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Pyrrolizidine Alkaloids—Genotoxicity, Metabolism Enzymes, Metabolic Activation, and Mechanisms

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ABSTRACT

Pyrrolizidine alkaloid-containing plants are widely distributed in the world and are probably the most common poisonous plants affecting livestock, wildlife, and humans. Because of their abundance and potent toxicities, the mechanisms by which pyrrolizidine alkaloids induce genotoxicities, particularly carcinogenicity, were extensively studied for several decades but not exclusively elucidated until recently. To date, the pyrrolizidine alkaloid-induced genotoxicities were revealed to be elicited by the hepatic metabolism of these naturally occurring toxins. In this review, we present updated information on the metabolism, metabolizing enzymes, and the mechanisms by which pyrrolizidine alkaloids exert genotoxicity and tumorigenicity.

Key Words: Genotoxicity; Pyrrolizidine alkaloids; Metabolism; Enzymes; DNA adduct; Mechanism; Metabolic activation.

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INTRODUCTION

Pyrrolizidine alkaloids are heterocyclic compounds, most of which derive from esters of basic alcohols, known as the necine bases. The hydrolyzed products of pyrrolizidine alkaloids are a necine base and a necic acid. The structures and numbering system of the several representative necine bases, platynecine, retronecine, heliotridine, and otonecine, from which the derived pyrrolizidine alkaloids have been mostly studied, are shown in Fig. 1, among which retronecine and heliotridine are enantiomers at the C7 position. In general, naturally occurring pyrrolizidine alkaloids are esterified necines, whereas unesterified pyrrolizidine alkaloids are rarely present in plants. In addition, except for the otonecine-type, pyrrolizidine alkaloid *N*-oxides are also natural plant constituents with quantities nearly equal to their corresponding parent pyrrolizidine alkaloids present in numerous plant species.

Pyrrolizidine alkaloids are common constituents of hundreds of plant species around the world, and pyrrolizidine alkaloid-containing plants are probably the most common poisonous plants affecting livestock, wildlife, and humans (Al et al., 1999; Asibal et al., 1989; Betz et al., 1994; Bicchi et al., 1985; Bourauel et al., 1998; Bull et al., 1968; Cheeke, 1988; Creeper et al., 1999; Edgar et al., 1992; IARC, 1976; Ingolfsdottir and Hylands, 1990; Liddel, 1998; Mattocks, 1971a,b; Phillipson, 1971; Schoental, 1976; Steenkamp et al., 2000; Stegelmeier et al., 1999). These compounds act as a constitutive plant defense mechanism, with sporadic phylogenetic distribution and a sporadic taxonomic occurrence. The defensive mechanism is thought to involve a response to the selective pressure of herbivory (Moll et al., 2002; Ober and Hartmann, 1999, 2000). It was reported that about 3% of the world's flowering plants contain toxic pyrrolizidine alkaloids (Smith and Culvenor, 1981). More than 660 pyrrolizidine alkaloids and *N*-oxide derivatives have been identified in over 6000 plants of the three families, *Boraginaceae*, *Compositae* (*Asteraceae*), and *Legumionsae* (*Fabaceae*), and about half of them exhibit toxic activities (Roeder, 2000; Stegelmeier et al., 1999). A number of pyrrolizidine alkaloids belonging to these three plant families were also shown to induce tumors in experimental animals.

Pyrrolizidine alkaloids are highly toxic to many animal species, including most domestic livestock, and have caused tremendous livestock loss (Araya and Fuentealba, 1990; Arzt and Mount, 1999; Bah et al., 1994; Baker et al., 1991; Chung and Buhler, 1995; Curran et al., 1996; Creeper et al., 1999; Hill et al., 1997; Mattocks, 1968, 1986; McLean, 1970; Noble et al., 1994; Seaman, 1978, 1987; W.H.O. (WHO), 1988). The toxic effects of pyrrolizidine alkaloids gained further attention when experimental animals, particularly rats, dosed with these compounds developed liver tumors and pulmonary lesions (Chan, 1993; Chan et al., 1994, 2003; Fu et al., 2001, 2002a; Harris

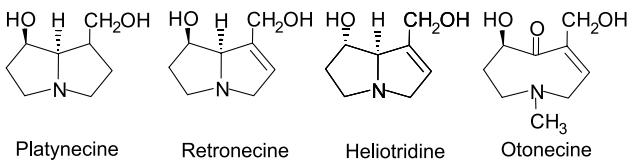

Figure 1. The common necine bases of pyrrolizidine alkaloids.

and Chen, 1970; Hirono et al., 1977, 1983; Huxtable, 1980; IARC, 1976; Kuhara et al., 1980; Mattocks, 1968, 1986; Mattocks and Cabral, 1982; Newberne and Rogers, 1973; Rao and Reddy, 1978; Schoental, 1975, 1976; Schoental and Cavanagh, 1972; Schoental and Head, 1957; Schoental et al., 1954, 1970; Svoboda and Reddy, 1972; W.H.O. (WHO), 1988; Woo et al., 1988). Because of their abundance and potent toxicities, including hepatotoxicity and carcinogenicity, the retronecine-, heliotridine-, and otonecine-type pyrrolizidine alkaloids received the most attention. The names and structures of the representative pyrrolizidine alkaloids of these three types are shown in Fig. 2.

There are numerous reports of human poisoning caused by pyrrolizidine alkaloids (Bull et al., 1968; Chauvin et al., 1994; Prakash et al., 1999; Stegelmeier et al., 1999; Woo et al., 1988). In general, humans are exposed to these toxic alkaloids through

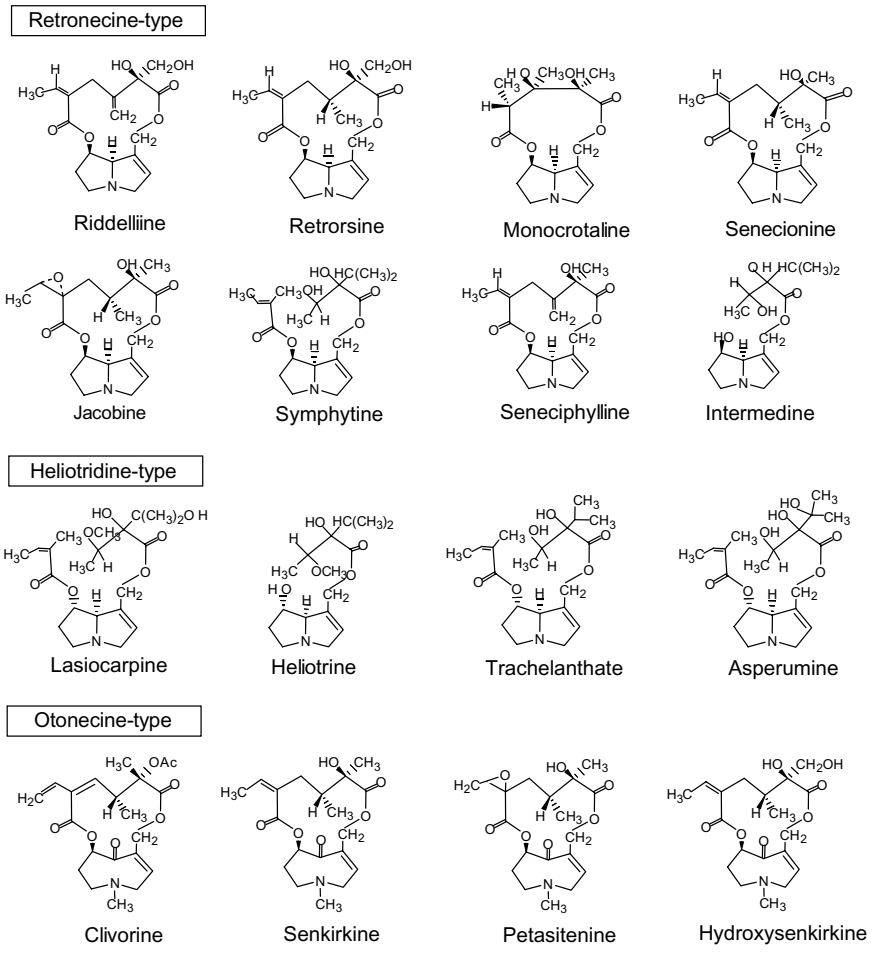

Figure 2. The names and structures of the representative retronecine-type, heliotridine-type, and otonecine-type pyrrolizidine alkaloids.

eating pyrrolizidine alkaloid-containing food (Hirono et al., 1979a, 1983; Prakash et al., 1999), contaminated staple food (Bull et al., 1968; Chauvin et al., 1994; Crews et al., 1997; Culvenor et al., 1981; Deinzer et al., 1977; Dickinson and King, 1978; Dickinson et al., 1976; Goeger et al., 1982; Hirono et al., 1979a; Molyneux and James, 1990; Prakash et al., 1999; Roeder, 1995; Selzer and Parker, 1951; Stegelmeier et al., 1999; Tandon et al., 1976, 1978; Willmot and Robertson, 1920; Woo et al., 1988), herbal teas, herbal medicines (Arseculeratne et al., 1981; Bach et al., 1989; Bull et al., 1968; Byron, 1998; Culvenor et al., 1986; Edgar et al., 1992; Larrey and Pageaux, 1995; Manteiga et al., 1997; Mehta et al., 1986; Roeder, 1995, 2000; Sommer, 1989; Spang, 1989; Seawright, 1995; Woo et al., 1988), and dietary supplements (Betz et al., 1994). Pyrrolizidine alkaloids were found to contaminate such human food sources as wheat, milk, and honey, and exposure through these routes is suspected to have caused worldwide human health problems (Bah et al., 1994; Betz et al., 1994; Byron, 1998; Edgar et al., 1992; Hirono et al., 1976; IARC, 1976; Mattocks, 1968; Prakash et al., 1999; Roeder, 1995; Rosberger et al., 1981; Winship, 1991).

Since the early 1990s, the use of dietary supplements has increased rapidly worldwide, and it was reported that some dietary supplements contain pyrrolizidine alkaloids (Betz et al., 1994). For this reason, human exposure to toxic pyrrolizidine alkaloids through this route is a concern.

The book *Chemistry and Toxicology of Pyrrolizidine Alkaloids*, written by Mattocks and published in 1986, along with several review articles, cover in detail the subjects of hepatotoxicity, carcinogenicity, chemical and physical properties, and synthesis of pyrrolizidine alkaloids (Roeder, 1995, 2000; Stegelmeier et al., 1999; Woo et al., 1988). In this review, we present updated information on the metabolism, metabolizing enzymes, and the mechanisms by which pyrrolizidine alkaloids exert genotoxicity and tumorigenicity.

PYRROLIZIDINE ALKALOID TOXICITY

Acute Toxicity and Chronic Toxicity

Intake of pyrrolizidine alkaloid-containing plants has poisoned humans and livestock, including horses, cattle, sheep, goats, swine, chickens, quails, and doves, worldwide (Afzelius and Schoental, 1967; Allen et al., 1970; Buckmaster et al., 1977; Bull et al., 1968; Cheeke, 1988; de Lanux-Van Gorder, 2000). Pyrrolizidine alkaloids, particularly those from plant species of genera *Senecio*, *Crotalaria*, *Heliotropium*, and *Amsinckia*, were found to exhibit acute toxicity, chronic toxicity, and genotoxicity. Acute poisoning causes massive hepatotoxicity with hemorrhagic necrosis. The potency of hepatotoxicity and acute toxicity varies markedly among pyrrolizidine alkaloids (Mattocks, 1986). Chronic poisoning takes place mainly in liver, lungs, and blood vessels, and in some instances kidneys, pancreas, gastrointestinal tract, bone marrow, and brain (Mattocks, 1986). Exposure over a long period of time causes cell enlargement (megalocytosis), veno-occlusion in liver and lungs, nuclei enlargement with increasing nuclear chromatin, loss of metabolic function, inhibition of mitosis, fatty degeneration, proliferation of biliary tract epithelium, liver cirrhosis, nodular hyperplasia, and adenomas or carcinomas (Bull et al., 1968; Mattocks, 1986; Roeder, 1995).

