 weeks after the first injection (Peterson *et al.* 1983). Rats in group 1 received weekly injections of thioacetamide at 60 mg/kg b.w.; group 2 received an initial injection of *S*-DHP at 76.5 mg/kg b.w., a second dose at 65 mg/kg b.w. four weeks later, and subsequent doses at 60 mg/kg b.w. every four weeks; group 3 received both thioacetamide and *S*-DHP (at the same doses and on the same schedule as in groups 1 and 2, with the first thioacetamide injection given one week after the first *S*-DHP injection); and group 4 received i.p. injections of saline solution. After the eighth week, mean body weight was lower in the *S*-DHP-treated group than in the

controls. In the control and thioacetamide groups, 10 rats per group died at 90 to 113 weeks of age [study weeks 80 to 103], and 18 rats died in each of the *S*-DHP groups at 33 to 106 weeks of age [study weeks 23 to 96]. Mortality was significantly higher in the *S*-DHP-exposed groups than in the control group. Kidney and liver damage and polyarteritis were the most common causes of early deaths. Interim sacrifices were conducted at 10, 21, and 31 weeks after the first injection (2 animals per group), 82 weeks (5 animals each from groups 1 and 4), and 104 weeks (3 animals each from groups 1 and 4). No neoplasias other than age-associated spontaneous testicular tumors were observed in the controls. While the authors noted that the complete absence of other tumors in the control group could be considered unexpected, they did not have reliable historical data on tumor incidence rates for rats at these ages. Seven tumors (2 bronchiologenic adenocarcinomas, 2 liver hepatomas, 1 liver cystic cholangioma, 1 adrenal pheochromocytoma, and 1 subcutaneous fibroma) occurred in 6 rats in the group exposed to thioacetamide alone. There were 11 tumors in 6 rats in the *S*-DHP-exposed group. These included tumors of the abdomen or abdominal wall (leiomyofibrosarcoma and fibrosarcoma), thorax and lung (bronchiologenic adenocarcinoma), pancreas (Islet cell carcinoma), adrenal gland (pheochromocytoma), liver (cystic cholangioma), forebrain (glioma), and gastrointestinal tract (adenocarcinoma or carcinoma). The group exposed to both thioacetamide and *S*-DHP had 6 tumors in 4 rats, including 2 liver hepatomas, 1 liver carcinoma, 1 osteogenic sarcoma of the hind leg, 1 pheochromocytoma, and 1 bronchiologenic adenocarcinoma. The total tumor incidence was significantly higher ($P < 0.02$) in all *S*-DHP- and/or thioacetamide-exposed groups combined than in the controls, but there were no significant differences among the exposed groups.

4.5 Plant materials and extracts

Dried plant materials (such as leaves, roots, flowers, and seeds) or extracts from plant materials containing PAs have caused tumors when administered to rats or chickens. In many cases, the PA content of these materials and extracts was not described. Although none of the studies reviewed below specifically identified riddelliine as a constituent of these plant materials or extracts, it may reasonably be assumed that certain plants probably contained some riddelliine, along with other PAs. Riddelliine has been detected

in at least 13 plant species (see Section 2 and Table 2-1). Molyneux *et al.* (1988) reported that *S. riddellii* (Riddell's groundsel), *S. longilobus* (threadleaf groundsel), *S. jacobaea* (tansy ragwort), and *S. vulgaris* (common groundsel) were responsible for most livestock PA poisonings in the western United States. The riddelliine content of these plants varies, but is highest in *S. riddellii* (≥ 96% of total PAs) and *S. longilobus* (8% to 21% of total PAs) (Molyneux *et al.* 1979). Relatively small amounts of riddelliine occur in *S. vulgaris* (3% of total PAs). *S. jacobaea* contains at least 8 PAs, including riddelliine, but the amounts were not reported (Molyneux *et al.* 1979, Molyneux *et al.* 1991).

Three studies were identified in which rats or chickens were exposed to *S. jacobaea* (Cook *et al.* 1950, Schoental *et al.* 1954, Campbell 1956) and one study in which rats were exposed to *S. longilobus* (Harris and Chen 1970). Several types of liver tumors were reported in rats given solutions of PAs extracted from *S. jacobaea* in drinking water (Cook *et al.* 1950) or by gavage (Schoental *et al.* 1954), in chickens injected with alkaloids extracted from *S. jacobaea* (Campbell 1956), and in rats (Harris and Chen 1970) and chickens (Campbell 1956) fed diets containing dried and milled (or ground) plant material containing PAs from *S. jacobaea* and *S. longilobus*, respectively. Results are summarized in Table 4-4.

Table 4-4. Neoplastic lesions observed in experimental animals exposed to plant materials and extracts from *Senecio jacobaea* or *S. longilobus*

Reference	Plant species (form)	Animal (N)	Exposure (duration)	Results
Cook *et al.* 1950	*S. jacobaea* (solution of alkaloids)	albino rat (11)	0.1 mg/mL in drinking water; reduced due to toxicity to 0.05 mg/mL (daily up to 11 mo)	hepatoma or cholangioma in 3 rats surviving ≥ 8 mo (sex not specified)
Schoental *et al.* 1954	*S. jacobaea* (solution of alkaloids)	Wistar rat (25)	solution containing 0.05 mg/mL, reduced to 0.03 mg/mL 3 days/wk; (most likely by gavage) (3 days weekly for life)	hepatoma in 2 male rats
Campbell 1956	*S. jacobaea* (solution of alkaloids, mainly seneciphylline)	chicken (18)	solution injected i.v. at dose of 35 mg/kg, reduced to 20 mg/kg after second injection	liver tumors in 2 males and 1 female on normal diet and 2 males and 1 female on deficient diet
Campbell 1956	*S. jacobaea* (dried and milled plant in diet)	chicken (21)	diet containing 1 mg/day, reduced to 0.5 mg/day (daily for 14 wk)	liver tumors in 3 males (hepatoma) and 1 female (liver-cell and bile-duct carcinoma)
Harris and Chen 1970	*S. longilobus* (dried and ground stems and leaves in diet)	Harlan rat (40 to 50)	0.5% to 0.75% in diet (daily and intermittent for up to 1 yr)	hepatocarcinoma in 4/23 (3 males and 1 female) and 16/47 (13 males and 3 females); angiosarcoma (hemangiosarcoma) in liver of 1 male in intermittent exposure groups surviving > 200 days

4.6 Summary

The carcinogenicity of riddelliine was investigated in B6C3F$_1$ mice and F344/N rats (administered by gavage for two years) and in Wistar rats (administered in drinking water for one year). The NTP stated that there was clear evidence of carcinogenic activity in B6C3F$_1$ mice (hemangiosarcoma in the liver in males and alveolar/bronchiolar adenoma or carcinoma in females) and F344/N rats (hemangiosarcoma in the liver in males and females). Hepatocellular adenoma and mononuclear-cell leukemia also were significantly increased in incidence in both sexes of F344/N rats and were considered treatment related. The tumor locations and types associated with riddelliine are summarized in Table 4-5.

Two riddelliine metabolites (see Sections 5.1) and have also been evaluated for carcinogenicity. The riddelliine metabolite *R*-DHP was tested for carcinogenicity in female mice following skin application and in male rats exposed by s.c. injection. DHH (*S*-DHP), another metabolite of riddelliine and an enantiomer of *R*-DHP, was tested for carcinogenicity in male rats by i.p. injection. *R*-DHP caused malignant skin tumors in mice and local rhabdomyosarcomas in rats. Male rats exposed to *S*-DHP by i.p. injection developed a variety of malignant tumors.

Four studies of the carcinogenicity of plant species known to contain riddelliine were reviewed, three in rats and one in chickens. Liver tumors were reported in all four studies.

Table 4-5. Summary of neoplastic responses in mice and rats exposed to riddelliine

Tumor location	Tumor type	Mice		Rats	
		Male	Female	Male	Female
Liver	hemangiosarcoma	✓		✓	✓
	hepatocellular adenoma			✓	✓
Lung	alveolar/bronchiolar adenoma or carcinoma combined		✓		
Hematopoietic	mononuclear-cell leukemia			✓	✓

✓ = increased incidences of tumors associated with riddelliine exposure.
Source: Chan *et al.* 2003, NTP 2003.

5 Other Relevant Data

This section discusses the relevant mechanistic and other information needed to understand the toxicity and potential carcinogenicity of riddelliine. It includes information on (1) absorption, distribution, metabolism, and excretion, (2) DNA adducts, (3) mechanistic studies and considerations, (4) genetic damage and related effects, (5) carcinogenicity, genotoxicity, and structure-activity relationships of riddelliine metabolites and other PAs, (6) toxicity, and (7) a summary.

5.1 Absorption, distribution, metabolism, and excretion

5.1.1 Absorption

Riddelliine and other PAs have been reported to be absorbed via oral ingestion and dermal exposure. Feeding experiments with domestic farm animals indicated that both riddelliine and riddelliine *N*-oxide are absorbed via the gastrointestinal tract (IARC 2002). Dermal absorption of PAs has been shown to result in less bioaccumulation than oral absorption. In a study comparing urinary excretion following dermal versus oral administration of a crude mixture of PA *N*-oxides, free alkaloids, and metabolites, Brauchli *et al.* (1982) reported that the percutaneous absorption of PA *N*-oxides was less than the gastrointestinal absorption by a factor of 20 to 50 when the excretion of *N*-oxides and metabolites in the urine was considered. However, it is possible that skin or scalp absorption of PAs could be increased by the presence of inflammation or lesions (Chojkier 2003). The possibility of absorption of PAs following inhalation exposure to plant dusts or fragments also has been proposed.

5.1.2 Distribution

Riddelliine fed to animals, particularly rats, is distributed to the liver, where pyrrolic metabolites are formed (Mattocks and White 1971). In pigs fed riddelliine, Schoch *et al*. (2000) detected pyrrolic metabolites in the blood and liver one day after exposure. Disposition studies have been reported for many riddelliine analogues, including monocrotaline, lasiocarpine, senecionine, seneciphylline, and retrorsine (Mattocks 1986, NTP 1993). Most of the PAs are distributed to the liver and kidneys; much smaller amounts have been detected in the lungs and spleen. In a study of a mixture of senecionine and seneciphylline in lactating rats, the highest tissue levels were found in

the liver and lungs, and in a study of rats administered a compound showing PA-like hepatotoxicity, [^{3}H]synthanecine A bis(*N*-ethylcarbamate), the highest concentrations were found in the liver and lungs (Mattocks 1986). Wilson *et al.* (1992) reviewed kinetic studies in rats administered senecionine or monocrotaline and reported that these compounds were eliminated rapidly from the plasma but were retained by red blood cells. Sequestration by red blood cells might represent an important transport mechanism for PAs.

5.1.3 Metabolism

Riddelliine must be metabolically activated to exert toxic effects This section describes the metabolic pathways for riddelliine, as determined *in vivo* in rodents and *in vitro* in rat and human liver microsomes, the enzymes responsible for riddelliine metabolism, and compares metabolism in humans, farm animals, and experimental animals.

Metabolic pathways

Riddelliine has three primary metabolic pathways: (1) hydrolysis of the ester group(s) to form the necine base, (2) oxidation of the necine base (of riddelliine) to the corresponding *N*-oxide (which also may be reduced to riddelliine), and (3) hydroxylation of riddelliine at the C-3 or C-8 positions of the necine base, followed by dehydration to form the corresponding dehydroriddelliine (pyrrolic) derivative (Figure 5-1). Dehydroriddelliine is hydrolyzed to form the racemic (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine (DHP), which is a 50/50 mixture of the optically pure dehydroretronecine (*R*-DHP or DHR) and dehydroheliotridine (*S*-DHP or DHH) enantiomers. A number of studies have shown that many PAs have the same metabolic pathways; thus, DHP is a common metabolite of many retronecine-, heliotridine-, and otonecine-type PAs (Fu *et al.* 2001, IARC 2002, Fu *et al.* 2002b, Wang *et al.* 2005a, Wang *et al.* 2005b, Xia *et al.* 2006). The pyrrolic metabolites (dehydroriddelliine and DHP) are formed almost entirely in the liver, are highly reactive, and readily bind to tissue constituents (Mattocks 1986). Although these metabolites may be found in other tissues, they likely originate in the liver. Levels of pyrroles in extrahepatic tissues reflect the chemical stability of the compound, which controls the distance it can travel from the liver before breaking down.

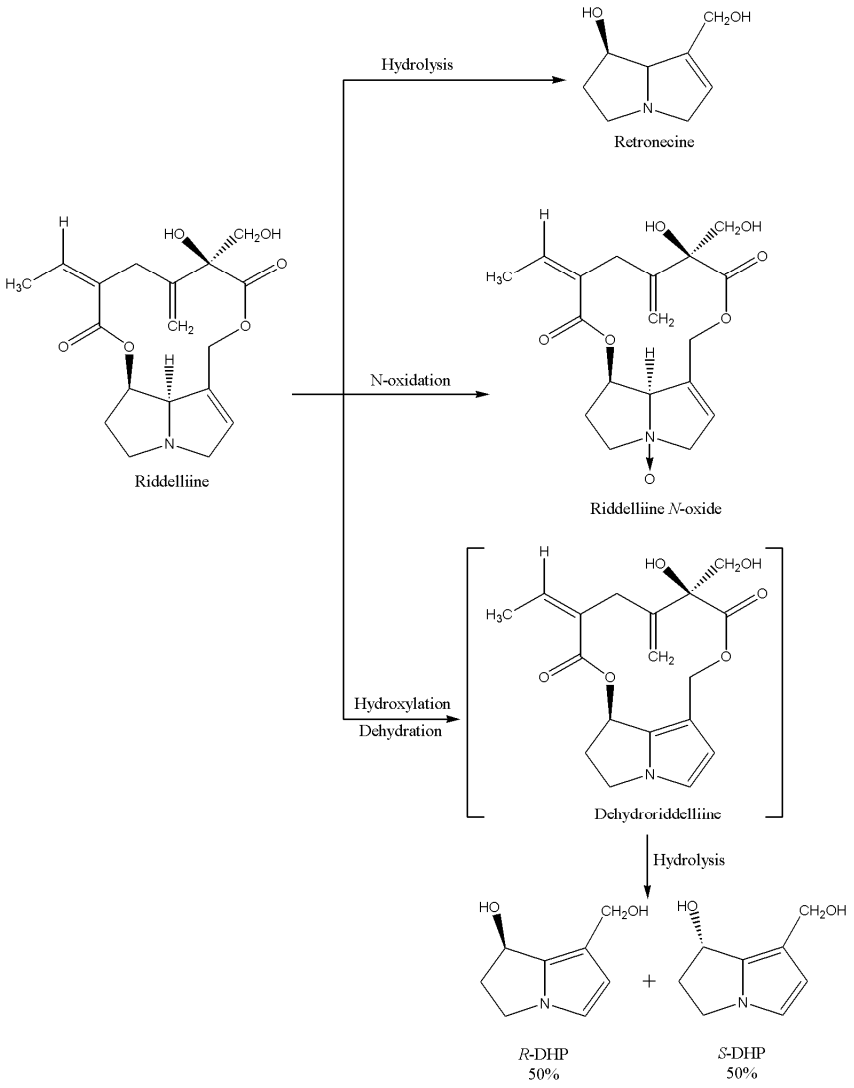

Figure 5-1. The three primary metabolic pathways for riddelliine

Source: Fu *et al*. 2002b, used with permission.