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Genotoxicity

Upon metabolic activation, pyrrolizidine alkaloids exhibit a variety of genotoxicities, including DNA binding, DNA cross-linking, DNA-protein cross-linking, sister chromatid exchange, chromosomal aberrations, mutagenicity, teratogenicity, and carcinogenicity (Brink, 1982; Bull et al., 1968; Carballo et al., 1992; Chan, 1993; Chan et al., 1994; Cook et al., 1950; Coulombe et al., 1999; Crout, 1972; Culvenor and Jags, 1979; Eastman et al., 1982; Fu et al., 2001; Galloway et al., 1987; Griffin and Segall, 1986; Harris and Chen, 1970; Hincks and Coulombe, 1989; Hincks et al., 1991; Hirono et al., 1977, 1978, 1979b, 1983; IARC, 1976; Kim et al., 1993, 1995, 1999; Kuhara et al., 1980; Mattocks, 1968, 1986; Mattocks and Bird, 1983; Mattocks and Cabral, 1982; MacGregor et al., 1990; Mirsalis et al., 1993; Newberne and Rogers, 1973; Pereira et al., 1998; Petry et al., 1984; Rao and Reddy, 1978; Reed et al., 1988; Rubiolo et al., 1992; Schoental, 1970, 1972, 1975, 1976; Schoental and Cavanagh, 1972; Schoental and Head, 1957; Schoental et al., 1954, 1970, 1971; Svoboda and Reddy, 1972; White and Mattocks, 1972; W.H.O. (WHO), 1988; Yang et al., 2001a). Mutagenicity induced by pyrrolizidine alkaloid-containing plant extracts and pure pyrrolizidine alkaloids was extensively studied in different biological systems, including *Drosophila melanogaster*, *Salmonella typhimurium*, and *Escherichia coli* (Brink, 1969, 1982; Bruggeman and van der Hoeven, 1985; Candrian et al., 1984; Carballo et al., 1992; Frei et al., 1992; Zeiger et al., 1988). The pyrrolizidine alkaloids found to be mutagenic in *Salmonella typhimurium* TA100 in the presence of the S9 activation enzyme system, including clivorine, heliotrine, lasiocarpine, senkirkine, retrorsine, seneciphylline, and riddelliine, are also tumorigenic in experimental animals (see next section) (Fu et al., 2001; Mattocks, 1986; Rubiolo et al., 1992; Wehner et al., 1979; Yamanaka et al., 1979; Zeiger et al., 1988).

Tumorigenicity

The pyrrolizidine alkaloids that exhibit the most potent genotoxicity and tumorigenicity are the macrocyclic diester pyrrolizidine alkaloids, namely, the retronecine-, heliotridine-, and otonecine-type pyrrolizidine alkaloids.

Shown in Table 1 are the pyrrolizidine alkaloids that were tested to be tumorigenic in experimental animals. These pyrrolizidine alkaloids are mainly from three plant families. The structures of these compounds are shown in Fig. 2.

GENERAL CONSIDERATION OF METABOLISM

Similar to the majority of other types of toxic chemicals, pyrrolizidine alkaloids require metabolic activation to exert toxicities. Metabolism and determination of metabolic activation pathways leading to toxicity, particularly genotoxicity, have been extensively studied (Buhler and Kedzierski, 1986; Chan, 2001; Chung and Buhler, 1994; Chung et al., 1995; Dueker et al., 1992a; Eastman and Segall, 1981; Fu et al., 2001, 2002b; Jago et al., 1970; Kasahara et al., 1997; Kedzierski and Buhler, 1985, 1986a; Lame et al., 1991; Lin et al., 1998a,b, 2000a, 2002; Mattocks, 1968, 1986; Mattocks and Cabral, 1982; Mattocks and White, 1971; Miranda et al., 1981, 1991a; Reid et al., 1998;

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Table 1. Carcinogenicity of the naturally occurring pyrrolizidine alkaloids and the synthetically prepared pyrrolic metabolites determined in rats.

Pyrrolizidine alkaloid	Family	Major plant genus	Tumor type	References
Otonecine-type pyrrolizidine alkaloids				
Clivorine	Compositae	*Ligularia*	Hemangioendothelial sarcoma, liver adenoma	(Kuhara et al., 1980)
Senkirkine	Compositae	*Tussilago*, *Senecio*, *Petasites*	Hemangioendothelial sarcoma, liver adenoma	(Hirono et al., 1976, 1979b)
Hydroxysenkirkine	Compositae	*Senecio*	Brain and spinal cord tumor	(Schoental and Cavanagh, 1972)
Petasitenine	Compositae	*Senecio*	Liver hemangioendothelial sarcoma, liver adenoma	(Furuya et al., 1976; Hirono et al., 1977)
Heliotridine-type pyrrolizidine alkaloids				
Heliotrine	Boraginaceae	*Heliotropium*	Pancreatic islet cell tumor, hepatoma	(Schoental, 1975)
Lasiocarpine	Boraginaceae	*Heliotropium*	Liver carcinoma, skin carcinoma, pulmonary adenoma	(Rao and Reddy, 1978; Rao et al., 1983; Svoboda and Reddy, 1972, 1974)
Retronecine-type pyrrolizidine alkaloids				
Intermedine	Boraginaceae	*Amsinckia*	Islet cell adenoma, bladder papillary tumor	(Schoental et al., 1970, 1971)
Jacobine	Compositae	*Senecio*	Liver tumor	(Cook et al., 1950; Schoental et al., 1954)
Lycopasamine	Boraginaceae	*Amsinckia*	Islet cell adenoma, bladder papillary tumor	(Schoental et al., 1970, 1971)

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Monocrotaline	Leguminosae	*Crotalaria*	Liver carcinoma, pulmonary adenoma, adrenal adenoma	(Allen et al., 1975; Shumaker et al., 1976)
Retrorsine	Compositae	*Senecio*	Liver carcinoma	(Harris and Chen, 1970; Schoental and Head, 1957; Schoental et al., 1954, 1971)
Retronecine	Leguminosae	*Crotalaria*	Spinal cord tumor	(Schoental and Cavanagh, 1972)
Riddelliine	Compositae	*Senecio*	Hepatocarcinoma	(Brandange et al., 1970; Harris and Chen, 1970; Schoental and Head, 1957)
Seneciphylline	Compositae	*Senecio*	Hemangioendothelial sarcoma, liver adenoma	(Harris and Chen, 1970; Hirono et al., 1983)
Symphytine	Boraginaceae	*Symphytum*	Liver tumor	(Hirono et al., 1978, 1979b)
Senecionine[a]	Compositae	*Senecio*	Liver tumor	(Hirono et al., 1976; Schoental and Head, 1957; Schoental et al., 1954)
Isatidine (Retrorsine *N*-oxide)	Compositae	*Senecio*	Liver carcinoma,	(Schoental and Head, 1957; Schoental et al., 1954, 1971)
Synthetic pyrrolic metabolites				
Dehydro-heliotridine		—	Liver cystadenoma, lung adenocarcinoma, pancreas islet cell tumor	(Peterson et al., 1983)
Dehydro-monocrotaline		—	Skin tumor	(Mattocks and Cabral, 1982)
Dehydro-retronecine		—	Rhabdomyosarcoma, skin tumor	(Allen et al., 1975; Johnson et al., 1978; Mattocks and Cabral, 1982; Shumaker et al., 1976)

[a]Not pure, extracted from the *Senecio* plants.

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Segall et al., 1985; Williams et al., 1989a; Yan et al., 2002; Yang et al., 2001a,b). Since the retronecine-, heliotridine-, and otonecine-type pyrrolizidine alkaloids exhibit the highest toxicity and tumorigenicity in experimental animals (Table 1), metabolism of pyrrolizidine alkaloids of these three types has been the most studied.

Retronecine-type and heliotridine-type pyrrolizidine alkaloids possess a similar necine base. The difference is at the C7 position, with the retronecine-type pyrrolizidine alkaloids possessing an *R* absolute configuration, and the heliotridine-type pyrrolizidine alkaloids possessing an *S* stereochemistry (Fig. 1). In general, there are three principal metabolic pathways for the metabolism of both retronecine- and heliotridine-type pyrrolizidine alkaloids. The first pathway is hydrolysis of the ester functional groups linked to the C7 and C9 positions to form the necine bases and the necic acids. The second pathway is *N*-oxidation of the necine bases to the corresponding pyrrolizidine alkaloid *N*-oxides. The third metabolic pathway is oxidation via two steps, hydroxylation of the necine base at the C3 or C8 position to form the corresponding 3- or 8-hydroxynecine derivatives followed by spontaneous dehydration to produce the corresponding dehydropyrrolizidine (pyrrolic ester) derivatives (Fu et al., 2001; Lin et al., 2002). To illustrate these metabolic pathways, metabolism of riddelliine by rat liver microsomes is shown in Fig. 3.

Otonecine-type pyrrolizidine alkaloids have a necine base structurally different from the retronecine- and heliotridine-type pyrrolizidine alkaloids (Fig. 1). As such, there are

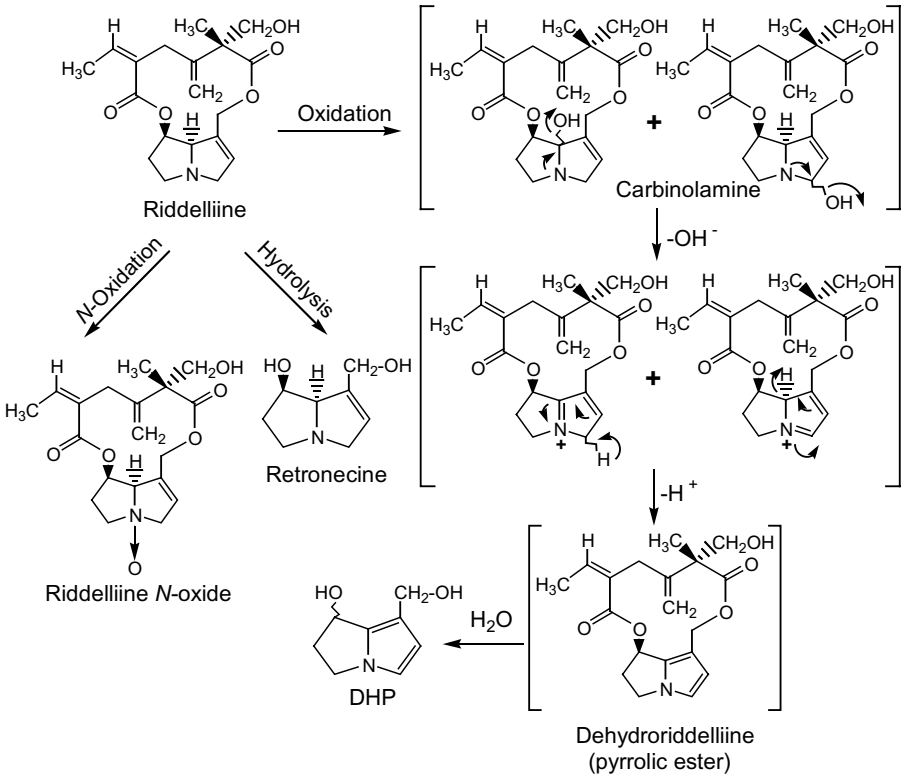

Figure 3. Principal phase I metabolism pathways of riddelliine.

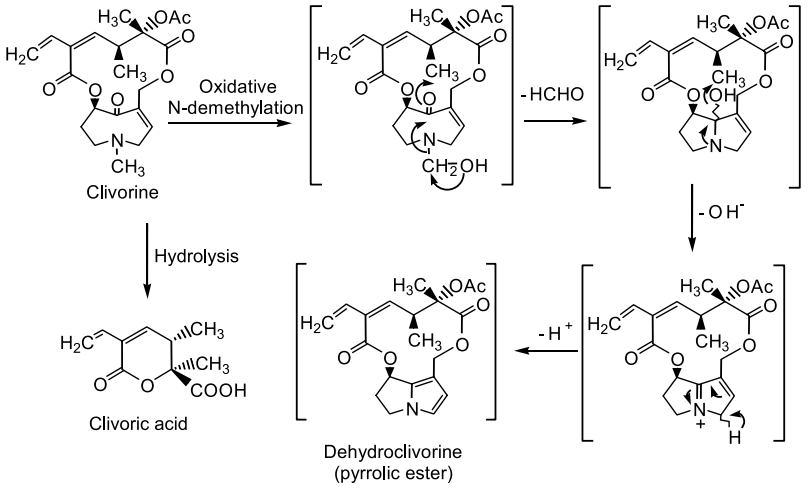

Figure 4. Principal phase I metabolism pathways of clivorine.

only two general principal metabolic pathways for this type of pyrrolizidine alkaloid (Lin et al., 1998a, 2000a, 2002). These two pathways are hydrolysis of the ester functional groups to form the corresponding necine bases and acids and formation of the corresponding pyrrolic esters (dehydropyrrolizidine alkaloids) through oxidative *N*-demethylation of the necine base followed by ring closure (by elimination of a formaldehyde molecule) and dehydration. The C7 position of the otonecine-type pyrrolizidine alkaloids possesses an *R* absolute configuration. Consequently, the resulting pyrrolic esters have a necine base identical to that of retronecine-type pyrrolizidine alkaloids. The metabolism of clivorine in the guinea pig shown in Fig. 4 is an example of the principal phase I metabolism of otonecine-type pyrrolizidine alkaloids.

As illustrated in Fig. 5, the dehydropyrrolizidine alkaloid (pyrrolic ester) metabolites are chemically and biologically reactive and tend to undergo further biotransformations. Once formed, the pyrrolic ester metabolites can rapidly bind with DNA, leading to DNA cross-linking, DNA-protein cross-linking, and DNA adduct formation (Fu et al., 2001; IPCS, 1989; Kim et al., 1995, 1999; Mattocks, 1986; Pereira et al., 1998; Reed et al., 1988; Robertson, 1982; White and Mattocks, 1972; Yang et al., 2001b). Thus, metabolic formation of pyrrolic ester metabolites has been shown to be the primary metabolic activation responsible for the adverse toxicities, including cytotoxicity, genotoxicity, and tumorigenicity of pyrrolizidine alkaloids (Dueker et al., 1992a; Huxtable and Wild, 1994; IARC, 1976; Lame et al., 1991; Mattocks, 1968, 1986; Mattocks and Bird, 1983; White and Mattocks, 1972; Williams et al., 1989b; Yang et al., 2001a,b). Because of high reactivity, pyrrolic ester metabolites can also react readily with water and other endogenous constituents, such as glutathione, to form the detoxified products (Fig. 5).

Retronecine-type and heliotridine-type pyrrolizidine alkaloids are enantiomers with respect to the C—O bond at the C7 position and are optically active. However, further hydrolysis of the pyrrolic ester metabolites generated from these two types of pyrrolizidine alkaloids, such as riddelliine, monocrotaline, retrorsine, jacobine, and lasiocarpine by rat liver microsomes did not form the corresponding optically active necine bases dehydroretronecine (DHR) (*R*-6,7-dihydro-7-hydroxy-1-hydroxymethyl-

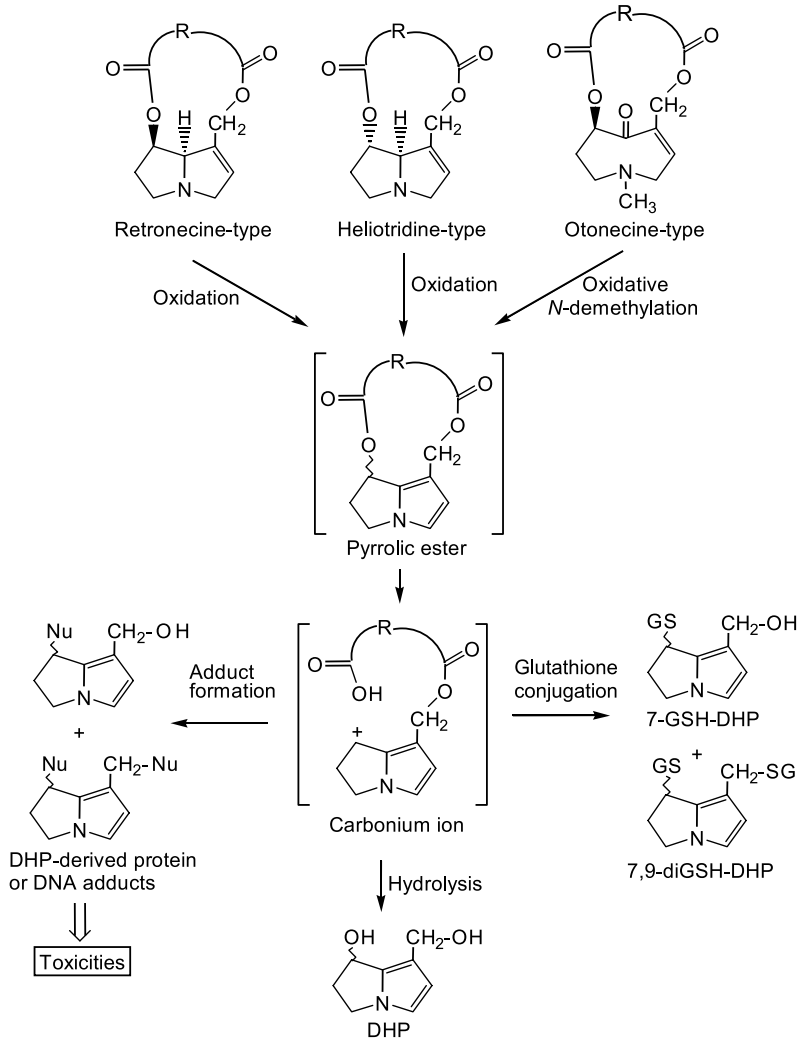

Figure 5. Further metabolic pathways of the dehydropyrrolizidine alkaloids formed from pyrrolizidine alkaloids of different types. Nu: nucleophilic biological macromolecules, such as –*S*-protein or –*N*-DNA.

5H-pyrrolizine) or dehydroheliotridine (DHH) (*S*-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine), respectively. Instead, the racemic mixture DHP [(+/−)6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine] with a 1:1 ratio was formed from metabolism (Buhler and Kedzierski, 1986; Bull et al., 1968; Dueker et al., 1992a; Fu et al., 2001; Jago et al., 1969, 1970; Kedzierski and Buhler, 1985, 1986a; Lame et al., 1991; Mattocks, 1986; Mattocks and White, 1971). As shown in Fig. 5, the common pathway for the formation of the racemic DHP metabolites involves the following: 1) oxidation or oxidative *N*-demethylation of retronecine- and heliotridine-type or otonecine-type pyrrolizidine alkaloids producing dehydropyrrolizidine alkaloids;

2) conversion of the dehydropyrrolizidine alkaloids (the reactive intermediate) to the carbonium ions with a positive charge located at the C7 position of the necine base; and 3) reaction of the carbonium ions with water, with attack at both sides of the necine plane, to form the racemic DHP.

Furthermore, the pyrrolic ester metabolites may bind with one or two molecules of glutathione (GSH) to form 7-glutathionyl-6,7-dihydro-1-hydroxymethyl-5H-pyrrolizine (7-GSH-DHP) or 7,9-diglutathionyl-6,7-dihydro-1-hydroxymethyl-5H-pyrrolizine (7, 9-diGSH-DHP), respectively. Similarly, these glutathione conjugates are racemic mixtures with a 1:1 ratio of two enantiomers at the C7 position (Fig. 5). The racemic mixture of 7-GSH-DHP generated by rat liver microsomal metabolism of clivorine was confirmed by NMR spectroscopic analysis (Lin et al., 2000b). This phase II glutathione conjugation catalyzed by glutathione *S*-transferases is generally considered to be the principal detoxification pathway (Lin et al., 1998a, 2000a; White, 1976; Yan and Huxtable, 1995a,b).

On the other hand, the hydrolysis process in all three types of pyrrolizidine alkaloids and the *N*-oxidation process in the retronecine-type and heliotridine-type of pyrrolizidine alkaloids are also generally considered detoxification pathways. Therefore, the metabolic kinetic between the pyrrolic ester formation and the detoxification pathways, such as hydrolysis, glutathione conjugation, and *N*-oxidation, appears to be crucial in determining the toxicity of pyrrolizidine alkaloids.

In the case of senecionine, in addition to the three general pathways described above, Segall and coworkers also found *trans*-4-hydroxy-2-hexenal as a hepatic metabolite of senecionine (Griffin and Segall, 1986; Segall et al., 1985). This metabolite was shown to be toxic, causing hepatic necrosis in vivo (Griffin and Segall, 1986, 1989; Segall et al., 1985; Winter et al., 1986). Like the other alkenals, this product can bind with deoxyguanosine to form DNA adducts (Winter et al., 1986). Experimental data suggested that this metabolite is formed from senecionine-induced lipid peroxidation (Miranda et al., 1981). Thus, formation of this compound from metabolism of senecionine has been considered an activation pathway (Segall et al., 1985). Induction of lipid peroxidation by pyrrolizidine alkaloids may be involved in pyrrolizidine alkaloid-induced toxicity and tumorigenicity.

METABOLIZING ENZYMES

Phase I Metabolizing Enzymes

Cytochrome P450 Enzymes

The hepatic cytochrome P450 (CYP450) metabolizing enzymes are involved in both *C*-oxidation and *N*-oxidation of the necine base to form the reactive pyrrolic ester metabolites and pyrrolizidine alkaloid *N*-oxides, respectively (Buhler and Kedzierski, 1986; Chung and Buhler, 1994, 1995; Dueker et al., 1992a; Eastman and Segall, 1981; Hayes et al., 1984; Kasahara et al., 1997; Lin et al., 2000a; Miranda et al., 1991a; Reid et al., 1998; Tepe and Williams, 1999a; Williams et al., 1989a; Yang et al., 2001a). The metabolic formation of the reactive pyrrolic ester metabolites is mainly catalyzed by cytochrome P450 monooxygenases, specifically the CYP3A and CYP2B isoforms

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in humans. Phenobarbital is an inducer of both CYP2B and CYP3A isozymes (Omiecinski et al., 1999). Compared to liver microsomes from untreated rats, the rate of metabolism of pyrrolizidine alkaloids, specifically of riddelliine, is higher with liver microsomes of rats pretreated with phenobarbital (Kasahara et al., 1997; Yang et al., 2001a).

That CYP3A enzymes are the major metabolizing enzymes responsible for metabolism of pyrrolizidine alkaloids was confirmed by incorporation of a specific CYP3A enzyme inhibitor in the metabolism in vivo. Both triacetyleandomycin and ketonoconazole are specific CYP3A enzyme inhibitors (Ervine et al., 1996; Ghosal et al., 1996). Recent studies from our laboratories found that rat liver microsomal metabolism of riddelliine, clivorine, and lasiocarpine in the presence of triacetyleandomycin resulted in reduced DHP formation (Xia et al., 2003a,b, unpublished data). These results indicate that the DHP formation from rat liver microsomal metabolism of riddelliine, clivorine, and lasiocarpine is primarily catalyzed by the hepatic CYP3A metabolizing enzyme. This finding is consistent with the report that metabolism of clivorine by liver microsomes of male Sprague Dawley rats and Dunkin Hartley guinea pigs of both sexes forms the reactive pyrrolic ester metabolites and is mainly catalyzed by the cytochrome CYP3A enzyme (Lin et al., 2002). Riddelliine, clivorine, and lasiocarpine represent the retronecine-, otonecine-, and heliotridine-type pyrrolizidine alkaloids. Thus, these results support that the CYP3A enzymes are the major metabolizing enzymes responsible for metabolic activation of most, if not all, of the toxic pyrrolizidine alkaloids.

Metabolism of retronecine- and heliotridine-type pyrrolizidine alkaloids to the corresponding *N*-oxides is catalyzed by both cytochrome P450 and flavin-containing monooxygenases (Chung and Buhler, 1995; Miranda et al., 1991a,b; Williams et al., 1989b). Buhler and coworkers reported that metabolism of senecionine to senecionine *N*-oxide was catalyzed by CYP2B and flavin-containing monooxygenases in untreated and phenobarbital-treated guinea pig (Chung and Buhler, 1995; Ramsdell and Buhler, 1987).