Metabolism of riddelliine *in vitro* by human liver microsomes formed DHP and riddelliine *N*-oxide (Xia *et al.* 2003). *In vitro* metabolism of riddelliine by liver microsomes of female and male F344 rats also generated DHP and riddelliine *N*-oxide as major metabolites (Yang *et al.* 2001a, Fu *et al.* 2002b). Riddelliine was metabolized more rapidly by liver microsomes in male than in female rats (Xia *et al.* 2003).

Williams *et al.* (2002) studied the toxicokinetics of riddelliine by administering a single dose of riddelliine orally at 10.0 mg/kg b.w. to F344 rats and B6C3F$_1$ mice. Six sequential blood samples were collected, and serum concentrations of riddelliine and its

metabolites were determined by LC-electrospray- (ES-) MS. Riddelliine was completely absorbed within 30 minutes after a gavage dose in all rats and mice and there was rapid and extensive conversion of riddelliine to riddelliine *N*-oxide. All animals produced small amounts of retronecine. No DHP was detected, presumably because the highly reactive DHP can bind to macromolecules in the blood, such as serum proteins or red blood cells. The half-times for elimination from serum increased in the following order: riddelliine < retronecine < riddelliine *N*-oxide (see Table 5-1). The half-times for elimination and distribution were similar for male and female rats. In addition, the internal exposure (calculated as area under the time-concentration curve from zero to infinity [$AUC_{0\text{-infinity}}$]) for riddelliine *N*-oxide was greater than that for riddelliine in male rats; however, this relationship was reversed for female rats.

The hydrolysis process in all types of PAs and the *N*-oxidation process in the retronecine- and heliotridine-type PAs are generally considered detoxification pathways. Plants that contain PAs generally also contain large amounts of PA *N*-oxides. PA *N*-oxides are major metabolites of PAs and also are generally regarded as detoxification products. However, recent studies have shown that metabolism of riddelliine *N*-oxide and other PA *N*-oxides by human or rat liver microsomes generates DHP and the corresponding parent PAs under both aerobic and hypoxic (under argon) conditions (Chou *et al.* 2003a, Wang *et al.* 2005c). Oxidative conditions inhibited reduction to the parent PA by 38% to 66% for human liver microsomal metabolism and 25% to 57% for the rat. DHP formation was reduced by 40% to 67% (human) and 25% to 68% (rat) under hypoxic conditions. Thus, the *N*-oxides of riddelliine and other PAs may be metabolically activated.

Table 5-1. Toxicokinetic determinations for riddelliine and metabolites

Molecule	Animal	Elimination $t_{1/2}$ (h)[a]	Distribution $t_{1/2}$ (h)[b]	$AUC_{0\text{-infinity}}$ (ng·h/mL)[a]
Riddelliine	Rat, male	4.2 ± 0.3	0.35	516 ± 80*
	Rat, female	4.2 ± 1.0	0.55	1,267 ± 395
	Mouse, male	3.2	0.34	1307
	Mouse, female	3.0	0.24	1064
Riddelliine *N*-oxide	Rat, male	7.0 ± 1.3	0.55	1,494 ± 367*
	Rat, female	11.9 ± 7.2	0.37	714 ± 405
	Mouse, male	15.4	0.35	1753
	Mouse, female	28.9	0.33	2746
Retronecine	Rat, male	8.2 ± 1.0	NA	88 ± 24
	Rat, female	6.7 ± 1.8	NA	135 ± 36
	Mouse, male	6.9	NA	128
	Mouse, female	8.1	NA	217

Source: Williams *et al.* 2002.
$AUC_{0\text{-infinity}}$ = area under the time-concentration curve from zero to infinity; NA = not applicable.
[a]Means ± SDs were determined from plots of data for serum from individual rats (N = 5, females; N = 3, males) and means without SDs were determined from plots of data averaged from six individual mice for each time point.
[b]A first-order distribution rate constant was determined from mean blood concentration-time plots.
*$P < 0.05$; significant sex difference.

Metabolizing enzymes

Metabolism of PAs to the reactive pyrrolic ester metabolites in rodents and humans is mainly catalyzed by CYP3A and CYP2B6 isozymes of cytochrome P450 (Chung and Buhler 1994, Chung *et al.* 1995, Kasahara *et al.* 1997, Reid *et al.* 1998, Tepe and Williams 1999, Lin *et al.* 2000, Yang *et al.* 2001a). These two isoforms are primarily responsible for the metabolism of PAs to dehydropyrrolizidines, whereas both cytochrome P450 and flavin-containing monooxygenase catalyze formation of the *N*-oxides (Fu *et al.* 2002b) (see Figure 5-2). The rate of metabolism of riddelliine by rat liver microsomes was increased 3.4- to 3.8-fold by pretreatment with phenobarbital, an inducer of CYP2B and CYP3A isozymes (Yang *et al.* 2001a).

When riddelliine was metabolized *in vitro* by human liver microsomes in the presence of the P450 3A4 enzyme inhibitor triacetyleandomycin, formation of DHP and riddelliine *N*-oxide were reduced 84% and 92%, respectively (Xia *et al.* 2003), indicating that the

P450 3A4 enzyme is principally responsible for the metabolism of riddelliine and for metabolic activation of most, if not all, toxic PAs.

Metabolism of PAs to the corresponding *N*-oxides is catalyzed by both cytochrome P450 and flavin-containing monooxygenase (Williams *et al.* 1989a, Miranda *et al.* 1991a, Miranda *et al.* 1991b, Chung *et al.* 1995). Buhler and co-workers reported that metabolism of senecionine to senecionine *N*-oxide was catalyzed by both CYP2B and flavin-containing monooxygenase in untreated and phenobarbital-treated guinea-pigs (Ramsdell and Buhler 1987, Chung *et al.* 1995). Enzymatic hydrolysis of the ester functional groups of PAs is catalyzed mainly by liver microsomal carboxylesterases (Eastman and Segall 1981, Buhler and Kedzierski 1986, Williams *et al.* 1989b, Miranda *et al.* 1991b, Chung and Buhler 1994, Chung *et al.* 1995, Kasahara *et al.* 1997, Reid *et al.* 1998, Yang *et al.* 2001a), but also can be catalyzed by liver cytosolic carboxylesterase (Mattocks 1982, 1986, Dueker *et al.* 1992, Kasahara *et al.* 1997).

Comparative metabolism

There is a wide range of susceptibilities to PAs among species (see Section 5.6). The rate of metabolic conversion of a PA to its active pyrrole, and the relative activity of detoxifying enzymes are important factors. Susceptible species such as rats, cattle, and horses have higher rates of pyrrole production than resistant species such as sheep and Japanese quail (Cheeke 1988). Some resistant species also have high activity of enzymes involved in detoxification and excretion. Hooper (1978) reported that susceptibility in laboratory animals varies with sex and age, and can be altered by various physical and chemical factors that influence hepatic metabolism. Some studies have indicated that metabolism by ruminal microflora in sheep can detoxify PAs prior to absorption. Duringer *et al.* (2004) reported that sheep ruminal fluid degraded PAs 10 times more efficiently than cattle ruminal fluid, which the authors noted appeared to be an important protective mechanism for sheep being less susceptible than cattle. Humans are highly susceptible to PA toxicity and cattle, horses, rats, and mice are similarly sensitive (Fu *et al.* 2002b). Mattocks (1986) concluded that humans are more susceptible to the acute effects of PAs than male rats and noted that only a few studies provided estimates of the amount of PAs ingested by humans.

Huan *et al.* (1998) investigated the roles of CYP3A and CYP2B isoforms in hepatic bioactivation and detoxification of senecionine in sheep and hamster microsomes (both resistant species). The rate of activation (formation of DHP) was much higher than the rate of detoxification (formation of the *N*-oxide) in hamsters, but the *N*-oxide was the major metabolite in sheep. CYP3A had a major role in the formation of pyrrolic metabolites in both species (> 90% in sheep and 68% in hamsters), and also was involved in *N*-oxidation (38.8% in sheep and 41.3% in hamsters). CYP2B had a more limited capacity for DHP formation (47% in sheep and 32% in hamsters) and *N*-oxidation (24.6% in sheep and 35.4% in hamsters). Huan *et al.* also reported that previous studies indicated that in rats CYP3A2 was primarily involved in biotransformation of senecionine to DHP while *N*-oxidation was catalyzed mainly by CYP2C11. CYP2B enzymes have been proposed to be important in bioactivation of senecionine in guinea-pigs, while CYP2C and CYP3A subfamily members had little influence. CYP3A4 was the major enzyme involved in bioactivation and detoxification of senecionine in human liver.

Duringer *et al.* (2004) compared hepatic *in vitro* metabolism of senecionine in sheep and cattle. There were no significant differences in the amount of DHP formed or the catalytic efficiency of the enzymes responsible for DHP formation between sheep and cattle. Thus, there was not a strong correlation between *in vitro* DHP formation and species susceptibility. However, sheep liver microsomes formed more *N*-oxide, had a higher *N*-oxide catalytic efficiency, and metabolized senecionine faster than cattle. P450 concentrations and isoforms had a large influence on DHP formation, and flavin-containing monooxygenases (FMOs) were important for *N*-oxide formation. CYP3A played a larger role in DHP formation in cattle, while FMO activity was greater in sheep. The ratio of DHP:*N*-oxide was about 4.5 times higher in cattle than in sheep. Gender differences also were reported. Castrated male cattle or sheep had a higher DHP:*N*-oxide ratio than females of the same species. However, the differences in hepatic metabolism alone did not account for all the variation in susceptibility. As mentioned above, increased ruminal metabolism in sheep was also very important.

As previously discussed, human liver microsomes metabolize riddelliine to DHP and riddelliine *N*-oxide (Xia *et al.* 2003). CYP3A was demonstrated to be the principal

isoform responsible for metabolism of riddelliine in humans because formation of DHP and riddelliine *N*-oxide were reduced by 84% and 92%, respectively, in the presence of the P450 3A4 inhibitor triacetyleandomycin. The K_m and V_{max} values from human and rat liver microsomal preparations were similar (Table 5-2).

Table 5-2. Enzyme kinetic parameters for riddelliine oxidative metabolism to DHP and riddelliine *N*-oxide in rat and human liver microsomes

Samples	Kinetic parameters[a]			
	V_{max} (nmol/min per mg of protein)		K_m (mM)	
	DHP	Riddelliine *N*-oxide	DHP	Riddelliine *N*-oxide
Rat, female[b]	0.48 ± 0.03	0.30 ± 0.01	0.37 ± 0.05	0.44 ± 0.04
Rat, male[b]	1.12 ± 0.04	2.17 ± 0.08	0.28 ± 0.03	0.25 ± 0.03
Human, female[c]	1.70 ± 0.09	0.43 ± 0.03	0.66 ± 0.08	0.71 ± 0.12
Human, male[c]	0.95 ± 0.02	0.26 ± 0.01	0.24 ± 0.02	0.44 ± 0.06

Source: Xia *et al.* 2003.
[a]Kinetic parameters, represented as mean ± SD (3 replicates), were determined with GraphPad Prism software.
[b]Liver microsomes were prepared by combining liver tissues of 6 (female) or 5 (male) rats.
[c]Equal amounts of liver microsomal protein from 4 female human liver microsomes samples were combined; 1 male human liver microsome sample was used.

5.1.4 Excretion

In general, about 80% of ingested PAs are excreted unchanged in the urine and feces, with urine the more prevalent route (NTP 2003). Excretion of metabolized ^{14}C-labeled PAs senecionine and seneciphylline as CO_2 by lactating rats was less than 1% of the total dose (Eastman *et al.* 1982). The authors stated that higher rates of excretion via CO_2, approaching 10%, had been reported for lasiocarpine, another PA. Biliary excretion of some PAs and their metabolites may be as high as 25% (Mattocks 1986).

5.2 DNA adducts

5.2.1 Studies of DHP adduct formation in vitro

Figure 5-2 shows the proposed pathway of metabolic activation of riddelliine leading to DNA adduct formation based on metabolism studies with rat and human liver microsomes and studies of DNA adduct formation *in vitro* and *in vivo*.

A common mechanism likely exists for DNA adduct formation for the PAs, including riddelliine, that form DHP as a metabolite. As shown in Figure 5-2, two possible

pathways lead to DHP-derived DNA adduct formation from metabolism of riddelliine and other PAs *in vitro* and *in vivo*: (1) a dehydro-PA, e.g., dehydroriddelliine, binds covalently to DNA to form dehydro-PA-derived DNA adducts, which are hydrolyzed to DHP-derived DNA adducts, and (2) dehydro-PAs hydrolyze to form DHP, which binds to DNA. At present, it is not known which pathway predominates. Because dehydro-PAs are highly unstable, and DHP is the most stable pyrrolic compound (Galloway *et al.* 1987, Huxtable *et al.* 1996), it has been proposed that more binding occurs through DHP than through dehydro-PAs (Figure 5-2) (Yang *et al.* 2001a, Fu *et al.* 2002b, Xia *et al.* 2004).