Metabolic activation and detoxification of senecionine by sheep and hamster liver microsomes were mainly catalyzed by the CYP3A enzymes. The CYP2B enzyme was also involved, but to a much lesser extent (Huan et al., 1998a). A marked sex difference in the metabolic oxidation of the senecionine, especially with respect to the *N*-oxide formation, was determined in rats (Williams et al., 1989a). The overall results suggested the potential involvement of the male-specific CYP450 UT-A (CYP2C11) and CYP450 PCN-E (CYP3A). However, CYP3A is not the major enzyme for senecionine metabolism in guinea pigs (Chung and Buhler, 1994). Metabolism of senecionine to the pyrrolic ester metabolite by the liver microsomes of untreated and phenobarbital-treated guinea pig was mainly catalyzed by the CYP2B enzyme (Chung and Buhler, 1995).

Flavin-containing Monooxygenases

Flavin-containing monooxygenases and cytochrome P450 enzymes were found to be involved in the biotransformation of pyrrolizidine alkaloids to the corresponding *N*-oxide metabolites. Also, the relative contribution of the flavin-containing monooxygenases and cytochrome P450 enzymes on the *N*-oxide formation was determined to

be species and tissue dependent (Williams et al., 1989b). For example, metabolism of senecionine to senecionine *N*-oxide by microsomes of pig liver, lung, and kidney is mainly catalyzed by flavin-containing monooxygenases (Miranda et al., 1991b; Williams et al., 1989b). In contrast, metabolism of senecionine by rat liver microsomes to senecionine *N*-oxidation is mainly catalyzed by cytochrome P450 but not by the purified rabbit lung flavin-containing monooxygenases (Williams et al., 1989b).

Carboxylesterases

For metabolism of pyrrolizidine alkaloids, enzymatic hydrolysis of the ester groups leading to the corresponding necine base and necic acid moieties is considered to be a major detoxification pathway. Enzymatic hydrolysis of the ester functional groups is mainly catalyzed by liver microsomal carboxylesterases (Buhler and Kedzierski, 1986; Chung and Buhler, 1994; Chung et al., 1995; Eastman and Segall, 1981; Kasahara et al., 1997; Miranda et al., 1991a; Reid et al., 1998; Williams et al., 1989a). Hydrolysis can also be catalyzed by liver cytosolic carboxylesterases (Dueker et al., 1992a; Kasahara et al., 1997; Mattocks, 1982, 1986). Hepatic microsomal carboxylesterases consist of multiple isozymes, which exhibit drastically different immunological properties and substrate specificities. It was determined that there are three rat liver carboxylesterase isozymes, RL1, RH1, and RL2, and two guinea pig liver carboxylesterase isozymes, GPL1 and GPH1 (Hosokawa et al., 1990).

The carboxylesterases are highly substrate specific (Hincks et al., 1991; Kim et al., 1993; Mattocks, 1982). The GPH1 was able to hydrolyze seneciphylline, monocrotaline, and a mixture of senecionine and integerrimine, but GPL1 showed no activity toward these compounds. The enzyme substrate specificity may explain the resistance of guinea pig pyrrolizidine alkaloid intoxication (Dueker et al., 1992b). Steric hindrance around the ester functional groups of the molecule can drastically affect substrate specificity and can even inhibit the enzymatic hydrolysis process (Mattocks, 1982). In general, the allylic ester group at the C7 position in some macrocyclic diester pyrrolizidine alkaloids (e.g., senecionine, retrorsine, and riddelliine) is more easily hydrolyzed than the ester group at the C9 position. This is because the allylic ester is less sterically hindered, and the double bond may enhance the hydrolysis.

Monocrotaline was metabolized by guinea pig liver microsomes, with hydrolysis of the ester functional group catalyzed by carboxylesterases accounting for 92% of the total metabolism (Dueker et al., 1992a). On the other hand, the rat liver microsomes did not display esterase activity on monocrotaline, resulting in the high susceptibility of rats to monocrotaline-induced toxicity (Chesney and Allen, 1970; Dueker et al., 1992a; Lame et al., 1991). These results support guinea pig resistance to toxicity induced by monocrotaline and several other pyrrolizidines, such as retrorsine (White et al., 1973) and senecionine (McLean, 1970), although the guinea pig is susceptible to jacobine (Swick et al., 1982).

Phase II Metabolizing Enzymes—Glutathione *S*-Transferases

In the hepatic metabolism of pyrrolizidine alkaloids of different types, conjugation of the reactive toxic pyrrolic ester metabolites with glutathione to form glutathione conjugates can occur either enzymatically or nonenzymatically. Although the majority

of the reported formation of glutathione conjugates from different pyrrolizidine alkaloids occurred either in vivo or in the presence of liver microsomes, the production of 7-GSH-DHP was also demonstrated nonenzymatically in a buffer solution containing dehydroretronecine and glutathione (Robertson et al., 1977). The enzymes mediating the glutathione conjugation of pyrrolizidine alkaloids in the liver are generally recognized as both cytosolic and microsomal glutathione-*S*-transferases (GSTs) (Castagnoli et al., 1997; Mattocks, 1986; Prakash et al., 1999; Stegelmeier et al., 1999). The extent of contribution of the GST catalysis to glutathione conjugation varies with the different pyrrolizidine alkaloid structures. In an in vivo study of glutathione conjugation of jacobine in guinea pig microsomes, the rate of conjugation mediated by glutathione-*S*-transferases was found to be no different from the nonenzymatic rate (Dueker et al., 1994).

Glutathione *S*-transferases are a large family of structurally related proteins, with different GST isozymes catalyzing different substrates, but often with an overlapping specificity (Jakobsson et al., 2000; Sheehan et al., 2001; Whalen and Boyer, 1998). The GST-catalyzed glutathione conjugation requires the ubiquitous endogenous tripeptide glutathione (γ-glutamyl-cysteinyl-glycine) as the cosubstrate. In view of the studies of GST-mediated glutathione conjugation of different pyrrolizidine alkaloids, there is a lack of information on the type and isozyme of GSTs responsible for such biotransformation.

The formation of 7-GSH-DHP at least partially mediated by GSTs was revealed in the perfused rat liver with monocrotaline (Mattocks et al., 1991; Nigra and Huxtable, 1992; Yan et al., 1995), seneciphylline (Yan et al., 1995), retrorsine (Yan et al., 1995), and trichodesmine (Yan et al., 1995), and also in the in vivo microsomal metabolisms of jacobine (Dueker et al., 1994; Miranda et al., 1980), senecionine (Dueker et al., 1994; Huan et al., 1998b; Miranda et al., 1980; Reed et al., 1992), seneciphylline (Huan et al., 1998b), monocrotaline (Huan et al., 1998b), and clivorine (Lin et al., 1998a, 2000a, 2002, 2003) Furthermore, the excretion of 7-GSH-DHP in the urine of rats treated with monocrotaline and senecionine (Estep et al., 1990a), and in the bile of rats treated with monocrotaline (Lame et al., 1990; Yan and Huxtable, 1995a) was reported.

Hepatic GSH content was reported to affect the glutathione detoxification of pyrrolizidine alkaloids. In a study of retrorsine toxicity to rats (White, 1976), the acute LD_{50} value of retrorsine in the control animals (42 mg/kg) was significantly increased (83 mg/kg). There was a 100% increase in hepatic GSH level when the rats were pretreated with cysteine, and there was a 75% reduction of hepatic GSH level when pretreatment was with chloroethanol. The influences of the hepatic GSH level on the metabolism of monocrotaline were also investigated in the perfused rat liver (Nigra and Huxtable, 1992; Yan and Huxtable, 1995b). Reduction of GSH levels in the liver of rats pretreated with different chemicals had no effect on the release of total pyrrolic metabolites of monocrotaline. However, the biliary release of 7-GSH-DHP significantly decreased, while the tissue-bound pyrroles in the liver significantly increased. The results indicate that pyrrolizidine alkaloid-induced hepatotoxicity may be more pronounced if the GSH content in the liver is low. A comparison of formation rates of 7-GSH-DHP conjugates and their relation to the extrahepatic toxicities was conducted in the isolated rat liver perfused with four different retronecine-type pyrrolizidine alkaloids (Yan et al., 1995). The rates of formation and release into the bile of the conjugates of four pyrrolizidine alkaloids were found to have the order of retrorsine>seneciphyllin>trichodesmine>monocrotaline, which is related to the reactivity

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of dehydropyrrolizidine alkaloids (pyrrolic esters) toward nucleophilic constituents in the liver to form DHP-derived adducts. Dehydropyrrolizidine alkaloids are the reactive intermediates responsible for toxicity, and it was found that more stable intermediates (with longer half-lives) were able to reach target organs to induce extrahepatotoxicity (Cooper and Huxtable, 1999). Therefore, the higher reactivity and the larger portion of nucleophilic reactions of the pyrrolic esters, together with the smaller portion of the release of pyrrolic esters into the circulation, will result in low extrahepatic toxicities of the parent pyrrolizidine alkaloids.

Enzyme Modulation by Pyrrolizidine Alkaloids

It was determined that the metabolizing enzyme activities, including those of the cytochrome P450 enzymes (Guengerich, 1977), of animals fed pyrrolizidine alkaloids can be significantly altered. Rats dosed with monocrotaline displayed increased activities of hepatic succinate dehydrogenase, acid ribonuclease, acid phosphatase, gammaglutamyl transpeptidase, and 5′-nucleotidase, and reduced activities of glucose-6-phosphatase and cytochrome P450 enzymes (Dwivedi et al., 1991). Rats fed heliotrine were found to have reduced contents of cytochrome P450 and b_5 enzymes (Khakimov, 1985). The heliotrine-induced decrease in cytochrome P450 content was due to hepatocyte membrane destruction (Dobrynina et al., 1987). Acute hepatitis induced by heliotrine was accompanied by uncoupling of oxidative phosphorylation in liver mitochondria (Gizatullina et al., 1991). Rats treated with retrorsine had increased expressions of hepatic CYP1A1, 1A2, 2E1, and 2B1/2 enzymes (Gordon et al., 2000a). Rats treated with heliotrine had decreased liver cytochrome P450 enzymes and NADPH- and NADH-dependent flavoprotein activities and *O*-dealkylating and hydroxylation activities (Savin et al., 1983).

Rats fed seneciphylline had significantly increased activities of liver epoxide hydrase and glutathione *S*-transferase but reduced cytochrome P450 and related monooxygenase activities (Kakrani and Kalyani, 1984). Senecionine had no effect on epoxide hydrase formation, but it reduced the activities of glutathione *S*-transferase, aminopyrine demethylase, and arylhydrocarbon hydroxylase (Kakrani and Kalyani, 1984).

Effects of pyrrolizidine alkaloids on the activity of glutathione *S*-transferase in different strains of rats were reported. It was found that the activity of hepatic GSTs of young male Long Evans rats treated with jacobine (Miranda et al., 1980) and young male Swiss Albino rats treated with seneciphylline (Kakrani and Kalyani, 1984) were significantly elevated. However, monocrotaline (Miranda et al., 1980) and senecionine (Kakrani and Kalyani, 1984) markedly diminished the activity of hepatic cytosolic GSTs in young male Long Evans and albino rats, respectively. In contrast, two other studies showed significant elevations of activity of the hepatic microsomal GSTs in adult male Sprague Dawley (SD) rats treated with monocrotaline (Yan and Huxtable, 1996a, 1996b).

Glutathione conjugation with the toxic pyrrolic ester metabolites formed is generally recognized as one of the detoxification pathways. In addition to GST enzymes mediating such conjugation, the endogenous GSH is an essential substance involved in the reaction. In vivo studies revealed that tissue GSH levels also change in the case of pyrrolizidine alkaloid poisoning. At 24 hours after administration of a toxic dose of monocrotaline, trichodesmine (Yan and Huxtable, 1995a), or retrorsine

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(Lin et al., 1999) to the male SD rat, the hepatic GSH levels significantly increased. Furthermore, the elevations of GSH contents in the liver and lung of the male SD rat correlated with the high formation rates of toxic pyrrolic metabolites, such as the tissue-bound pyrroles, after administration of a toxic dose of monocrotaline (Yan and Huxtable, 1996b). However, in a study of the isolated rat liver perfused with monocrotaline at a concentration markedly higher than the toxic dose, GSH depletion was demonstrated (Yan and Huxtable, 1995c). Therefore, the elevation of the GSH contents, especially in the liver, is coupled with increased activity of GSTs in the early stage of nonsevere pyrrolizidine alkaloid poisoning and may represent a self-defense mechanism in preventing pyrrolizidine alkaloid intoxication in the body.

Human Metabolizing Enzymes

Metabolism of pyrrolizidine alkaloids by human liver microsomes of both sexes was studied. Miranda et al. reported that metabolism of senecionine by human liver microsomes formed a pyrrolic ester metabolite and senecionine *N*-oxide. Metabolism was mainly catalyzed by CYP3A4 isozyme (Miranda et al., 1991a). The metabolic studies of clivorine in the pooled human liver microsomes and cDNA-expressed isozymes also revealed that CYP3A4 is the key enzyme mediating the metabolic activation of clivorine to DHP and DHP-derived conjugates and adducts (Lin et al., 2001, unpublished results). To date, all the pyrrolizidine alkaloids studied, including senecionine, riddelliine, lasiocarpine, heliotrine, and clivorine, are the representative retronecine-, heliotridine-, and otonecine-type pyrrolizidine alkaloids. Apparently, the results obtained from these studies support that the isozymes in the CYP3A subfamily, in particular CYP3A4 isozyme, are the primary metabolizing enzymes responsible for the metabolic activations of all different types of pyrrolizidine alkaloids by human liver microsomes. Recently, Xia et al. (2003a) conducted a comparative study on the metabolism of riddelliine by human and rat liver microsomes and found that the results from human liver microsomal metabolism to form DHP and riddelliine *N*-oxide are comparable to those obtained from rat liver microsomal metabolism. Similar results were obtained from metabolism of lasiocarpine and heliotrine by male and female human liver microsomes, producing DHP (or DHH) and the corresponding *N*-oxides as metabolites (Xia et al., unpublished data).

Metabolism of riddelliine by male and female human liver microsomes in the presence of calf thymus DNA produced the same set of eight DHP-derived DNA adducts as those formed from rat liver microsomal metabolism (Xia et al., 2003a). Thus, based on the similar metabolism pattern and DNA adduct profile from human and rat liver microsomal metabolism, the mechanistic data on liver tumor induction obtained for riddelliine in laboratory rodents is relevant to humans.

MECHANISM LEADING TO TUMOR FORMATION

It has long been determined that pyrrolizidine alkaloids can induce liver tumors in experimental rats (as shown in Table 1). The pursuit of mechanistic understanding of carcinogenesis by pyrrolizidine alkaloids occurred over the past several decades, but has so far failed. It was recently that our mechanistic study determined that riddelliine

induced liver tumors through a genotoxic mechanism mediated with DHP-derived DNA adduct formation (Yang et al., 2001a). These DHP-derived DNA adducts are potential general biomarkers of pyrrolizidine alkaloid tumorigenicity. We hypothesize that these DNA adducts are also responsible for the other genotoxicities, including mutagenicity and teratogenicity, of pyrrolizidine alkaloids. The following presents this mechanism and other possible activation pathways.

Formation of DNA Cross-linking and DNA–Protein Cross-linking

The pyrrolic ester (dehydropyrrolizidine alkaloid) and the DHP metabolites have two functional groups, each at the C7 and C9 positions, and have been demonstrated to be capable of binding to DNA and protein to form DNA cross-linking and DNA–protein cross-linking (Coulombe et al., 1999; Hincks and Coulombe, 1989; Hincks et al., 1991; Kim et al., 1995, 1999; Pereira et al., 1998; Reed et al., 1988; Tepe and Williams, 1999b). These studies were mainly conducted in cultured bovine kidney epithelial cells.

The level of DNA-protein cross-linking formation was found to correlate with animal toxicity induced by pyrrolizidine alkaloids (Kim et al., 1995). Because most of the tested pyrrolizidine alkaloids are tumorigens, formation of DNA cross-linking and DNA-protein cross-linking may lead to tumor formation. Further investigation is warranted for the confirmation of this mechanistic pathway.

To study on a structure–activity relationship basis, Coulombe and coworkers compared the capability of inducing DNA cross-linking of eight representative pyrrolizidine alkaloids in cultured bovine kidney epithelial cells in the presence of an external metabolizing system (Hincks et al., 1991; Kim et al., 1993). The compounds examined include five macrocycles (seneciphylline, senecionine, riddelliine, retrorsine, monocrotaline), two open diesters (heliosupine and latifoline), and one necine base (retronecine). The relative potency in causing DNA cross-linking and DNA-protein linking of these compounds was found to have the order of seneciphylline > riddelliine > retrorsine > senecionine > heliosupine > monocrotaline > latifoline > retronecine. In general, the level of DNA cross-linking was higher than the DNA-protein cross-linking (Hincks et al., 1991). The results also indicated that the stereochemical orientation of the ester linkage had no effect on cross-linking cellular DNA (Hincks et al., 1991). When cocultured with an NADPH-generating system in the presence of rat liver S9, seneciphylline, senecionine, riddelliine, and retrorsine, all with an α, β-unsaturated double bond, showed a dose-dependent inhibition of colony formation and induction of megalocytosis. Monocrotaline, a macrocyclic pyrrolizidine alkaloid without an α,β-unsaturated double bond, and heliosupine and latifoline, both having noncyclic diesters, slightly inhibited colony formation and had no effect on cellular morphology. Retronecine did not inhibit colony formation or induce cell morphology. These results clearly indicate that pyrrolizidine alkaloids with different structural features exhibit markedly different pyrrolizidine alkaloid-induced cytopathological effects (Kim et al., 1993).

Formation of Endogenous DNA Adducts

Segall and coworkers reported that *trans*-4-hydroxy-2-hexenal was formed from hepatic microsomal metabolism of senecionine and that it caused hepatic necrosis in

vivo (Griffin and Segall, 1986, 1989; Segall et al., 1985; Winter et al., 1986). In primary cultures of rat hepatocytes, both senecionine and *trans*-4-hydroxy-2-hexenal exhibited positive cytotoxic responses (Griffin and Segall, 1986). It was demonstrated that reaction of *trans*-4-hydroxy-2-hexenal with deoxyguanosine resulted in two pairs of diastereomeric adducts (Winter et al., 1986). The first pair of the diastereomeric adducts consisted of 3-(2-deoxy-β-D-erythro-pentofuranosyl)-5,6,7,8-tetrahydro-8*R*-hydroxy-6*S*[1-(*R* and *S*)hydroxypropyl]pyramido[1,2-a]purine-10-(3H)one, and the second pair was 3-(2-deoxy-β-D-erythro-pentofuranosyl)-5,6,7,8-tetrahydro-8*S*-hydroxy-6*R*-[1-(*R* and *S*)hydroxypropyl]pyramido[1,2-a]purine-10-(3H)one (Winter et al., 1986). As such, metabolism of senecionine leading to the *trans*-4-hydroxy-2-hexenal formation has been considered an activation pathway (Segall et al., 1985). The overall results suggest that *trans*-4-hydroxy-2-hexenal may be a tumogenic metabolite of senecionine and also possibly of other pyrrolizidine alkaloids. However, the mechanism of forming this metabolite has not been fully determined. It can be formed from enzymatic degradation of the senecionine molecule or from senecionine-induced lipid peroxidation. Miranda et al. (1981) reported that monocrotaline-induced toxicity was inhibited by the antioxidant butylated hydroxyanisole. These findings suggest that induction of lipid peroxidation by pyrrolizidine alkaloids may be involved in pyrrolizidine alkaloid-induced toxicity and tumorigenicity. This warrants further investigation.

Chemical carcinogens may exert tumorigenicity through secondary mechanisms, such as oxidative stress, hypomethylation/hypermethylation, induction of lipid peroxidation and formation of endogenous DNA adducts, induction of peroxisome proliferation, and modulation of endocrine disruptors. Besides lipid peroxidation, it has not been reported whether the other secondary mechanisms are involved in the metabolic activation of pyrrolizidine alkaloids leading to carcinogenicity.

Formation of Exogenous DNA Adducts

Riddelliine is the first pyrrolizidine alkaloid for which a mechanism of induction of liver tumors was determined in experimental animals (Yang et al., 2001a). To study the DNA adduct formation in vitro and in vivo, a ^{32}P-postlabeling/HPLC was developed (Yang et al., 2001b). Subsequently, it was determined that reaction of the synthetically prepared DHP with calf thymus DNA formed eight DHP-derived DNA adducts. Two of these adducts were identified as enantiomers of DHP-derived 7′-deoxyguanosin-N^2-yl adducts (Yang et al., 2001a), and the other six adducts were characterized as DHP-modified dinucleotides (Fig. 6a) (Chou et al., 2003a). Metabolism of riddelliine by liver microsomes of mice and rats of both sexes in the presence of calf thymus DNA also resulted in the same set of DHP-derived DNA adducts (Fig. 6b). A similar DNA adduct profile was detected in the livers of F344 female rats administered riddelliine (Fig. 6c) (Yang et al., 2001a). Similar results on the DHP-derived DNA adduct formation were also found from metabolism of other tumorigenic pyrrolizidine alkaloids, including retrorsine, monocrotaline, lasiocarpine, heliotrine, and clivorine in vitro and in vivo. The details of these results will be discussed in the following sections (Chou, unpublished data; Xia, unpublished data; Xia et al., 2003b).

A dose–response relationship was obtained between the dose administered to the rats and the levels of the eight DHP-derived adducts (Fig. 7) (Yang et al., 2001a). Results of 2-year tumorigenicity studies conducted by the National Toxicology Program

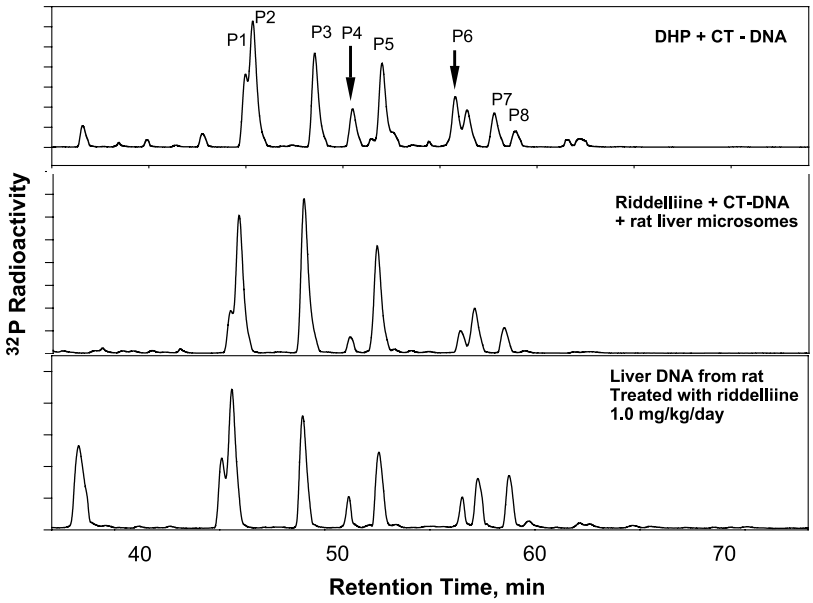

Figure 6. ^{32}P-Postlabeling/HPLC analysis of DHP-derived DNA adducts from (a) modified DNA from reaction of DHP with calf thymus DNA (CT-DNA); (b) metabolism of riddelliine by female rat liver microsomes in the presence of calf thymus DNA; and (c) livers of rats orally gavaged with 1.0 mg/kg/day 5 days/week beginning at weaning and continuing until sacrifice at 3 months (Yang et al., 2001a). The eight chromatographic peaks designated as P1, P2, P3, P4, P5, P6, P7, and P8, respectively, are the identified DHP-derived DNA adducts. The chromatographic peaks P4 and P6 are DHP-3′-dGMP adducts, and the other six adducts are DHP-derived dinucleotides (Chou et al., 2003a).

showed that riddelliine induced mainly liver hemangiosarcomas in male and female F344 rats and male B6C3F$_1$ mice (Chan et al., 2003). To examine the relationship between DNA adduct levels and the incidence of hemangiosarcomas, the levels of DHP-derived DNA adduct in purified rat and mouse liver endothelial cells, the cells of origin for the hemangiosarcomas, were determined (Chou et al., 2003a). F344 rats and B6C3F$_1$ mice were treated by gavage 5 days per week for 2 weeks with riddelliine at 1.0 mg/kg for rats and 3.0 mg/kg for mice. One, 3, 7, and 28 days after the last dose, liver parenchymal and endothelial cell fractions were isolated, and the quantities of DHP-derived DNA adduct levels were determined by ^{32}P-postlabeling/HPLC. The DHP-derived DNA adduct levels in the endothelial cells were significantly greater than in the parenchymal cells (Fig. 8). Furthermore, the DNA adduct levels in rat endothelial cells were greater than in the mouse endothelial cells (Chou et al., 2003a). Thus, these results clearly indicate that the riddelliine-induced cell-specific DNA adducts in liver cells correlate with the potency of riddelliine for the induction of liver hemangiosarcomas, and that the eight DHP-derived DNA adducts are responsible for part, if not all, of the liver tumor development.