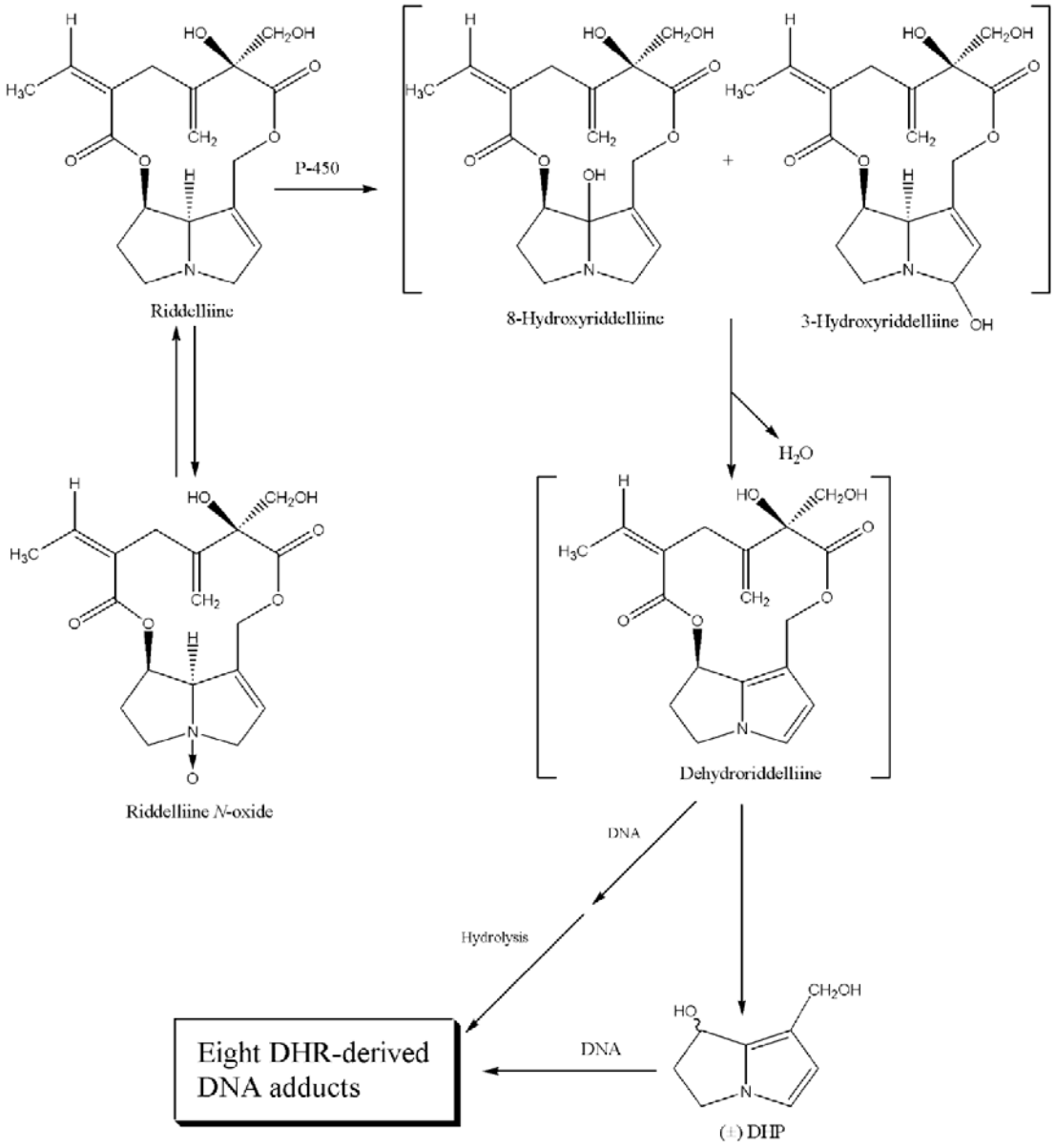

Figure 5-2. Pathway for metabolic activation of riddelliine leading to DNA adduct formation

Sources: adapted from Yang *et al.* 2001a, Chou *et al.* 2003a, used with permission.

Binding of DHP to DNA may be a key step leading to DHP's genotoxicity and tumorigenicity. Studies of DHP-derived DNA adducts formed *in vitro* and *in vivo* are discussed below and summarized in Table 5-3. Studies of DNA adduct formation and their relationship to tumorigenicity is discussed in Section 5.4.

Yang *et al*. (2001a, 2001b) developed a ^{32}P-postlabeling/HPLC method for detection and quantification of DHP-derived DNA adducts formed *in vitro* or *in vivo*. (See Table 5-3

for details on the experimental conditions.) The HPLC chromatograms of DHP-derived DNA adducts from the DHP-modified calf thymus DNA assayed by ^{32}P-postlabeling/HPLC are shown in Figure 5-3, along with the assignments of individual peaks as determined by LC-ES/MS analysis (Chou *et al.* 2003b). A set of eight DHP-derived adduct peaks was formed from the reaction of DHP with calf thymus DNA or from rat or human liver microsomal metabolism of riddelliine in the presence of calf thymus DNA (Yang *et al.* 2001a, b); the adducts formed by rat and human microsomes were similar (Xia *et al*. 2003). Among the set of DHP-derived DNA adduct peaks, two (P4 and P6) were identified as epimers of DHP-2'-deoxyguanosine 3'-monophosphate (adduct I and II in Figure 5-3), and the remaining adducts were characterized as DHP-modified dinucleotides (structures were not identified). Four of the adduct peaks (P1, P2, P3, and P5) each corresponded with a single DHP-modified dinucleotide, while the remaining two peaks (P7 and P8) each consisted of a mixture of 4 DHP-modified dinucleotides (Yang *et al.* 2001b, Chou *et al.* 2003b). The formation of these adducts appears to occur as the result of DNA binding to the carbonium ion at the C-7 position of the necine base (Fu *et al.* 2004).

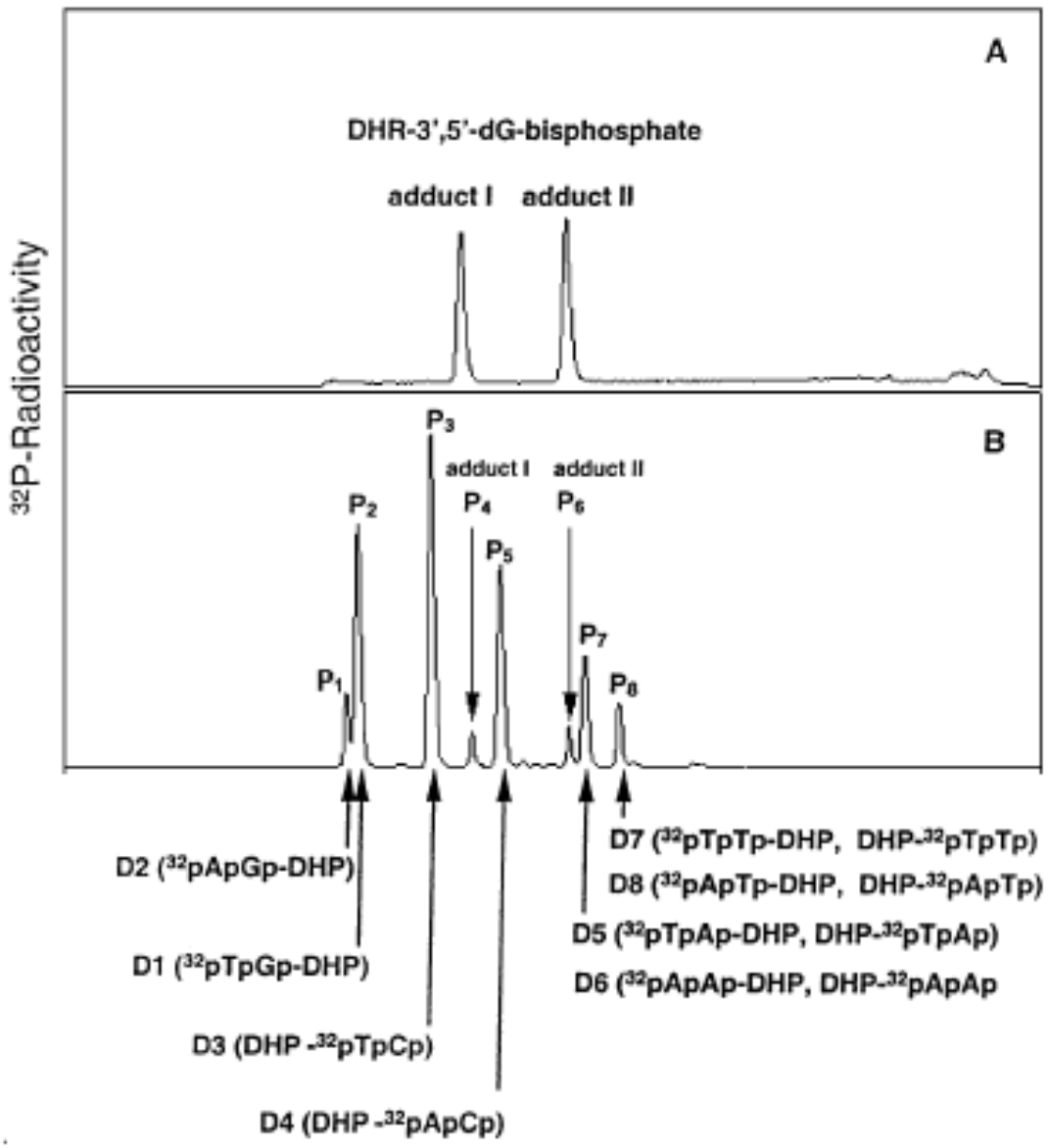

Figure 5-3. ^{32}P-postlabeling chromatograms of DHP-derived DNA adducts from DHP-modified calf thymus DNA

^{32}P-postlabeling chromatograms of epimeric DHP-3',5'-dG-biphosphate adducts (top panel) or DHP-modified calf thymus DNA (bottom panel) with assignment of individual peaks to the respective DHP-modified dinucleotides. Note: D5 and D6 both point to P7 and D7 and D8 both point to P8.
Source: Chou *et al.* 2003b, used with permission.

The structures of DHP-derived DNA adducts were studied by Wickramanayake *et al.* (1985) who investigated alkylation of nucleosides (guanosine, adenosine, deoxyadenosine, uridine, and deoxythymidine) and nucleotides (deoxyguanosine, deoxyadenosine, deoxythymidine, and deoxyuridine 5'-monophosphates) by dehydroretronecine (*R*-DHP), which can be formed by metabolism of riddelliine and

other PAs. Reaction of *R*-DHP with the nucleosides and nucleotides under mild basic conditions resulted in formation of adducts with N^2 of guanosine and deoxyguanosine, N^6 of adenosine, and O^2 of thymidine and deoxythymidine, all reacting with *C*7 of the necine base to form monoalkylated covalent adducts (see Figure 5-4 for representative structures). The formation of DNA adducts by PAs and other compounds also has been reviewed by Wiessler (1994), who reported that dehydro-PAs can act as bifunctional alkylating agents through successive reactions. The reactivity of the 7 and 9 positions of the necine base is dependent on steric hindrance by the ester function. However, Niwa *et al.* (1991) reported that alkylation of deoxynucleosides by dehydromonocrotaline resulted in formation of seven adducts, five of which resulted from nucleophilic attack at C9 of the necine base and the other two at C7.

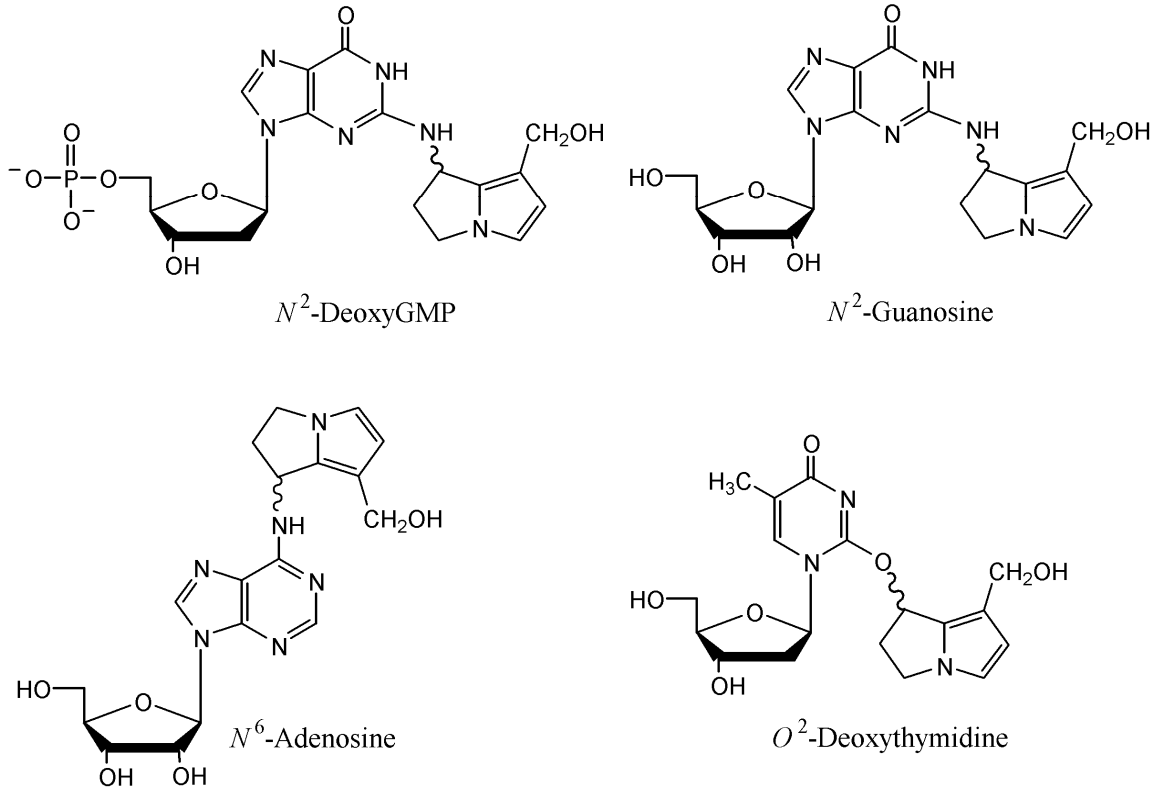

Figure 5-4. Alkylation of nucleosides and nucleotides by dehydroretronecine
Source: Adapted from Wickramanayake *et al.* 1985, Wiessler 1994.