There are two possible pathways leading to the DHP-derived DNA adduct formation from metabolism of pyrrolizidine alkaloids in vitro and in vivo. The first

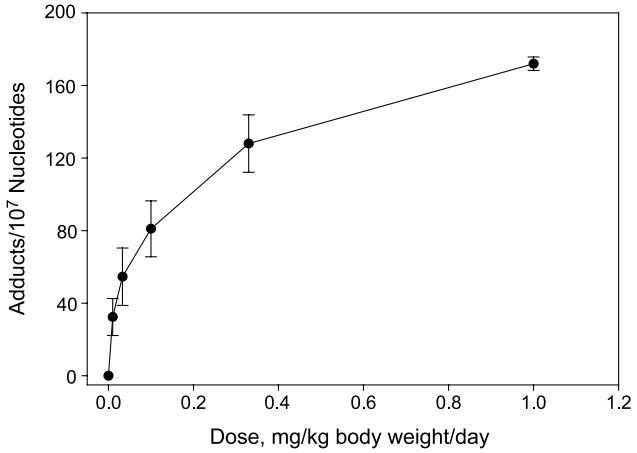

Figure 7. Dose–response of total DHP-derived DNA adduct formation in liver DNA of female rats fed riddelliine for 6 months at the doses of 0, 0.01, 0.033, 0.1, 0.33, and 1.0 mg/kg body weight/day (Yang et al., 2001a).

pathway is that dehydropyrrolizidine alkaloids covalently bind to cellular DNA to form dehydropyrrolizidine alkaloid-derived DNA adducts, which are subsequently hydrolyzed to DHP-derived DNA adducts. The second pathway is that dehydropyrrolizidine alkaloids are hydrolyzed to form DHP (IARC, 1976; Kim et al., 1999; Mattocks, 1968, 1986), which subsequently binds to DNA. At present, it is not known which pathway predominates (Fig. 9).

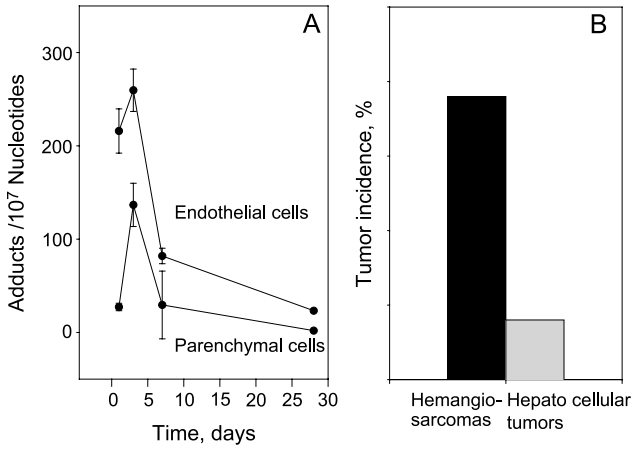

Figure 8. DHP-derived DNA adduct formation and removal in liver cells of female rats fed riddelliine for 2 weeks (Panel A), and tumor incidence of female rats treated with riddelliine for 2 years (Panel B) (Chou et al., 2003a).

Metabolism of riddelliine by male and female human liver microsomes generated DHP and riddelliine *N*-oxide (Xia et al., 2003a). When metabolism of riddelliine was in the presence of calf thymus DNA, the eight DHP-derived DNA adducts were formed (Xia et al., 2003a). These results suggest that human exposure to pyrrolizidine alkaloids may also result in the formation of these exogenous DNA adducts in vivo.

DHP-Derived DNA Adducts—Potential Biomarkers

We demonstrated that metabolism of riddelliine, retrorsine, and monocrotaline (retronecine-type), lasiocarpine and heliotrine (heliotridine-type), and clivorine (otonecine-type) as well as the necine base retronecine all form the same set of

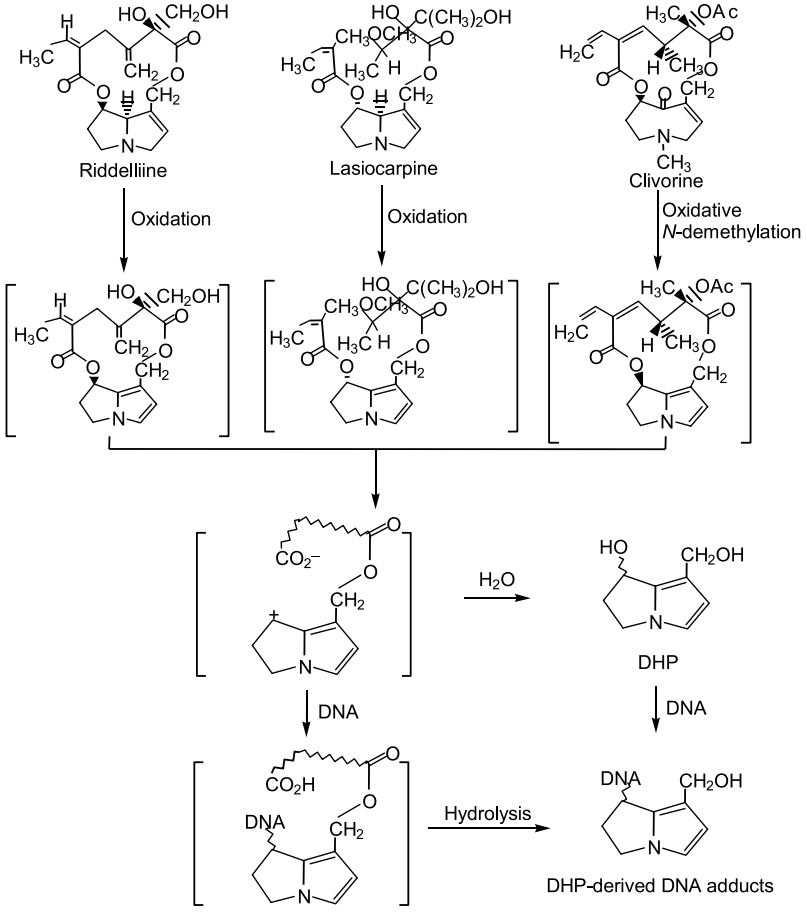

Figure 9. A proposed general mechanism leading to an identical set of DHP-derived DNA adducts formation from metabolism of the three types of carcinogenic pyrrolizidine alkaloids. For convenience, lasiocarpine, riddelliine, and clivorine are used as examples for the heliotridine-type, retronecine-type, and otonecine-type pyrrolizidine alkaloids, respectively.

DHP-derived DNA adducts in vitro and in vivo. Thus, the formation of the DHP-derived DNA adducts may be a common pathway for all three types of tumorigenic pyrrolizidine alkaloids in vitro and in vivo (Chou, unpublished data; Xia, unpublished data; Xia et al., 2003b). A general mechanism is proposed and shown in Fig. 9. This set of eight DHR-derived DNA adducts provides a potential biomarker for exposure to all carcinogenic pyrrolizidine alkaloids.

The DHP-derived DNA adducts in the blood of rats fed riddelliine were also detected in vivo (Yan et al., 2002). The results indicate that leukocyte DNA can bind with the DHP metabolite to form DHP-derived DNA adducts. These results strongly suggest that DHP-derived DNA adducts in blood may be able to serve as noninvasive biomarkers for assessing the exposure to riddelliine and other tumorigenic as well as genotoxic pyrrolizidine alkaloids (Yan et al., 2002).

SPECIES AND GENDER SPECIFICTY TOWARD METABOLISM AND TOXICITY

Pyrrolizidine alkaloid poisonings have been reported in humans and livestock, and the type and severity of pyrrolizidine alkaloid toxicity are influenced by factors including species, sex, and age (Castagnoli et al., 1997; Mattocks, 1986; Pan et al., 1993; Prakash et al., 1999; Stegelmeier et al., 1999). Significant species differences in susceptibility to the toxicity induced by pyrrolizidine alkaloids of different types were reported and studied in livestock and laboratory animals. In general, cattle, horses, pigs, chickens, ducks, rats, and mice resemble humans and are susceptible to pyrrolizidine alkaloid intoxication, whereas sheep, goats, rabbits, and guinea pigs are resistant to pyrrolizidine alkaloid toxicity, and young animals exhibit higher susceptibility than do adults (Castagnoli et al., 1997; Huan et al., 1998a; Mattocks, 1986; Prakash et al., 1999; Robertson, 1982; Stegelmeier et al., 1999; White et al., 1973).

As described above, among the major metabolic pathways, oxidative formations of the toxic pyrrolic metabolites are activation pathways, whereas hydrolysis, glutathione conjugation, and *N*-oxidation, particularly in the retronecine- and heliotridine-type pyrrolizidine alkaloids, are detoxification pathways. Thus, species difference in susceptibility to the pyrrolizidine alkaloid toxicities is mainly due to the variations in the balance between the metabolic activations to the toxic pyrrolic metabolites, like pyrrolic esters (dehydropyrrolizidine alkaloids) and the DHP-derived adducts, and the detoxification pathways to less or nontoxic metabolites such as hydrolyzed products, glutathione conjugates, and *N*-oxides (Castagnoli et al., 1997; Huan et al., 1998a; Mattocks, 1986; Prakash et al., 1999; Robertson, 1982; Stegelmeier et al., 1999; White et al., 1973). Furthermore, the hepatic biotransformation mainly contributes to this metabolic variation (Cheeke, 1994), and rumen metabolism also at least partially accounts for the ruminant animals (Stegelmeier et al., 1999; Wachenheim et al., 1992).

Most of the investigations on the species difference have been focused on the retronecine-type pyrrolizidine alkaloids. An early acute toxicity study of retrorsine in different species of the male animals performed in 1973 (White et al., 1973) demonstrated that the male rat (LD_{50} 34 mg/kg, i.p.) is highly susceptible, followed by the mouse (LD_{50} 65 mg/kg), hamster (LD_{50} 81 mg/kg), and fowl (LD_{50} 85 mg/kg),

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while quail (LD_{50} 279 mg/kg) and guinea pig (LD_{50} > 800 mg/kg) are resistant. The acute hepatotoxicity induced by retrorsine in animals was also evaluated and correlated to the amount of the bound pyrroles in the liver at 2 hours after administration of retrorsine. A recent study on the in vitro microsomal metabolism of senecionine in eight animal species did not show a strong correlation between the formations of pyrrolic metabolites and susceptibility of these animals to senecionine intoxication, but the correlation was not made on the specific toxic tissue-bound pyrroles in the liver (Huan et al., 1998b). Furthermore, a study using a well-established trapping technique to quantify the dehydropyrrolizidine alkaloid (pyrrolic ester) formed in the in vitro microsomal metabolism of pyrrolizidine alkaloids revealed that the ability of microsomal biotransformation of pyrrolizidine alkaloids to the pyrrolic esters correlates with the acute toxicity of the parent pyrrolizidine alkaloids in the rat (Huxtable and Wild, 1994). Based on the currently available data, it is generally recognized that the susceptible species show high formation rates for producing pyrrolic metabolites, especially the toxic pyrrolic esters and tissue-bound pyrroles.