5.2.2 Studies of DNA adduct formation in vivo

DNA adducts with a very similar pattern of adduct peaks have been reported to result from exposure of human, rat, and mouse liver tissue to riddelliine in *in vivo* and *in vitro*

studies as discussed above. Following the same exposure regimen as in the two-year carcinogenicity bioassays, NTP conducted a study of DNA adduct formation *in vivo* in female F344 rats, using the ^{32}P-postlabeling method (Yang *et al.* 2001a). A total of 72 rats were assigned to 12 experimental groups (6 rats per group) and administered riddelliine by gavage at a dose of 0.01, 0.033, 0.1, 0.33, or 1.0 mg/kg b.w. per day, five days per week, beginning at weaning and continuing for three or six months. The results shown in Figure 5-5 indicate a positive dose-response trend in the frequency of DHP-derived adducts in the livers of rats fed riddelliine for 3 or 6 months.

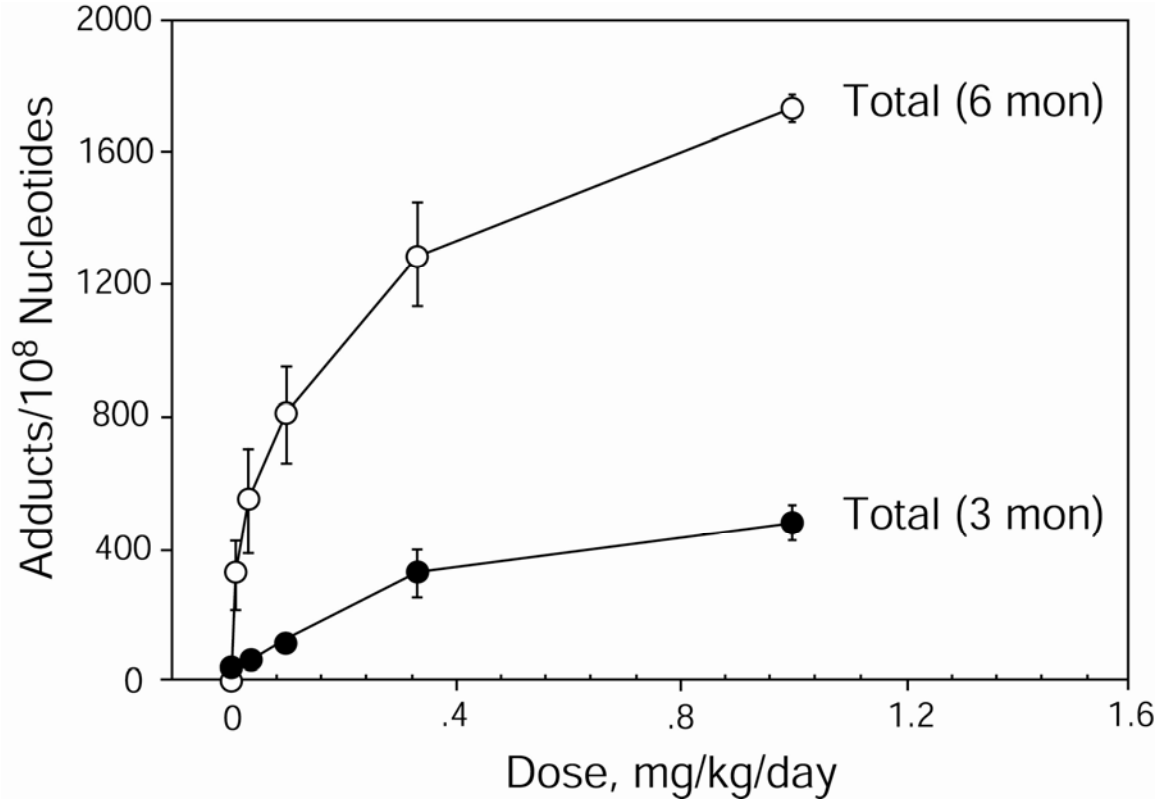

Figure 5-5. Dose-response of total DHP-derived DNA adducts in liver DNA of female rats fed riddelliine

Dose-response relationship of total riddelliine-derived DNA adduct formation in liver of female rats fed riddelliine for 3 and 6 months. [Note: the scale on the x axis as reported in the original publication incorrectly read 0, 4, 8, 12, and 16 mg/kg per day.]

Source: Adapted from Yang *et al*. 2001a, used with permission.

Chou *et al.* (2003c, 2004) reported that DNA adduct levels were significantly higher in rat and mouse liver endothelial cells isolated from animals exposed to riddelliine by gavage compared with parenchymal cells from the same animals. The relationship

between DNA adduct formation in these cells and tumorigenicity of riddelliine is discussed in Section 5.4.1. DHP-derived DNA adducts that formed the same HPLC profile as adducts identified in the livers of rats exposed to riddelliine by gavage were also reported to be formed in the livers of female F344 rats exposed to three dietary supplements (comfrey root extract, comfrey compound oil, coltsfoot root extract) or an extract of a Chinese herbal plant, flos farfara (*Tussilago farfara* or Kuan Tong [Dong] Hua, see Table 2-2), by gavage (Chou and Fu 2006). Comfrey is known to contain PAs such as intermedine, symphytine, and lycopsamine, and coltsfoot root extract and flos farfara root extract contain senkirkine and senecionine.

Table 5-3. Studies in which DHP-derived DNA adducts were detected via ^{32}P-postlabeling or mass spectrometry following exposure to riddelliine or its metabolites

Test system	Dose, test agent, route (*in vivo*), exposure duration	Reference
In vitro		
Nucleosides (guanosine, adenosine, deoxyadenosine, uridine, and deoxythymidine) and nucleotides (deoxyguanosine 5'-monophosphate, deoxyadenosine 5'-monophosphate, deoxythymidine 5'-monophosphate, and deoxyuridine 5'-monophoshphate)	NR, *R*-DHP, several hours	Wickramanayake *et al.* (1985)[a]
Calf thymus DNA incubated with *R*-DHP	NR, *R*-DHP, several hours	Yang *et al.* 2001a, Chou *et al.* 2003b
Rat liver microsomes + calf thymus DNA, female & male	0.1 mM riddelliine; 30 min	Xia *et al.* 2003
Human liver microsomes + calf thymus DNA, female & male	0.1 mM riddelliine; 30 min	Xia *et al.* 2003
In vivo		
F344 rat liver, female	0.01–1.0 mg/kg b.w. riddelliine, gavage; 3–6 mo	Yang *et al.* 2001a
F344 rat liver, male	1 mg/kg b.w. riddelliine, gavage; 2 wk	Chou *et al.* 2003c, 2004
B6C3F$_1$ mouse liver, female & male	3 mg/kg b.w. riddelliine, gavage; 2 wk	Chou *et al.* 2003c, 2004
F344 rat liver, female	1 mg/kg b.w. riddelliine, gavage; 3 d	Chou and Fu 2006

NR = not reported.
[a]Adducts were characterized by liquid secondary ion mass spectrometry in this study; all others used ^{32}P-postlabeling.

5.3 Genetic damage and related effects

DNA adduct formation may play a role in the genotoxicity of riddelliine. Riddelliine has been tested for genotoxicity in a number of *in vitro* and *in vivo* test systems, and the genetic and related effects of riddelliine have been reviewed (IARC 1976, 2002, WHO 1988, Prakash *et al.* 1999, Chan *et al.* 2003, NTP 2003).

5.3.1 Prokaryotic systems

Riddelliine is mutagenic in *Salmonella typhimurium* TA100 in the presence of S9 metabolic activation, but is not mutagenic in TA97, TA98, and TA1537, either with or without metabolic activation (Zeiger *et al.* 1988, NTP 1993, Chan *et al.* 1994). The TA100 strain detects base-pair substitutions, while the other three strains detect frameshift mutations. Table 5-4 summarizes the results of tests in prokaryotic systems.

Table 5-4. Results of genotoxicity testing of riddelliine in prokaryotic systems

Test system	End point (concentration)	Results		Reference
		+S9	–S9	
S. typhimurium TA97, TA98, TA1537	reverse mutation (100–5,000 μg/plate)	–	–	Zeiger *et al.* 1988, NTP 1993, Chan *et al.* 1994
S. typhimurium TA100	reverse mutation (100–5,000 μg/plate)	+	–	Zeiger *et al.* 1988, NTP 1993, Chan *et al.* 1994

5.3.2 Mammalian in vitro systems

Riddelliine has been tested for genetic effects in several mammalian *in vitro* systems, including Chinese hamster V79 cells, CHO cells, rat hepatocytes, BALB/c-3T3 fibroblasts, and bovine kidney epithelial cells. DNA intrastrand cross-linking that was protease sensitive [and thus may have represented protein-associated cross-links] was induced in cultured bovine kidney epithelial cells, but no single-strand breaks were detected in the study (Hincks *et al.* 1991) (see Section 5.5.3 for discussion of cross-linking by other PAs in comparison with riddelliine). Berry *et al.* (1996) reported that riddelliine induced HGPRT mutations in Chinese hamster V79 lung cells in the presence of primary hepatocytes and induced unscheduled DNA repair synthesis (UDS) in rat hepatocytes. Riddelliine induced sister chromatid exchange (SCE) and chromosomal aberrations in Chinese hamster ovary (CHO) cells (Galloway *et al.* 1987, NTP 1993).

Although SCE tests were positive both with and without metabolic activation, the response was stronger in the presence of S9. Chromosomal aberrations occurred only with metabolic activation. Riddelliine also induced cell transformation in mouse BALB/c-3T3 fibroblast cells (Matthews *et al.* 1993). Table 5-5 summarizes the results of tests in mammalian *in vitro* systems.

Table 5-5. Results of genotoxicity testing of riddelliine in mammalian *in vitro* systems

Test system	End point (concentration)	Results		Reference
		+S9	–S9	
V79 cells	HGPRT mutations (0.5–50 μM)	+[c]	NT	Berry *et al.* 1996
Rat hepatocytes	UDS (0.2–5 μM)	NT	+	
Bovine kidney epithelial cells	DNA-intrastrand cross-links (50–500 μM)	NT	+	Hincks *et al.* 1991
	DNA single-strand breaks (50–500 μM)	NT	–	
CHO cells	SCEs (3–300 μg/mL)[a]	+	+	Galloway *et al.* 1987
CHO cells	Chromosomal aberrations (300–600 μg/mL)[b]	+	–	Galloway *et al.* 1987
BALB/c-3T3 cells	Cell transformation (NR)	NT	+	Matthews *et al.* 1993

[a]Dose range 3 to 30 μg/mL (with S9) and 30 to 300 μg/mL (without S9).
[b]Dose range 300 to 498 μg/mL (with S9) and 402 to 600 μg/mL (without S9).
[c] Hepatocyte-mediated.
NR = not reported; NT = not tested, SCE = sister chromatid exchange, UDS = unscheduled DNA repair synthesis.

5.3.3 Mammalian in vivo *systems*

This section presents information from mammalian *in vivo* studies, including studies on unscheduled DNA synthesis (UDS), S-phase synthesis, and micronucleus formation in rats and mice. Studies on mutational frequency in transgenic rats and mutations and gene expression in tumor suppressor genes or oncogenes are discussed in Section 5.4.

The results for UDS, S-phase synthesis, and micronucleus formation in rats and mice are summarized in Table 5-6. Several of the studies cited in this section discuss the same set of genetic toxicology data from the 2- and 13-week prechronic studies conducted by the NTP (1993). Genotoxicity studies related to the prechronic studies include the 5- and 30-day gavage studies in B6C3F$_1$ mice (at doses from 3.3 to 25 mg/kg b.w.) and F344 rats (at doses from 0.33 to 25 mg/kg b.w.) (Mirsalis *et al.* 1993, NTP 1993, Chan *et al.* 1994)

and the 4- (at doses from 3.3 to 25 mg/kg b.w.) and 13-week (at doses from 10 to 25 mg/kg b.w.) gavage studies (NTP 1993, Chan *et al.* 1994, Witt *et al.* 2000).

Mirsalis (1987) reported increased UDS and S-phase synthesis in the hepatocytes of rats (sex and strain not reported) following a single dose of riddelliine at 50 or 100 mg/kg b.w. Nyska *et al.* (2002) examined S-phase synthesis in hepatocytes of male F344 rats given riddelliine at daily doses of 1.0 or 2.5 mg/kg b.w. for eight days or six weeks (30 doses); S-phase synthesis was increased in hepatocytes and liver endothelial cells after eight days and in endothelial cells (but not hepatocytes, which had fewer S-phase nuclei) after six weeks. The NTP (1993) measured UDS and S-phase DNA synthesis in cultured hepatocytes from F344/N and $B6C3F_1$ mice after treatment by gavage for 5 and 30 days (Mirsalis *et al.* 1993, NTP 1993, Chan *et al.* 1994). Similar to Nyska (2002) and Mirsalis (1987) they reported that riddelliine increased S-phase DNA synthesis in rats (both males and females). In $B6C3F_1$ mice, an increase in S-phase synthesis was only observed in male mice at the lowest dose (3.3 mg/kg) after 30 days. The high variability of S-phase synthesis in the female mice prevented the interpretation of the results (NTP 2003).

An increase in UDS was observed in at least one dose group in male rats and male and female mice at both time points and in female rats after 5 days of treatment. The increase was assessed by statistically (Dunn's or Shirley test) comparing the percentage of cells showing evidence of UDS in treated animals compared with the control animals (NTP 2003). Mirsalis *et al.* (1993), analyzing the same data set, concluded that riddelliine did not induce an increase in UDS in rat hepatocytes but did induce an equivocal response in male mice (both time points) and a positive response in female mice (after 30 days). Mirsalis *et al.* (1993) stated that for a UDS response to be considered positive, 20% of cells must be in repair (this is an indication of the extent of the response throughout the liver) and the net grains/nucleus must be greater than zero.

Micronucleated polychromatic erythrocytes (PCEs) were not increased in male or female $B6C3F_1$ mice administered riddelliine orally at doses of up to 25 mg/kg b.w. for 4 to 13 weeks (NTP 1993, Witt *et al.* 2000) or in male or female F344 rats or $B6C3F_1$ mice administered riddelliine orally at doses up to 25 mg/kg b.w. for 5 or 30 days (Mirsalis *et*

al. 1993, Chan *et al.* 1994). However, male B6C3F$_1$ mice administered a single gavage dose of 150 mg/kg b.w. or greater had increased incidences of micronucleated PCEs in peripheral blood and bone marrow (Chen *et al.* 1994). In another study, Swiss mice given a single 70-mg/kg b.w. i.p. dose of riddelliine, had an increased frequency of micronucleated PCEs (MacGregor *et al.* 1985).