Except for jacobine (Chung and Buhler, 1995), studies on guinea pig resistance to the retronecine-type pyrrolizidine alkaloids, including the most frequently studied monocrotaline (Cheeke and Pierson-Goeger, 1983; Dueker et al., 1992a) and retrorsine (White et al., 1973), demonstrated that metabolic rates for hydrolysis detoxification pathways are significantly high, due to the high esterase activity toward pyrrolizidine alkaloids in live guinea pigs. Therefore, the high metabolic rates for the hydrolysis detoxifications have been regarded as one of the main reasons responsible for the resistance of the guinea pig to the toxicities of the retronecine-type pyrrolizidine alkaloids (Dueker et al., 1992a; Hosokawa et al., 1990; Prakash et al., 1999; Stegelmeier et al., 1999). In the case of metabolism of jacobine in the guinea pig, the activity of esterases catalyzing hydrolysis was lower, which was considered one of the causes of jacobine intoxication in guinea pig (Chung and Buhler, 1995; Stegelmeier et al., 1999).

The low rates of pyrrolic metabolite production in sheep may explain the resistance to senecionine in sheep (White et al., 1973). Studies on the toxicity of jacobine in sheep suggested that detoxification of the parent pyrrolizidine alkaloid by the ruminal biotransformations mediated by bacteria is most likely one of the reasons for the resistance to pyrrolizidine alkaloids in sheep (Wachenheim et al., 1992). Moreover, a high activity of hepatic microsomal epoxide hydrolase was determined in sheep. This enzyme extensively catalyzes the hydrolysis of senecio pyrrolizidine alkaloids, and thus was suggested to also be accountable for sheep resistance to senecio pyrrolizidine alkaloids (Swick et al., 1983).

Cui (1999) and Lin et al. (2001, 2002, 2003) studied the species difference in susceptibility to the hepatotoxicity induced by otonecine-type pyrrolizidine alkaloids. As illustrated in Fig. 10, the in vitro metabolic activation of clivorine in the male rat and humans of both sexes was similar but different from that in the guinea pig. The higher activation rates for the generation of the reactive pyrrolic ester followed by the formation of the toxic tissue-bound pyrroles mainly contribute to the high susceptibility of humans of both sexes and the male rat to clivorine hepatotoxicity. This indicates that humans may also be susceptible to otonecine-type pyrrolizidine alkaloid intoxication (Cui, 1999; Lin et al., 2001, 2002). Similar to the metabolism of the retronecine-type

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pyrrolizidine alkaloids in the guinea pig (Hosokawa et al., 1990; Prakash et al., 1999), significantly higher metabolic rates for two hydrolysis pathways were found in guinea pig hepatic metabolism of clivorine (Lin et al., 2003). These include the direct hydrolysis of clivorine to clivoric acid, which is considered a nontoxic hydrophilic metabolite and is readily excreted, and the hydrolysis of the reactive pyrrolic ester to produce less toxic DHP. Furthermore, a significantly low formation rate for the toxic tissue-bound pyrroles was observed in the guinea pig. The higher metabolic rates for the hydrolysis in combination with a lower rate for the formation of toxic DHP-derived adducts play a key role in guinea pig resistance to clivorine intoxication (Lin et al., 2003).

Gender difference in the susceptibility to pyrrolizidine alkaloids of different types was also reported. Most of the studies on the delineation of the mechanisms underlying such difference are conducted in rats and mice. The male rat was reported to be more susceptible than the female rat to retronecine-type pyrrolizidine alkaloids, such as riddelliine (Chan, 1993, 2001; Chan et al., 1994), senecionine (Candrian et al., 1985; Chung and Buhler, 1994; Williams et al., 1989a), seneciphilline (Candrian et al., 1985),

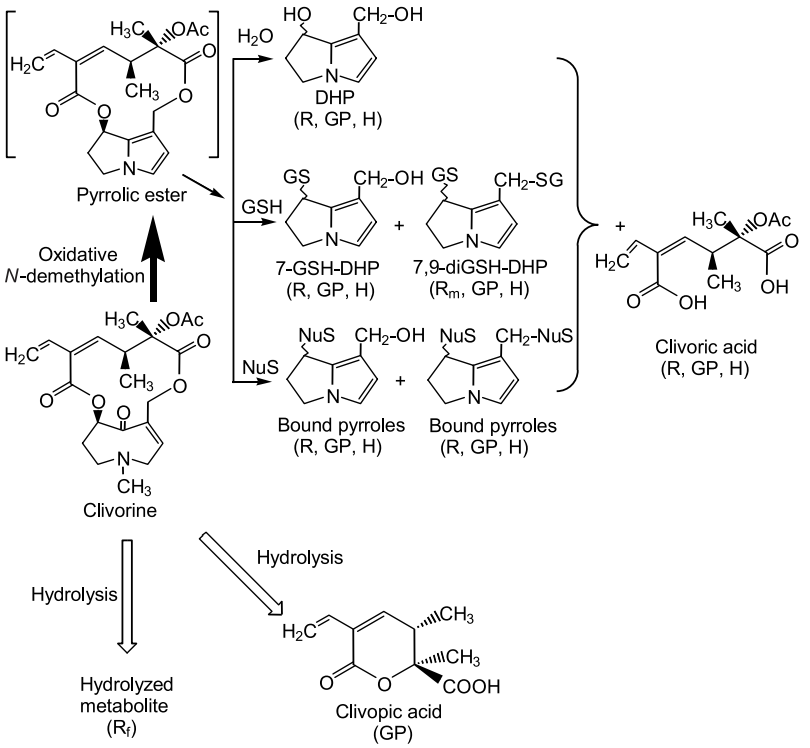

Figure 10. Microsomal metabolisms of clivorine in rat (R), guinea pig (GP), and human (H). NuS: nucleophilic biological macromolecules. R_m: the male rat. R_f: the female rat. ➡ The predominant pathways in the male rat and humans of both sexes. ⇨ The predominant pathways in the female rat and guinea pigs of both sexes.

retrorsine (Mattocks, 1972), and monocrotaline (Mattocks, 1972). Most of the studies try to link this gender difference to the metabolic profiles of pyrrolizidine alkaloids.

The National Toxicology Program 2-year toxicity study of riddelliine in F344/N rats revealed that the male rat is much more sensitive to pyrrolizidine alkaloid intoxication than the female rat, shown by the high mortality of the male rats administered riddelliine (1 mg/kg/day, 5 days per week). The study had to be terminated at week 72, whereas the same study in the female rats lasted for 2 years (Chan, 2001). In this study, the tumorigenicity of riddelliine in B6C3F1 mice was also investigated, and it is interesting to note that only the male mice developed liver tumors. The kinetics of two metabolic pathways, *N*-oxidation and DHP formation, were evaluated but could not be related to such a gender difference (Williams et al., 2002). This indicates that other factors including the formation of the toxic metabolites, such as the pyrrolic ester, bound pyrroles, and DHP-derived DNA adducts, which are believed to directly cause genotoxicities, may be responsible for the observed sex difference in tumorigenicity.

The study on monocrotaline and retrorsine correlated their acute hepatotoxicity to the amount of pyrrolic metabolites found in the livers of rats (Mattocks, 1972). The induction of cytochrome P450 by phenobarbital increased the susceptibility of the female rats. On the other hand, pretreatment of the male rats with cytochrome P450 inhibitor SKF 525A decreased both the toxicity and the formation of pyrrolic metabolites in the liver of the male rats. Studies on senecionine linked the gender difference in SD rats to the variations in the hepatic metabolism and the enzymes involved (Chung and Buhler, 1994; Williams et al., 1989a). The formation rates for the pyrrole-related metabolites DHP (reflecting the activation pathway) and *N*-oxide (reflecting the detoxification pathway) in the male rats were higher and were mediated by the male-specific CYP3A (cytochrome P450 PCN-E) and CYP2C11 (P450 UT-A), respectively (Williams et al., 1989a). Using the specific antibodies, *N*-oxidation was significantly reduced after inhibition of CYP2C11, whereas the rates for both *N*-oxidation and DHP formation decreased with CYP3A inhibition (Williams et al., 1989a). Furthermore, with an induction of CYP3A1, the female rats exhibited significant increase in both DHP and senecionine *N*-oxide production (Chung and Buhler, 1994). These results demonstrated that the male-specific isozymes CYP3A and CYP2C11 catalyzed biotransformations that might result in the marked gender difference in senecionine toxicity in rats.

Results of the studies on the otonecine-type pyrrolizidine alkaloid clivorine also evidenced similar gender differences in SD rats and explained the mechanism responsible for such gender difference (Lin et al., 2001, 2003). The male rat is more susceptible to clivorine intoxication, because clivorine is predominantly metabolized via the metabolic activation pathway leading to toxicity. This metabolic activation is primarily mediated by CYP3A1 and CYP3A2, the well-known isozymes expressed in the male rat but with significantly lower levels in the female rat (Imaoka et al., 1991; Mahnke et al., 1997; Ribeiro and Lechner, 1992; Waxman et al., 1985). In the female rat, clivorine was predominantly hydrolyzed by microsomal carboxylesterases, and the formation of the toxic pyrrolic ester via the metabolic activation was the minor pathway (Fig. 10). Therefore, the lack of CYP3A1 and CYP3A2 activities leading to a significantly low metabolic activation rate is considered as one of the main reasons that the female rat is less susceptible to clivorine intoxication.

To date, there are no reports on a gender difference in the susceptibility of humans to pyrrolizidine alkaloid intoxication. It is unlikely that such gender difference occurs in humans, because there are no evidences of exhibiting significant variations of human expressions of CYP3A4, the key enzyme responsible for the metabolic activation of pyrrolizidine alkaloids in humans. However, it is well known that the abundance of CYP3A4 in the liver varies significantly in individuals and in different ethnic groups, which may result in marked variations in the metabolic profile and thus different susceptibilities toward pyrrolizidine alkaloid intoxication. Furthermore, variations in the activity of CYP3A4 occur in concurrent therapy from drug–drug or herb–drug interactions (Herrlin et al., 2000; Ioannides, 2002; Wandel et al., 2000). These variations may significantly affect the CYP3A4-related pyrrolizidine alkaloid intoxication in patients concurrently taking pyrrolizidine alkaloid-containing herbal remedies with medicinal drugs.

STUDY OF REPRESENTATIVE PYRROLIZIDINE ALKALOIDS

Retronecine-Type Pyrrolizidine Alkaloids

Riddelliine

Riddelliine is a 12-membered macrocyclic diester pyrrolizidine alkaloid with an α,β-unsaturated double bond linked to the ester group at the C7 position of the necine base. Riddelliine, isolated from plants of the genera *Senecio*, *Crotalaria*, and *Amsinckia*, produced in plants growing in range lands of the western United States, is one of the most studied pyrrolizidine alkaloids (Chan, 1993; Chan et al., 1994, 2003; Fu et al., 2002b; Mattocks, 1968, 1986). Riddelliine exhibits a variety of toxic activities, including acute toxicity, mutagenicity (Chan et al., 1994; Zeiger et al., 1988), sister chromatid exchanges, chromosomal aberrations (Galloway et al., 1987), unscheduled DNA synthesis (MacGregor et al., 1985; Mirsalis, 1987; Mirsalis et al., 1983), DNA cross-linking, DNA-protein cross-linking (Hincks et al., 1991; Kim et al., 1995) inhibition of colony formation, and megalocytosis (Kim et al., 1993). Livestock were poisoned by grazing plants containing riddelliine (Chan et al., 1994; Mattocks, 1968). The riddelliine-containing plants may contaminate human food sources used as staple food with the plants or the seeds, or as residues present in milk and honey (Chan et al., 2003). Due to its genotoxicity and potential for human exposure, riddelliine was nominated by the U.S. Food and Drug Administration to the National Toxicology Program (NTP) for genotoxicity and carcinogenicity testing (Hill et al., 1997; Seaman, 1978). It was found from the NTP 2-year carcinogenicity bioassay that riddelliine induced mainly liver hemangiosarcomas in male and female F344 rats and male $B6C3F_1$ mice (Chan, 2001; Chan et al., 2003).