Table 5-6. Results of genotoxicity testing of riddelliine in mammalian *in vivo* systems

Test system	Dose (mg/kg b.w.)	LEC	Results	Reference
Unscheduled DNA synthesis				
Rats (sex and strain not reported)	50 and 125; single dose	50	+	Mirsalis 1987
Male and female F344 rat hepatocytes	0.3–3.3; 5 and 30 d[a]	1 (5 d)	– +	Mirsalis *et al.* 1993 NTP 1993, Chan *et al.* 1994[b]
Male and female $B6C3F_1$ mouse hepatocytes	0.33–25; 5 and 30 d	10 (5 d)	+ (F) + (M) equiv (M)	Mirsalis *et al.* 1993, NTP 1993, Chan *et al.* 1994 NTP 1993, Chan *et al.* 1994[b] Mirsalis *et al.* 1993
S-phase synthesis				
Rats (sex and strain not reported)	50 and 125; single dose	50	+	Mirsalis 1987
Male and female F344 rat hepatocytes	0.3–3.3; 5 and 30 days[a]	0.3 (5 and 30 d)	+	Mirsalis *et al.* 1993, NTP 1993, Chan *et al.* 1994
Male and female $B6C3F_1$ mouse hepatocytes	3.3–25; 5 and 30 days	3.3 (30 d)	+/–[c]	Mirsalis *et al.* 1993, NTP 1993, Chan *et al.* 1994
Male F344 rat parenchymal (hepatocytes) and nonparenchymal (endothelial) cells	1.0 and 2.5; 8 or 30 doses	2.5	+[d]	Nyska *et al.* 2002
Micronucleus formation in PCEs				
Male and female F344 rat PCEs	0.3–3.3; 30 days	NAP	–	Mirsalis *et al.* 1993
Male and female $B6C3F_1$ mouse PCEs	3.3–25; 5 or 30 days	NAP	–	Mirsalis *et al.* 1993
Male and female $B6C3F_1$ mouse PCEs	0.3–25; 4 weeks 10–25; 13 weeks 75–300; single dose	NAP NAP 150	– – +	NTP 1993, Chan *et al.* 1994, Witt *et al.* 2000
Swiss mouse (sex not reported) PCEs	70	70	+	MacGregor *et al.* 1985

equiv = equivocal, LEC = lowest effective concentration, NAP = not applicable.

[a]Mirsalis *et al.* (1993) reported the dose for rats in the 5-day feeding study to range from 3.3 to 25 mg/kg b.w.

[b]Mirsalis *et al.* (1993), Chan *et al.* (1994), and NTP (1993) used the same data set, but in some cases interpreted the results differently. NTP (1993) reported a dose-related positive trend for UDS in hepatocytes from female rats treated for 5 days but not 30 days.

[c]NTP (2003) stated that the high variability in S-phase synthesis in control mice in the NTP (1993) study confounded interpretation of the results of that study.

[d]The numbers of S-phase nuclei in hepatocytes were significantly ($P < 0.05$) increased after 8 doses but were significantly ($P < 0.01$) decreased after 30 doses.

5.4 Mechanistic studies and considerations

The mechanisms responsible for PA-induced carcinogenesis in experiment animals are not completely understood, but a number of papers suggest that a genotoxic mechanism is involved. Fu *et al.* (2002b) reviewed three potential mechanisms leading to tumorigenicity in experimental animals: (1) formation of exogenous DNA adducts, (2) formation of endogenous DNA adducts, and (3) formation of DNA-DNA and DNA-protein cross-links. Other investigators have also conducted mechanistic studies for riddelliine. Hong *et al.* (2003) investigated K-*ras* mutations and p53 protein expression in riddelliine-induced hemangiosarcomas, and Nyska *et al.* (2002) examined the role of cytotoxicity, hypoxia, and VEGF-stimulated proliferation. These potential mechanisms are discussed below.

5.4.1 Formation of exogenous DNA adducts

As described in Section 5.2, riddelliine is metabolized to DHP, which can bind DNA, and may be a key step leading to riddelliine's genotoxicity and tumorigenicity. This section describes studies that evaluated the relationship of adducts to mutations and the relationship of adducts to tumors.

Female transgenic Big Blue rats received riddelliine by gavage at a dose of 0.1, 0.3, or 1.0 mg/kg b.w., five days a week for 12 weeks, and were sacrificed one day after the last administration. The DNA from liver endothelial cells was examined. The mutation frequency in the transgenic *cII* gene was determined, and the mutant genes were sequenced (Mei *et al.* 2004a). Riddelliine induced a significant dose-dependent increase in the mean mutation frequency, from 30×10^{-6} in the control group to 103×10^{-6} in the high-dose group. The mutational spectra from the riddelliine-exposed and control rats also differed significantly (Table 5-7), with G·C to T·A transversions predominant in riddelliine-treated rats and G·C to A·T transitions predominant in controls. The authors concluded that riddelliine was genotoxic in rat liver and that the types of mutations induced by riddelliine were consistent with riddelliine-induced formation of DNA adducts involving G·C base pairs.

Table 5-7. Independent *cII* gene mutations in liver endothelial cells of Big Blue rats exposed to riddelliine

Type of mutation	Control		Riddelliine***	
	Number	%	Number	%
G·C → C·G	2	4	4	5
G·C → A·T	30	55	22	26
G·C → T·A	5	9	29	35
A·T → T·A	3	5	4	5
A·T → C·G	3	5	5	6
A·T → G·C	3	5	4	5
Frameshift	8	15	8	10
Complex mutation	1	2	0	0
Total mutants screened	55	100	83	100

Source: Mei *et al.* 2004a.
***Mutational spectra significantly different ($P < 0.001$) from controls by the Adams and Skopek (1987) test.

In another study by Mei *et al.* (2004b), the cell specificity of riddelliine mutagenicity in rat liver was studied in female transgenic Big Blue rats administered riddelliine by gavage at 0.3 mg/kg b.w., five days per week for 12 weeks. This study followed the observation of Chou *et al*. (2003c, 2004) that liver endothelial cells of riddelliine-exposed mice and rats contained higher levels of DNA adducts than did the liver parenchymal cells (hepatocytes), suggesting that the tumor specificity was due to higher levels of DNA damage in the cells that form liver hemangiosarcomas. Mei *et al*. (2004b) collected the collagenase-perfused livers from the rats, separated fractions containing the parenchymal (hepatocytes) and non-parenchymal (mainly endothelial) cells by a series of low-speed centrifugations, and enriched the fractions by Percoll gradient centrifugation. They found that mutagenicity was higher in the non-parenchymal (mainly endothelial cells) than in parenchymal cells. In comparisons between control and riddelliine-exposed rats, the *cII* mutation frequencies differed significantly for endothelial cells, but not for parenchymal cells (see Table 5-8).

DNA sequencing indicated that the riddelliine-induced mutations were primarily G·C to T·A transversions (17%, compared with 9% in the controls); however, in contrast to the findings of Mei *et al.* (2004a), the overall mutational spectra did not differ significantly

between the riddelliine-exposed rats and the controls. The authors concluded that the relatively high mutagenicity of riddelliine in rat liver endothelial cells may be partially responsible for the tumorigenic specificity of this agent (Mei *et al.* 2004b).

Table 5-8. Frequencies of *cII* mutations in the liver cells of Big Blue rats exposed to riddelliine and in non-exposed controls

Group	Cells	Total plaques screened (× 10^3)	Total mutant plaques	Mutation frequency (× 10^{-6}) mean ± SD[a]
Control	parenchymal	1,019	34	35.2 ± 5.7
	endothelial	1,054	41	39.5 ± 3.8
Riddelliine	parenchymal	1,374	55	37.5 ± 9.3
	endothelial	788	50	67.0 ± 17.1*

Source: Mei *et al.* 2004b.
[a]The means were based on 3 replicates.
*Significantly different ($P < 0.05$) from the control group by ANOVA followed by the Holm-Sidak test.

DHP-derived DNA adduct levels were measured in purified rat and mouse liver endothelial cells (the cells of origin for liver hemangiosarcoma) to examine the relationship between DNA adduct levels and the incidence of liver hemangiosarcoma (Chou *et al.* 2003c, 2004). F344 rats and B6C3F_1 mice were given riddelliine by gavage, five days per week for two weeks, at 1.0 mg/kg b.w. for rats and 3.0 mg/kg b.w for mice. On days 1, 3, 7, and 28 after the last dose, liver parenchymal and endothelial cell fractions were isolated, and DHP-derived DNA adduct levels were determined by ^{32}P-postlabeling/HPLC. Eight adducts were detected in DNA isolated from both cell types. The adduct profile was very similar to that obtained when DHP was reacted with calf thymus DNA (see Figure 5-3). Similar adduct patterns were obtained with both sexes of both species. Peak adduct levels occurred 3 days after the last dose in all cases. Adduct levels were higher in endothelial cells than in parenchymal cells (hepatocytes) at all time points (Figure 5-6) and were higher in rat endothelial cells than in mouse endothelial cells even though mice were exposed to a higher dose. In addition, adducts were 2.1- to 3.6-fold more persistent in endothelial cells than in parenchymal cells for both rats and mice. However, adduct persistence was greater in rats than mice and was greater in females than in males. The adduct pattern in rats (levels and persistence) is consistent with the preferential induction of liver hemangiosarcoma as opposed to hepatocellular adenoma. A similar pattern, but lower adduct levels, was observed in mice and is consistent with

the lower tumor incidence in mice compared with rats. Peak adduct levels were higher in male mice but persistence was greater in female mice. Hemangiosarcoma incidence was increased only in male mice (see Section 4.1); [therefore, these data suggest that peak adduct levels may be more important than persistence in tumorigenesis].

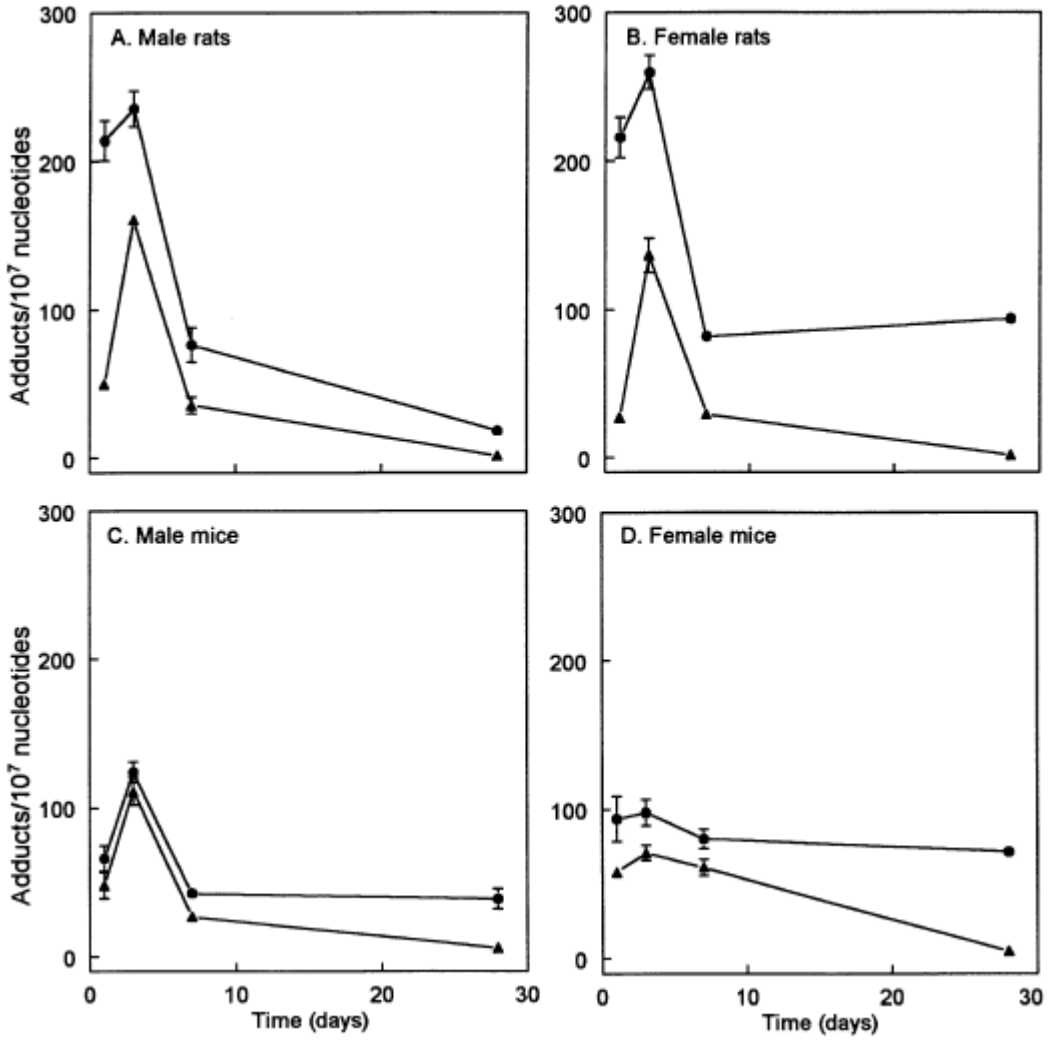

Figure 5-6. DHP-derived DNA adduct levels in the livers of F344 rats and B6C3F$_1$ mice

DNA adduct levels were determined 1, 3, 7, and 28 days after the last treatment of rats and mice by gavage with 1.0 mg/kg b.w. (rats) or 3.0 mg/kg b.w. (mice) of riddelliine. The data are presented as the mean ± s.e.m. of 3 or 4 animals per time point. Filled circle = endothelial cells (upper curve in each panel); filled triangle = parenchymal cells (hepatocytes) (lower curve in each panel).

Source: Chou *et al.* 2003c, 2004, used with permission.

[Studies of riddelliine metabolism in rat and human liver microsomes and findings of dose-related riddelliine-induced cell-specific adduct formation in liver DNA suggest that

DHP-derived DNA adduct formation may be a step in the mechanism of tumorigenicity.] As previously discussed, riddelliine metabolism in human microsomes, the pathways, DNA adduct profiles, and metabolizing enzymes are very similar to those observed in rat liver *in vitro* and *in vivo* (Yang *et al.* 2001a). [Because riddelliine induced hemangiosarcomas in the liver of male and female rats and male mice (Chan *et al.* 1994, Chan *et al.* 2003) and DHP-derived DNA adducts may be a step in hemangiosarcoma induction, the results for human liver microsome metabolism suggest that riddelliine can be highly genotoxic to humans and that the genotoxic mechanism may be mediated by the DHP-derived DNA adducts. However, the relationship between DNA adduct levels and the incidence of hepatocellular tumors is not entirely consistent. For example, in mice, DNA adducts also were measured in parenchymal cells at a dose of 3.0 mg/kg b.w. (see Figure 5-7), but the incidence of hepatocellular neoplasms at this dose was decreased compared with controls (see Table 4-1)].

5.4.2 Formation of endogenous DNA adducts

Fu *et al.* (2002b) reported that there is some evidence that secondary mechanisms may be involved in PA-induced toxicity and tumorigenicity. *trans*-4-Hydroxy-2-hexanal has been identified as a metabolite of senecionine (Segall *et al.* 1985) but not riddelliine (Fu *et al.* 2002b). *trans*-4-Hydroxy-2-hexanal is highly toxic and may be formed from enzymatic cleavage or from senecionine-induced lipid peroxidation. Furthermore, other α,β-aldehydes are mutagenic, form DNA adducts, and are carcinogenic. Miranda *et al.* (1981, 1982) found that the antioxidant, butylated hydroxyanisole, protected mice from the acute toxic effects of monocrotaline, and protected rats from the chronic toxic effects of mixed PAs derived from *S. jacobaea*. Fu *et al.* concluded that these findings suggest that lipid peroxidation and formation of endogenous DNA adducts may be involved in PA-induced toxicity and tumorigenicity.

5.4.3 Formation of DNA-DNA and DNA-protein cross-links

DNA-DNA and DNA-protein cross-links formed by PAs have been proposed to contribute to the toxic, carcinogenic, and anti-carcinogenic actions of these compounds (Kim *et al.* 1995). Although formation of cross-links has been demonstrated for riddelliine and a number of other PAs in *in vitro* studies (see Sections 5.3.2 and 5.5.3),

confirmation of this mechanism for the tumorigenicity of PAs is not available (Fu *et al.* 2002b)

*5.4.4 Beta-catenin and p53 protein expression and K-*ras *and* beta-catenin *gene mutations*

Hong *et al.* (2003) examined 12 riddelliine-induced hemangiosarcomas in the liver from a two-year diet study in mice and 15 spontaneous subcutaneous hemangiosarcomas for alterations in the genes for the K-*ras* and beta-catenin proteins and expression of the beta-catenin and p53 proteins. Of the 12 riddelliine-induced hemangiosarcomas in the liver, 7 (58%) had K-*ras* codon 12 GTT mutations, and 9 (75%) showed strong staining for p53 protein in malignant endothelial cells (the cells of origin for hemangiosarcomas). No beta-catenin protein was detected in riddelliine-induced hemangiosarcomas in the liver, and no genetic alterations in the *beta-catenin* gene were found. Spontaneous liver hemangiosarcomas from control mice lacked both detectable p53 and beta-catenin protein expression and K-*ras* mutations. The authors concluded that K-*ras* mutations and p53 protein expression in riddelliine-induced hemangiosarcomas in the liver most likely resulted from the chemical's genotoxic effects. Nyska *et al.* (2002) detected increased p53 protein expression by immunohistochemistry in endothelial cells in the liver of male F344 rats given riddelliine at a daily dose of 1.0 or 2.5 mg/kg b.w. for six weeks (30 doses) (see Section 5.3.5 for a description of other endpoints measured in this study).

5.4.5 Endothelial-cell proliferation

Nyska *et al.* (2002) proposed a potential mechanism for the pathogenesis of hemangiosarcoma in the liver of animals exposed to riddelliine. As illustrated in Figure 5-7, the riddelliine metabolite dehydroretronecine interacts with DNA in endothelial cells, resulting in cellular damage to these cells. The ensuing nuclear and cytoplasmic enlargement of endothelial cells causes sinusoidal obstruction and local hypoxia, which in turn stimulates vascular endothelial growth factor (VEGF) synthesis by anoxic hepatocytes. The VEGF-stimulated proliferation of endothelial cells could result in "fixation" of the DNA adducts into mutations, leading to development of hemangiosarcoma. VEGF is a specific and effective growth factor for stimulation of endothelial-cell function in vasculogenesis and angiogenesis and has been implicated as a major factor in malignant endothelial-cell transformation in the development of

angiosarcoma (Moyer *et al.* 2004). Smith *et al.* (2004) applied a predictive mathematical model to data taken from riddelliine-exposed rats in the Nyska *et al.* (2002) study. Replication and apoptotic rates were estimated and compared for hepatocytes and endothelial cells. The estimated replication rates were found to be significantly higher for endothelial cells, thus supporting the proposed mechanism described by Nyska *et al.* (2002).

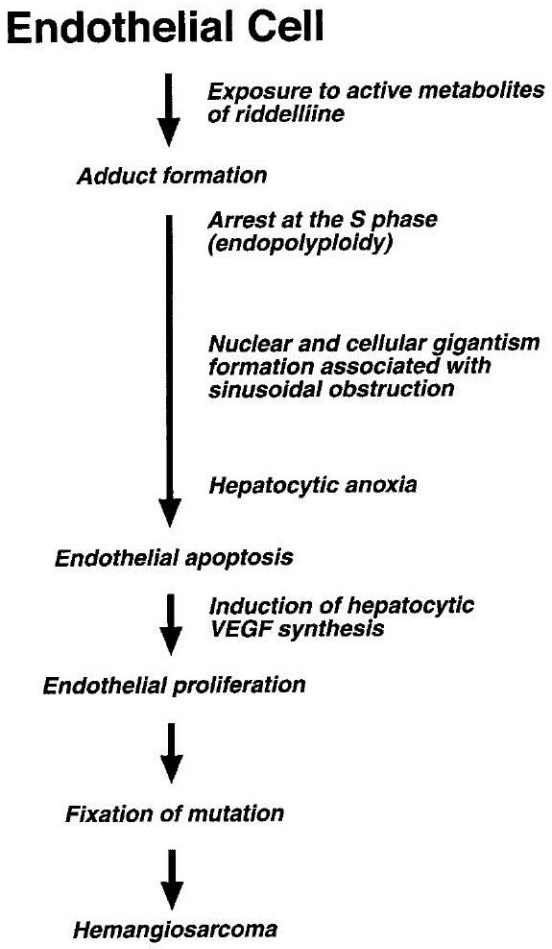

Figure 5-7. Proposed mechanism for induction of liver hemangiosarcoma by riddelliine in rats
Source: Nyska *et al.* 2002.

The proposed model was based on the findings from the Nyska *et al.* study, and supported by the study by Moyer *et al.* Both reports were based on F344 male rats exposed by daily gavage to vehicle (corn oil) or 1.0 or 2.5 mg/kg b.w. per day of

riddelliine for either 8 consecutive days or 30 days (5 doses/week, excluding weekends, for 6 weeks). The Nyska *et al.* study demonstrated that the riddelliine exposure (based on a comparison of animals exposed to 30 doses of riddelliine with untreated animals) is associated with specific damage to hepatic endothelial cells, including, karyomegaly, cytomegaly, decreased apoptosis, increased mitosis, and more S-phase nuclei, and *p53* mutation (as assessed by immunopositivity). Hepatocytes from riddelliine-exposed animals had increased hypertrophy, fatty degeneration, decreased apoptosis, fewer S-phase nuclei and reduced mitosis, and expressed higher VEGF immunopositivity compared with controls. The endothelial proliferation and eventual mutation and hemangiosarcoma development were proposed to be promoted through VEGF induction.

Moyer *et al.* expanded on the role of VEGF expression in hepatocytes and found that although VEGF mRNA expression occurred in the hepatocytes of both control and treatment groups, qualitative differences were noted. VEGF expression in treated animals occurred in clustered, focal hepatocytes and bile duct epithelium, while VEGF mRNA expression in controls was distributed evenly across all hepatocytes. They also reported that hepatic sinusoidal endothelial cells expressed the high affinity tyrosine receptor VEGFR2 receptor (KDR/flk-1; kinase domain region [KDR] in the human, and fetal liver kinase-1 [flk-1] in rodents), and immunohistochemical detection of phosphorylation of specific tyrosine residues of KDR/flk-1 was consistent with activation of the receptor. The authors proposed that riddelliine damages both hepatocytes and endothelial cells resulting in dysregulated VEGF synthesis by hepatocytes and activation of KDR/flk-1 in endothelium, leading to sustained endothelial-cell proliferation and development of hepatic hemangiosarcoma.

5.5 Carcinogenicity, genotoxicity, and structure-activity relationships of riddelliine metabolites and analogues

It is beyond the scope of this document to conduct a complete literature review of the carcinogenic and genotoxic effects of riddelliine metabolites and analogues; therefore, this section provides a brief overview of these effects and illustrates the similarity with riddelliine. Carcinogenicity and genotoxicity data were available for several riddelliine metabolites and a number of analogues. In addition, extracts from various plants known to contain PAs have been tested for genotoxic effects. The chemical structures of the metabolites and many of the analogues discussed in this section are provided in Sections 1.3 and 1.4.

5.5.1 Carcinogenicity

The carcinogenicity of DHP, which is a racemic mixture of *R*-DHP and *S*-DHP, is summarized in Section 4.4. *R*-DHP has been shown to induce rhabdomyosarcoma and skin tumors in rats (Allen *et al.* 1975, Shumaker *et al.* 1976, Johnson *et al.* 1978, Mattocks and Cabral 1982), and limited data have shown a possible association between *S*-DHP and total tumors in rats (Peterson *et al.* 1983). A single spinal cord tumor was reported in one of ten rats injected with retronecine as newborns (Schoental and Cavanagh 1972) but the study lacked controls, and no other CNS tumors have been reported for riddelliine metabolites. Schoental and Cavanagh also reported 5 pituitary tumors and 1 mammary tumor in female rats from the same litter.

Other PAs also share the reactive metabolite DHP in common with riddelliine (see Section 5.1.3). Studies in which rats were exposed to other PAs have shown liver tumors to be the most common tumor type; however, neoplastic responses also were reported for other organs, including tumors of the CNS, lung, bladder, pancreas, skin, testes, pituitary, and adrenal gland (Table 5-9). Campbell (1956) reported that liver tumors developed in 6 of 18 chickens that received weekly i.v. injections of seneciphylline hydrochloride at 20 to 35 mg/kg b.w. for up to 8 weeks. Chickens fed a protein- and choline-deficient diet did not show a greater tendency to develop liver tumors.

Table 5-9. Neoplastic lesions observed in rats exposed to various PAs other than riddelliine or plants containing these PAs

PA or plant	Tumor types	References (route of administration)
Heliotrine	Pancreatic islet cell tumor, hepatoma, testicular tumor	Schoental 1975 (gavage)
Heliotropium ramosissimum (Heliotrine)	Spinal cord tumor	Schoental and Cavanagh 1972 (feed)
Heliotropium supinum (PAs not reported)	Renal lipomatous tumor	Schoental *et al.* 1971 (gavage)
Lasiocarpine	Liver tumor (including carcinoma), skin tumor (including carcinoma), pulmonary adenoma, intestinal tumor (including carcinoma)	Svoboda and Reddy 1972 (i.p.) Svoboda and Reddy 1974 (i.p.) Rao and Reddy 1978 (feed) Rao *et al.* 1983 (feed)
Clivorine	Hemangioendothelial sarcoma[a], liver adenoma, testicular interstitial-cell tumor	Kuhara *et al.* 1980 (drinking water)
Hydroxysenkirkine	Cerebral tumor	Schoental and Cavanagh 1972 (i.p.)
Petasitenine	Liver hemangioendothelial sarcoma, liver adenoma	Hirono *et al.* 1977 (drinking water)
Farfugium japonicum (petasitenine & senkirkine)	Liver hemangioendothelial sarcoma, liver adenoma, adrenal cortical adenoma, pheochromocytoma, urinary bladder papilloma, testicular interstitial-cell tumor	Hirono *et al.* 1983 (feed)
Senkirkine	Liver adenoma	Hirono *et al.* 1979 (i.p.)
Tussilago farfara (common name is coltsfoot) (senkirkine)	Liver hemangioendothelial sarcoma, liver tumor (including carcinoma), urinary bladder papilloma	Hirono *et al.* 1976 (feed)
Senecio cannabifolius (seneciphylline, acozine & senecicannabine)	Liver hemangioendothelial sarcoma, liver adenoma, adrenal cortical adenoma, pheochromocytoma, testicular interstitial-cell tumor, pituitary adenoma	Hirono *et al.* 1983 (feed)
Amsinckia intermedia (intermedine & lycopsamine)	Islet cell tumor (including adenocarcinoma), bladder papillary tumor, renal lipomatous tumor, uterine tumor	Schoental *et al.* 1970 (gavage) Schoental *et al.* 1971 (feed)
Senecio jacobaea extract (jacobine, jacodine & jaconine)	Liver tumor	Cook *et al.* 1950 (drinking water) Schoental *et al.* 1954 (drinking water)

Monocrotaline	Liver tumor (including carcinoma), pulmonary adenoma, adrenal adenoma, renal adenoma, rhabdomyosarcoma, leukemia	Allen *et al.* 1975 (s.c) Shumaker *et al.* 1976 (s.c) Newberne and Rogers 1973 (gavage)
Retrorsine	Liver tumor (including carcinoma)	Schoental *et al.* 1954 (drinking water) Schoental 1957 (drinking water) Schoental *et al.* 1971 (gavage)
Senecio longilobus (retrorsine)	Liver tumor (including carcinoma)	Harris and Chen 1970 (feed)
Retrorsine *N*-oxide) (also known as isatidine)	Liver tumor (including carcinoma)	Schoental *et al.* 1954 (drinking water) Schoental 1957 (drinking water)
Symphytine	Liver tumor (including hemangioendothelial sarcoma)	Hirono *et al.* 1979 (i.p.)
Symphytum offcinale (common name is comfrey) (symphytine)	Liver tumor[b]	Hirono *et al.* 1978 (feed)

[a]Hemangioendothelial sarcoma is an alternative name for hemangiosarcoma.
[b]Urinary bladder tumors also developed but the authors could not draw any conclusions because one control had a tumor as well.

5.5.2 Genotoxicity

The data reviewed indicate that the genotoxic effects of riddelliine metabolites and analogues are similar to those reported for riddelliine. Rat liver microsomes converted riddelliine *N*-oxide to the genotoxic DHP metabolite, and incubation of rat liver microsomes with riddelliine *N*-oxide in the presence of calf thymus DNA produced the same set of DHP-derived DNA adduct peaks found in liver DNA of F344 rats fed riddelliine or the *N*-oxide (Chou *et al.* 2003a, 2003c, 2004). In rats given riddelliine *N*-oxide at 1.0 mg/kg b.w. for three consecutive days, the level of DNA adducts was 39.9 ± 0.6 per 10^7 nucleotides, which was lower by a factor of 2.6 than in rats given the same dose of riddelliine. These results indicate that riddelliine *N*-oxide, through its conversion to riddelliine, is a potential genotoxic carcinogen. The riddelliine metabolite, DHP, can bind to calf thymus DNA to form DHP-modified DNA adducts (Yang *et al.* 2001a). The DHP enantiomer, *R*-DHP, also was reported to be mutagenic in *S. typhimurium*, to induce sister chromatid exchange in human lymphocytes without exogenous metabolic activation, and to induce DNA-DNA and DNA-protein cross-links (IARC 2002).

There are hundreds of riddelliine analogues; therefore, as mentioned above, a complete review of the genetic toxicology of these compounds is beyond the scope of this document. However, many of the PAs are metabolically activated to a common metabolite, DHP, which forms DNA adducts and cross-links (see Sections 5.3). For example, Chou and Fu (2006) detected DHP-derived DNA adducts in female Sprague-Dawley rats exposed to various PA-containing plants or extracts (e.g., comfrey root extract, comfrey compound oil, coltsfoot root extract) for 3 consecutive days. Fu *et al.* (2004) reviewed the metabolism and toxicity of the PAs and reported a variety of genotoxic effects, including DNA binding, DNA cross-linking, DNA-protein cross-linking, sister chromatid exchange, chromosomal aberrations, micronuclei and mutagenic effects in *Salmonella typhimurium* and *Drosophila melanogaster*. Mutagenic effects have been reported both for PA-containing plant extracts and for pure PAs. Several different PAs induced reverse mutations in *S. typhimurium* TA100 in the presence of metabolic activation.

IARC (1976, 1983) reported a number of genetic and related effects of other PAs (hydroxysenkirkine, isatidine, jacobine, lasiocarpine, monocrotaline, petasitenine, retrorsine, seneciphylline, senkirkine, and symphytine) including induction of mutations in mammalian cells *in vitro*, induction of recessive sex-linked lethal mutations in *D. melanogaster*, induction of several types of suppression mutations in *Aspergillus nidulans*, inhibition of DNA synthesis in rat liver, cross-linking of DNA *in vitro*, unscheduled DNA synthesis in rat hepatocytes and transformed cryopreserved hamster embryo cells, and chromosomal aberrations and forward mutations to 8-azaguanine resistance in V79 Chinese hamster cells.

5.5.3 Structure-activity relationships for genotoxicity, tumorigenicity, and toxicity of PAs

Several studies have examined the structure-activity relationships among the PAs. Frei *et al.* (1992) investigated the genotoxic potencies of 16 PAs in the wing-spot test of *D. melanogaster*. The PAs tested did not include riddelliine but did include several other macrocyclic diester-type PAs (senecionine, retrorsine, jacoline, seneciphylline, monocrotaline, and senkirkine), as well as several open diester and monoester types. Genotoxicity varied widely, but in general, the macrocyclic diester types were the most

genotoxic, and the monoester types were the least genotoxic. There was a good correlation between hepatotoxicity in rodent studies and genotoxicity in the wing-spot test which suggests that PAs are bioactivated along similar pathways in the mammalian liver and somatic cells in *Drosophila*. There also was an apparent correlation between the genotoxic potential in the wing-spot test and the carcinogenic potential in mammals.

Fu *et al.* (2002b) reviewed mechanisms leading to genotoxicity and tumorigenicity of PAs of the retronecine, heliotridine, and otonecine types. They noted that the base of platynecine-type PAs does not contain a double bond as in the other types listed above, and the platynecine-type PAs are not genotoxic.

Kim *et al.* (1993) also reported that macrocyclic PAs with α,β–unsaturation (riddelliine, seneciphylline, senecionine, and retrorsine) showed a dose-dependent inhibition of colony formation (50 to 300 μM) and induced megalocytosis at 500 μM in cultured bovine kidney epithelial cells. Megalocytes are common in livers of PA-exposed animals. Saturated macrocyclic diesters and open diesters induced a slight inhibition of colony formation but had no effect on cellular morphology.

The toxicity of PAs has been attributed to their ability to form DNA cross-links (Kim *et al.* 1995). Hincks *et al.* (1991) compared the ability of eight PAs, representing three major structural classes (macrocyclic diesters, open diesters, and the necine base), to cross-link cellular DNA in cultured Madin-Darby bovine kidney epithelial cells. Cells were exposed to the PAs (50 to 500 μM) for 2 hours in the presence of an external metabolizing system (rat liver S9). All PAs induced DNA cross-links, most of which were DNA-DNA cross-links.The rank order of DNA cross-linking was seneciphylline > riddelliine > retrorsine > senecionine > heliosupine > monocrotaline > latifoline > retronecine.

In a similar experiment, DNA cross-linking activity of chemically activated PAs from four different structural classes (α,β–unsaturated macrocyclic diesters, α,β–saturated macrocyclic diesters, necine base, and *N*-oxides) was investigated in Madin-Darby bovine kidney cells (Kim *et al.* 1999). Cells were treated with 500 μM activated pyrroles or *N*-oxide for 2 hours. Cross-links were determined by alkaline elution, and the extent of

protein involvement in cross-linking was determined by proteinase treatment. [DNA-DNA cross-links are proteinase K resistant. The elution of labeled DNA will increase if proteins are involved in cross-linking.]. The unsaturated macrocyclic diester pyrroles (dehydroriddelliine and dehydrosenecionine), and the saturated macrocyclic diester pyrrole (dehydromonocrotaline) formed significantly more cross-links in cell culture than retronecine or indicine *N*-oxide. The rank order for DNA cross-linking potency was dehydrosenecionine > dehydroriddelliine = dehydromonocrotaline > dehydroretronecine > indicine *N*-oxide. The proportion of total cross-links that were DNA-DNA cross-links was 67%, 53%, 36%, and 8% for dehydrosenecionine, dehydromonocrotaline, dehydroriddelliine, and dehydroretronecine, respectively. Proteinase K-resistant cross-links were not detectable for indicine *N*-oxide.

Kim *et al.* reported that there appeared to be "some correlation between the rank order of cross-linking and animal toxicity." No statistical analysis of correlation was reported, but the authors noted that senecionine, the parent compound of the potent cross-linker dehydrosenecionine, had three to six times the acute toxicity in rats compared with monocrotaline, the parent compound of dehydromonocrotaline, which was less potent as a cross-linker. In a study of porcine pulmonary artery endothelial cells exposed to monocrotaline pyrrole (dehydromonocrotaline) *in vitro*, cross-links were formed in a dose-dependent manner that the authors considered consistent with monocrotaline's ability to inhibit cell proliferation (Wagner *et al.* 1993). The formation of DNA-DNA and DNA-protein cross-links increased dose dependently at 4 hours post administration in male Sprague-Dawley rats exposed to monocrotaline or jacobine *in vivo* by i.p. injection, but the DNA-DNA intrastrand cross-links returned to basal levels by 96 hours after injection (Petry *et al.* 1984, 1986).

5.6 Toxicity

5.6.1 Human toxicity

In humans, both acute and chronic toxicity has occurred from ingesting foods contaminated with PAs, particularly herbal products (see Section 2.3.2) and grains and flours (see Section 2.3.3) (Selzer and Parker 1951, Tandon *et al.* 1978, Culvenor 1983, Huxtable 1989a, Mayer and Luthy 1993, Steenkamp *et al.* 2000, Conradie *et al.* 2005).

The available data are consistent with the animal data and indicate that the liver is the primary target organ. A common lesion is occlusion of the central and sublobular hepatic veins resulting in veno-occlusive disease (Rietjens *et al.* 2005). Veno-occlusive disease was first described in the 1950s in Jamaican children with centrilobular cirrhosis (Bras *et al.* 1954, Rollins 1986). These children experienced sudden onset of right upper quadrant pain, enlarged liver, and ascites. Liver biopsies revealed sublobular venous occlusion by intimal proliferation and fibrosis with an absence of thrombotic occlusion. Further investigation revealed that these children had a history of ingesting a tea known as "bush tea" made from local plants. The bush teas were made from leaves of *Crotalaria* or *Senecio* and contained PAs (Huxtable 1989a). Other symptoms of PA poisoning may include weakness, abdominal pain and swelling, diarrhea, vomiting, hepatomegaly, and ascites (Stewart and Steenkamp 2001).

Veno-occlusive disease was also reported in two infants (a 2-month-old boy and a 6-month-old girl) in the United States who had consumed herbal tea prepared from *S. longilobus*, a plant known to contain PAs, including riddelliine, seneciphylline, senecionine, and retrorsine. The 2-month-old boy developed ascites, splenomegaly, hepatomegaly, and centrilobular hepatic necrosis and died after 6 days in the hospital. The 6-month-old girl initially showed signs of recovery but developed extensive liver fibrosis after 2 months and cirrhosis after 8 months.

As reviewed in Section 2.3.3, contamination of wheat with the seeds of *Heliotropium popovii* has resulted in large outbreaks of veno-occlusive disease in Afghanistan (7,800 cases) and Tajikistan (3,906 cases) (Tandon *et al.* 1978, Mayer and Luthy 1993). Veno-occlusive disease has also consistently been associated with ingestion of comfrey teas (Ridker *et al.* 1985, Weston *et al.* 1987, Bach *et al.* 1989, McDermott and Ridker 1990). In 20 cases of veno-occlusive disease in South African children thought to be caused by exposure to traditional remedies (see Section 2.3.2), Steenkamp *et al.* (2000) confirmed the presence of PAs in the urine of 4 children for whom an on-admission urine specimen was available. Also in South Africa, retrorsine was determined to be present in the traditional herbal remedies administered to two sets of twin infants (a boy and a girl in each set) with veno-occlusive liver disease (Conradie *et al.* 2005).

At least one case of human embryotoxicity has been reported (Roulet *et al.* 1988). In this case, the mother drank one cup of herbal tea daily throughout her pregnancy. The tea contained 0.6 mg senecionine per kg dry weight. The mother showed no signs of toxicity; however, the infant was born with fatal veno-occlusive disease. Toxicity is exacerbated by chronic, small doses, and infants are particularly susceptible. Mild cases of poisoning may resolve without long-term sequelae; however, in severe cases, liver failure from cirrhosis and veno-occlusive disease commonly occurs months to years after exposure. Culvenor (1983) estimated that a daily dose of > 1 mg/day for 2 weeks, or > 0.1 mg/day for longer periods could cause liver disease in humans.

5.6.2 Animal toxicity

Riddelliine and other PAs are toxic to farm animals, causing liver disease in cattle, and "walking disease" in horses, characterized by aimless wandering and cirrhosis of the liver (Johnson *et al.* 1985b). Several investigators have reported on the toxic effects in cattle or horses (Vardiman 1952, Cheeke 1984, Johnson and Molyneux 1984, Johnson *et al.* 1985b, Molyneux *et al.* 1988, Craig *et al.* 1991, Molyneux *et al.* 1991), and sheep or goats (Harris *et al.* 1957, Cheeke 1984). Chronic terminal hepatopathy may develop in cattle and horses after consuming 5% to 10% of their body weight in PA-containing plants (Lodge-Ivey *et al.* 2005).

The toxicity of riddelliine also has been demonstrated in experimental studies with exposure of calves to riddelliine-containing plants. *S. riddellii* produced typical signs of PA-induced liver damage when fed to calves at a daily total alkaloid dose of 15 mg/kg b.w. in the feed for 20 days (Johnson *et al.* 1985b). Molyneux *et al*. (1988) also reported liver damage in a calf fed dried *S. riddellii* leaves mixed in chopped alfalfa hay providing 30 mg/kg b.w. riddelliine to the animal for three 20-day periods interspersed with 30- and 60-day nonexposure periods.

In another study, both liver damage and pulmonary edema occurred when calves were administered 45 mg/kg b.w. of PAs (4.5 mg/kg of riddelliine and 40.5 mg/kg of riddelliine-*N*-oxide) in the feed for 20 days (Molyneux *et al.* 1991). Calves fed tansy ragwort, either continuously or for 60 days followed by a return to normal feed, developed terminal hepatopathy with the onset of a moribund state or sudden death at 11

to 17 weeks and 27 to 51 weeks, respectively (Craig *et al.* 1991). Johnson and Molyneux (1984) fed cattle threadleaf groundsel (*S. longilobus*) by gavage, mixed in alfalfa hay, or pelleted in feed. The minimum lethal dose in cattle that were dosed by gavage was approximately 200 mg of PAs per kg b.w. in a 15-day period (13 mg PAs/kg per day), while cattle that consumed up to 600 mg of PA per kg in hay or pellets for 20- to 100-day periods were not affected or were minimally affected.

Species differences in sensitivity to PA toxicity have been related to differences in metabolic activation of the PAs to their corresponding pyrrole metabolites. Sheep, guinea-pigs, rabbits, gerbils, and hamsters are resistant, whereas rats, cattle, horses, and chickens are highly susceptible (Cheeke and Pierson-Goeger 1983, Cheeke 1984, Rietjens *et al.* 2005). Lodge-Ivey *et al.* (2005) reported that a consortium of bacteria isolated from the rumen of sheep were capable of detoxifying PAs found in *S. jacobaea*, and this is believed to be a primary protective factor against PA toxicity in sheep. Japanese quail (Buckmaster *et al.* 1977) and rabbits (Pierson *et al.* 1977) were resistant to chronic intoxication when fed *S. jacobaea* but were susceptible to injected PAs. No mortality occurred in Japanese quail fed a diet containing 10% *S. jacobaea* for up to one year; however, changes in liver histology were noted (Buckmaster *et al.* 1977). The LD_{50} of i.p. injected *Senecio* alkaloid was 115 mg/kg in quail. Eggs from quail hens were fertile and yielded normal chicks. No gross lesions or changes in serum protein levels occurred in rabbits fed *S. jacobaea* for 263 days; however, microscopic changes in the liver were observed (Pierson *et al.* 1977). Two rabbits injected with 150 mg PA per kg died in less than 24 hours.

As discussed in Section 4.3, exposure of laboratory animals to riddelliine increased the incidences of liver, kidney, and spleen lesions in rats and mice and bone marrow, lung, stomach, and lymph node lesions in rats (NTP 2003). After the liver, the lungs are the next most common site of toxic action of PAs in experimental animals, but not all PAs affect the lungs (Mattocks 1986). *Crotalaria spp.* are generally pneumotoxic in horses and pigs, but *C. retusa* has been reported to produce only hepatic disease in horses (Hooper 1978). In contrast, *Senecio spp.* are primarily hepatotoxic, but *S. jacobaea* can produce pulmonary disease in pigs. As in the liver, lung damage is caused by the pyrrolic

ester metabolites, and the primary site of damage is the pulmonary vasculature. Eleven-membered macrocyclic diesters such as monocrotaline are known to be particularly active in the lungs; however, hepatic activation is required in order for lung injury to occur (Wilson *et al.* 1992). Monocrotaline pyrrole caused pulmonary vascular damage, pulmonary hypertension, and right ventricular hypertrophy in rats (Ganey *et al.* 1986, Ganey *et al.* 1988).

Some studies have reported that pulmonary lesions in rats were observed at doses that were equal to or greater than the doses required to induce liver damage (Mattocks 1986); however, others have reported that chronic exposure to lower doses of monocrotaline has caused pulmonary damage in the absence of hepatotoxicity (NTP 2003). Monocrotaline also has caused pulmonary arterial hypertension and right ventricular hypertrophy in non-human primates, but not in humans (Stewart and Steenkamp 2001). The mechanism of pulmonary toxicity is thought to involve delivery of long-lived pyrrole metabolites to the lungs by erythrocytes (Wilson *et al.* 1992).

Some data suggest that male rats and mice may be more sensitive to riddelliine toxicity than females (NTP 2003). However, no sex-related differences were observed in the kinetics of two metabolic pathways, *N*-oxidation and DHP formation (Williams *et al.* 2002), indicating that other factors may be responsible for the observed sex difference in tumorigenicity, including formation of the toxic metabolites, such as the pyrrolic ester, bound pyrroles, and DHP-derived DNA adducts, which are believed to directly cause toxicity. In rats, Yan *et al.* (2002) found levels of DHP-derived DNA adducts in the blood 48 to 168 hours after riddelliine administration to be 4-fold higher in females than in males.

5.7 Summary

5.7.1 Absorption, distribution, metabolism, and excretion

Riddelliine and other PAs are absorbed primarily via ingestion (though dermal absorption can occur), distributed to the liver, and excreted in the urine and feces. Riddelliine has three primary metabolic pathways: (1) hydrolysis of the ester group(s) to form the necine base, (2) oxidation of the necine base (of riddelliine) to the corresponding *N*-oxide (which

may be reduced to riddelliine), and (3) hydroxylation of the necine base (of riddelliine), followed by dehydration to form the corresponding dehydroriddelliine (pyrrolic) derivative. This pyrrolic derivative is then hydrolyzed to form the racemic (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine (DHP), which is a 50/50 mixture of the optically pure *R*- and *S*- enantiomers. Metabolism of PAs to the reactive pyrrolic ester metabolites in rodents and humans is mainly catalyzed by CYP3A and CYP2B6 isozymes of cytochrome P450. Metabolism of PAs to the corresponding *N*-oxides is catalyzed by both cytochrome P450 and flavin-containing monooxygenase.

5.7.2 DHP adducts

DHP can bind to DNA, which may be a key step leading to its genotoxicity and tumorigenicity. A set of eight DHP-derived adduct peaks has been detected in DNA reacted with riddelliine in the presence of rat microsomes. Dose-dependent DHP adduct formation has also been detected in livers of rats and mice exposed to riddelliine. Adduct levels were higher in endothelial cells than in parenchymal cells in rats and were more persistent in endothelial cells than in parenchymal cells in both rats and mice. The kinetic parameters (V_{max} and K_m) for formation of DHP are comparable in rat and human microsomes, and the same profile of DHP adduct peaks is detected, demonstrating that this pathway occurs in humans.

5.7.3 Genetic damage and related effects

Riddelliine induced mutations in a *S. typhimurium* strain (TA100) that detects base-pair substitutions (in the presence of metabolic activation) but not in three other *S. typhimurium* strains that detect frameshift mutations (with or without metabolic activation). In addition to mutations, riddelliine also induced other types of genetic damage in mammalian experimental studies. *In vitro*, riddelliine increased the frequency of sister chromatid exchange and chromosomal aberrations in Chinese hamster ovary cells, cell transformation in BALB/c-3T3 fibroblast cells, and DNA cross-linking, but not DNA strand breaks in bovine kidney epithelial cells. In rats exposed *in vivo*, riddelliine induced S-phase synthesis in hepatocytes and endothelial cells and increased p53 expression in endothelial cells but did not induce micronucleus formation in polychromatic erythrocytes. In mice, riddelliine caused unscheduled hepatocyte DNA

synthesis (in females only), but did not induce micronucleus formation. Mutations in the k-*ras* gene and *p53* gene expression were detected in hemangiosarcomas from mice treated with riddelliine.

5.7.4 Mechanistic studies and considerations

Formation of DHP-DNA adducts by riddelliine may be a key step leading to its genotoxicity and tumorigenicity. In addition to the formation of exogenous (DHP-DNA) adducts, the formation of endogenous DNA adducts and formation of DNA-DNA and DNA-protein cross-links have also been proposed as mechanisms of tumorigenicity.

The genotoxic effects of riddelliine have been reported to be greater in liver endothelial cells, the cells that form liver hemangiosarcomas, compared with liver parenchymal cells (hepatocytes). Liver endothelial cells of riddelliine-exposed mice and rats contained higher levels of DNA adducts than did the parenchymal cells. In addition, riddelliine induced a higher frequency of mutations in non-neoplastic endothelial cells (but not in parenchymal cells) in the *cII* gene mutation assay in transgenic Big Blue rats. The predominant mutations observed were G·C to T·A transversions, which are consistent with riddelliine-induced formation of DNA adducts involving G·C base pairs.

The apparent endothelial cell-specific toxicity of riddelliine metabolites has been shown by karyomegaly and cytomegaly in endothelial cells and accumulation of intravascular macrophages in many organs. Short-term exposure of rats to riddelliine increased apoptosis and S-phase nuclei in endothelial cells and hepatocytes. Increased levels of p53 protein were detected in endothelial cells, and vascular endothelial growth factor (VEGF), an endothelial cell-specific mitogen, was increased in hepatocytes. Development of hemangiosarcoma in the liver may have resulted from endothelial-cell DNA adduct formation, apoptosis, proliferation of endothelial cells, and mutations. Increased expression of VEGF protein also could have contributed by stimulating endothelial-cell proliferation.

5.7.5 Carcinogenicity and genotoxicity of metabolites and analogues

Metabolites and analogues of riddelliine have shown carcinogenic and genotoxic properties in experimental animals. Since many of the PAs share a common metabolic

activation pathway, the genotoxic and carcinogenic effects are similar to those observed with riddelliine. DHP-DNA adducts, mutations, clastogenic effects, liver tumors in rats and, to a lesser extent, tumors of other organs, including the CNS, lung, bladder, pancreas, skin, testes, pituitary, and adrenal gland, have been observed in studies with other PAs or plant extracts containing PAs.

Although the genotoxicity, tumorigenicity, and toxicity of PAs vary, the structure-activity relationships are not well defined. In general, the macrocyclic diester types are the most genotoxic and the monoester types the least. While the ability of PAs to form cross-links has been proposed to affect their toxicity, only limited data are available for this potential relationship.

5.7.6 Toxicity

The liver is the primary target organ in humans, experimental animals, and livestock. Veno-occlusive disease is a characteristic lesion in humans poisoned by PAs. Other common effects in humans include ascites, splenomegaly, hepatomegaly, centrilobular hepatic necrosis, and cirrhosis. Young children appear to be particularly susceptible since many of the case reports involve infants and young children. Livestock poisoned by ingesting PA-containing plants often develop fatal liver disease. [The available data indicate interspecies differences in susceptibility with sheep, guinea-pigs, gerbils, hamsters, and rabbits showing resistance, while rats, cattle, horses, and chickens are highly susceptible.] The lungs are the second most common site of PA toxicity, but not all PAs affect the lungs. The primary site of damage is the pulmonary vasculature. The 11-membered macrocyclic diesters such as monocrotaline are particularly active in the lung but only at doses that were equal to or greater than doses causing liver toxicity.

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Glossary of Terms

Adulterant: A foreign or inferior substance that makes another substance impure.

Boiling point: The boiling point of the anhydrous substance at atmospheric pressure (101.3 kPa) unless a different pressure is stated. If the substance decomposes below or at the boiling point, this is noted (dec). The temperature is rounded off to the nearest °C.

Contaminant: A substance inappropriately present in the environment that might cause harmful effects.

Density: The density for solids and liquids is expressed in grams per cubic centimeter (g/cm^3) and is generally assumed to refer to temperatures near room temperature unless otherwise stated. Values for gases are generally the calculated ideal gas densities in grams per liter at 25°C and 101.325 kPa.

Epimer: A type of isomer in which the difference between the two compounds is the relative position of the hydrogen group and hydroxyl group on the last asymmetric carbon atom of the chain.

Exogenous: Due to an external cause; not arising within the organism.

Fibroadenoma: A benign tumor derived from glandular epithelium, commonly occurs in breast tissue.

Hemangiosarcoma: A malignant tumor characterized by rapidly proliferating cells derived from the blood vessels and lining irregular blood-filled spaces.

Hemoptysis: the coughing up of blood or mucus containing blood from the respiratory tract.

Henry's Law constant at 25°C: The ratio of the aqueous-phase concentration of a chemical to its equilibrium partial pressure in the gas phase. The larger the Henry's law constant the less soluble it is (greater tendency for vapor phase).

Hepatectomy: Removal of the liver.

Hepatocytomegaly: The production of abnormal hepatocytes (the most common cell type) in the liver.

K_m: A kinetic parameter used to characterise an enzyme, defined as the concentration of substrate that permits half maximal rate of reaction.

Lipophilic: Having a strong affinity for fats.

Log octanol-water partition coefficient (log K_{ow}): The ratio of concentrations of a substance in octanol and in water, when dissolved in a mixture of octanol and water. For convenience, the logarithm of K_{ow} is used. The octanol/water partition coefficient of a substance is useful as a means to predict soil adsorption, biological uptake, lipophilic storage, and bioconcentration.

Melting point: The melting point of the substance at atmospheric pressure (101.3 kPa). When there is a significant difference between the melting point and the freezing point, a range is given. In case of hydrated substances (i.e., those with crystal water), the apparent melting point is given. If the substance decomposes at or below its melting point, this is noted (dec). The temperature is rounded off to the nearest °C.

Molecular weight: The molecular weight of a substance is the weight in atomic mass units of all the atoms in a given formula. The value is rounded to the nearest tenth.

Neoplasm: Tumor.

Negative log acid dissociation constant (pK_a): A measure of the degree to which an acid dissociates in water (a measurement of acid strength). The pKa is the negative logarithm (to the base 10) of the acid dissociation constant (Ka); the lower the pKa, the stronger the acid.

Optical rotation: Rotation of the plane of polarization of plane-polarized light, or of the major axis of the polarization ellipse of elliptically polarized light by transmission through a substance or medium.

Physical state: Substances may either be gases, liquids, or solids according to their melting and boiling points. Solids may be described variously as amorphous, powders, pellets, flakes, lumps, or crystalline; and the shape of the crystals is specified if available. Solids also may be described as hygroscopic or deliquescent depending upon their affinity for water.

Poly-3 test: A survival-adjusted statistical test that takes survival differences into account by modifying the denominator in the numerical (quantal) estimate of lesion incidence to reflect more closely the total number of animal years at risk. For analysis of a given tumor site, each animal is assigned either (1) a risk weight of one if the animal had a lesion at that site or if it survived until terminal sacrifice or (2) a risk weight that is the fraction of the entire study time that it survived, raised to the 3rd power, if the animal died prior to terminal sacrifice and did not have a lesion at that site. The resulting test is similar to the Cochran-Armitage trend test, with the adjusted tumor rates replacing the observed tumor rates in the test statistic (Portier and Bailer 1989, Bieler and Williams 1993). The Poly-3 test is based on the more general Poly-k test; however, Bailer and Portier (1988) showed that the Poly-3 test gave valid results if the true value of k was anywhere in the range of 1 to 5.

Polyarteritis: Simultaneous inflammation of a number of arteries.

Pyrrole: A heterocyclic aromatic organic compound consisting of a five-membered ring with 4 carbon and one nitrogen atom.

Relay toxicity: Toxicity in which a food animal relays a dietary toxicant to humans consuming the animal or its products, such as milk.

Rhabdomyosarcoma: A malignant tumor derived from skeletal muscle.

Solubility: The ability of a substance to dissolve in another substance and form a solution.

Tincture: An alcoholic extract of an herb or other material.

Transgenic: An animal that carries a foreign gene that has been deliberately inserted into its genome.

Vapor density, relative: A value that indicates how many times a gas (or vapor) is heavier than air at the same temperature. If the substance is a liquid or solid, the value applies only to the vapor formed from the boiling liquid.

Vapor pressure: The pressure of the vapor over a liquid (and some solids) at equilibrium, usually expressed as mm Hg at a specific temperature (°C).

Veno-occlusive disease: Blockage of the small veins in the liver, resulting in liver damage.

$\mathbf{V_{max}}$: The maximum initial velocity of an enzyme catalysed reaction.