Metabolism of riddelliine by liver microsomes of F344 female rats generated riddelliine *N*-oxide and DHP as major metabolites (Figs. 3 and 9) (Fu et al., 2002b; Yang et al., 2001a). Metabolism was enhanced when liver microsomes from phenobarbital-treated rats were used. Xia et al. (2003a) performed a comparative study on the metabolism of riddelliine by human and rat liver microsomes. It was found

that, from human liver microsomal metabolism, DHP and riddelliine *N*-oxide were the major metabolites, with the levels of 0.20–0.62 and 0.03–0.15 nmol/min/mg protein, respectively. These results are comparable to those obtained from rat liver microsomal metabolism. When metabolism was conducted in the presence of the CYP3A4 enzyme inhibitor, triacetyleandomycin, the formation of DHP and riddelliine *N*-oxide was reduced 84% and 92%, respectively. For DHP formation, the K_m and V_{max} values were determined to be 0.37 ± 0.05 mM and 0.48 ± 0.03 nmol/min/mg protein for female rats and 0.66 ± 0.08 mM and 1.70 ± 0.09 nmol/min/mg protein for female humans, respectively. These results are consistent with those reported on the metabolism of senecionine to DHP by male and female human liver microsomes (Miranda et al., 1991a).

Metabolism in the presence of calf thymus DNA resulted in eight DHP-derived DNA adducts that were identical to those obtained from the reaction of DHP with calf thymus DNA. Two of these adducts were identified as DHP-modified 7-deoxyguanosin-*N*(2)-yl epimers (DHP-3′-dGMP); the other six were DHP-derived dinucleotide adducts (Chou et al., 2003a; Yang et al., 2001a). A similar DNA adduct profile was detected in the livers of female F344 rats fed riddelliine, and a dose–response relationship was obtained for the level of the total (eight) DHP-derived DNA adducts in vivo. In rats and mice, riddelliine *N*-oxide and a hydrolyzed metabolite, retronecine, were the only circulating metabolites observed, presumably because the higher reactive DHP can bind to the macromolecules (e.g., proteins) in the blood (Williams et al., 2002). The DHP-derived DNA adducts were also identified in the blood of male and female rats treated with riddelliine (Yan et al., 2002). These results suggest that riddelliine induces liver tumors in rats through a genotoxic mechanism, and the eight DHP-derived DNA adducts are likely to contribute to liver tumor development. Thus, riddelliine represents the first pyrrolizidine alkaloid with which the levels of DNA adduct formation in vivo correlated with liver tumor potency. Thus, DNA adduct formation is responsible for liver tumors in rats fed riddelliine (Yang et al., 2001a). It was found that CYP2B and CYP3A isozymes are the major metabolizing enzymes responsible for riddelliine metabolism (Omiecinski et al., 1999). These results are consistent with those reported in the literature (Coulombe et al., 1999; Griffin and Segall, 1986; Kim et al., 1999; Lin et al., 2000a; Mattocks, 1986; Petry et al., 1986).

Metabolism of riddelliine *N*-oxide by liver microsomes of F344 female rats generated riddelliine and DHP as the major metabolites (Figs. 3 and 9) (Chou et al., 2003b). Metabolism of riddelliine *N*-oxide in the presence of calf thymus DNA resulted in formation of the same eight DHP-derived DNA adducts that have been shown to be responsible for riddelliine-induced liver tumor formation. The same DNA adduct profile was detected in livers of female F344 rats fed riddelliine *N*-oxide (Chou et al., 2003b). These results suggest that pyrrolizidine alkaloid *N*-oxides can induce liver tumors in experimental animals. Thus, the risk of human exposure to pyrrolizidine alkaloid *N*-oxides has to be assessed.

Retrorsine

Similar to riddelliine, retrorsine is a 12-membered macrocyclic diester pyrrolizidine alkaloid with an α,β-unsaturated double bond linked to the ester group at the C7 position of the necine base. Retrorsine has been identified in many plants, such as

Senecio spp., at different regions of the world. Because of being highly hepatotoxic, it poisoned livestock (Arzt and Mount, 1999; Gordon et al., 2000b; Habermehl et al., 1988). Retrorsine induced DNA cross-linking in cultured bovine kidney epithelial cells in the presence of an external metabolizing system (Hincks et al., 1991). When cocultured with an NADPH-generating system and rat liver S9 fraction, retrorsine showed a dose-dependent inhibition of colony formation and induction of megalocytosis (Kim et al., 1993). Retrorsine also exhibits clastogenic activity (Kevekordes et al., 2001; Muller et al., 1992). An increased number of micronuclei was found in the Hep-G2 cell line treated with retrorsine in the presence of the S9 fraction (Kevekordes et al., 2001).

Metabolism of retrorsine in rats in vivo formed isatinecic acid, pyrrolic metabolites, retrorsine *N*-oxide, and retronecine (Chu et al., 1993; Mattocks and White, 1973). Rats pretreated with phenobarbital displayed increased formation of pyrrolic metabolites and isatinecic acid. (Chu et al., 1993). Pyrrolic metabolites were detected in the bile of rats fed retrorsine (White, 1977).

Mice, hamsters, and rats are species susceptible to retrorsine-induced toxicity, while guinea pigs are the resistant species (Chu and Segall, 1991). It was found that mice, hamsters, and rats fed retrorsine excreted isatinecic acid and pyrrolic metabolites with quantities higher than those from guinea pigs (Chu and Segall, 1991). On the other hand, the levels of the urinary *N*-oxides were higher in guinea pigs than in mice, hamsters, and rats. These results suggested that a common metabolic pathway exists between the formation of isatinecic acid and pyrrolic metabolites and that the resistance of guinea pigs to pyrrolizidine alkaloid poisoning is attributed to the high formation rate yield of corresponding *N*-oxide derivatives and the relatively low formation rate of pyrrolic metabolites (Chu and Segall, 1991).

Liver microsomal metabolism of retrorsine in general produced DHP and retrorsine *N*-oxide (Chan et al., 1989; Couet et al., 1996; Kedzierski and Buhler, 1986b). The in vitro metabolism of retrorsine, monocrotaline, *Crotalaria* extract and *Eupatorium japonicum* extracts by mouse liver microsomes was compared (Chan et al., 1989). All produced pyrrolic metabolites and the corresponding *N*-oxide. While the rate of pyrrolic metabolite formation from retrorsine was much higher than the others, the rate of *N*-oxide formation was *Crotalaria* extract = monocrotaline > *Eupatorium japonicum* extract > retrorsine (Chan et al., 1989). Metabolism of retrorsine by human and rat liver microsomes produced DHP and retrorsine *N*-oxide (Couet et al., 1996). The generated pyrrolic ester, dehydrosenecionine, dehydromonocrotaline, and dehydroretronecine, were potent inducers of abnormal cellular morphology, and were also more active in the inhibition of colony formation than their parent compounds (Kim et al., 1993).

Retrorsine enhanced or induced expression of hepatic CYPs 1A1, 1A2, 2E1, and 2B1/2 in rats. These results suggest that one or more of these enzymes may be involved in retrorsine metabolism (Gordon et al., 2000a). Effects of senecionine, retrorsine, and seneciphylline on aminopyrine *N*-demethylase activity on the rat liver S-10 enzymes were studied. Both senecionine and seneciphylline inhibited the aminopyrine *N*-demethylase activity, while retrorsine exhibited as a competitive inhibitor (Eastman and Segall, 1981). Rats dosed with retrorsine increased glutathione concentration but decreased cytochrome P450 concentration in the liver (White, 1976). Rats treated with retrorsine, heliotrine, indicine, lasiocarpine, and senecionine formed sulfur-conjugated pyrrolic metabolites in blood and liver tissue (Mattocks and Jukes, 1992a).

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Lin et al. reported that rats pretreated with glycyrrhizin and glycyrrhetinic acid significantly inhibited retrorsine-induced hepatotoxicity (Lin et al., 1999). The conjugate 7-glutathionyl-dehydroretronecine, a pneumotoxic pyrrolic metabolite, was found in the bile when rat liver was perfused with monocrotaline or retrorsine, but was not formed from heliotrine, a pyrrolizidine alkaloid without a C7-ester function group (Mattocks et al., 1991).

The in vitro and in vivo metabolic activations of retrorsine *N*-oxide, in terms of DHP-derived DNA adduct formation were studied (Wang et al., unpublished data). The parent compound, retrorsine, and DHP were formed as the major metabolites, and a similar set of DHP-derived DNA were detected in the liver DNA of rats fed with retrorsine *N*-oxide by the ^{32}P-postlabeling/HPLC analysis. Because both retrorsine and DHP are tumorigenic, this result suggests that retrorsine *N*-oxide may be tumorigenic.

Senecio latifolius DC, an herbal plant, contains retrorsine and other toxic pyrrolizidine alkaloids. Steenkamp et al. (2001) found that the *Senecio latifolius* DC plant extracts caused gross morphological changes on human HuH-7 cells in a dose-dependent manner. It was proposed that chronic low-dose treatment with *Senecio latifolius* DC as well as the other pyrrolizidine alkaloid-containing traditional remedies may be teratogenic or carcinogenic to humans (Steenkamp et al., 2001).

Senecionine

Senecionine is a 12-membered macrocyclic diester pyrrolizidine alkaloid with an α,β-unsaturated double bond linked to the ester group at the C7 position of the necine base. Like riddelliine, retrorsine, monocrotaline, and lasiocarpine, senecionine is one of the most commonly studied hepatotoxic and tumorigenic pyrrolizidine alkaloids (Edgar et al., 1992; IARC, 1976; Mattocks, 1968, 1986; W.H.O. (WHO), 1988). Senecionine was detected in many plant species, including several *Senecio* spp. common in southern Brazil, Uruguay, and Paraguay (Arzt and Mount, 1999; Copper et al., 1996; Habermehl et al., 1988; Mattocks, 1968)]. Senecionine can be identified and quantitated by several different analytical methods, such as GC/MS, and competitive enzyme-linked immunoassay (Langer et al., 1996; Mroczek et al., 2002; Roeder and Pflueger, 1995; Zalkow et al., 1988). Like other pyrrolizidine alkaloids, senecionine has been found as a contaminant in the food chain, such as in honey of the Ragwort (*Senecio jacobaea*) (Crews et al., 1997).

Senecionine is hepatotoxic, genotoxic, and tumorigenic (Mattocks, 1968). It induced cross-link cellular DNA in cultured bovine kidney epithelial cells (Hincks et al., 1991). When cocultured with an NADPH-generating system and rat liver S9 fraction, senecionine showed a dose-dependent inhibition of colony formation and induction of megalocytosis (Kim et al., 1993). The pyrrolic metabolite was found to be more active in the inhibition of colony formation than the parent compound and was a potent inducer of abnormal cellular morphology (Kim et al., 1993).

Metabolism of senecionine was studied in many different enzymatic systems, including liver microsomes of rats and guinea pigs (Chung and Buhler, 1994; Kedzierski and Buhler, 1986a; Mattocks and Driver, 1987; Miranda et al., 1991b; Ramsdell and Buhler, 1987; Reed et al., 1992; Williams et al., 1989a; Winter et al., 1988a). Rat liver microsomal metabolism of senecionine produced DHP and senecionine *N*-oxide as the major metabolites (Reed et al., 1992). The use of a PRP-1

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column for HPLC separation was found to be highly effective for separation of these metabolites (Reed et al., 1992). Metabolism in the presence of glutathione resulted in the formation of a conjugate of DHP with glutathione (Reed et al., 1992). The *N*-oxidation of senecionine catalyzed by flavin-containing monooxygenase is considered a detoxification pathway. It was determined that the relative contribution of flavin-containing monooxygenase and cytochrome P450 to the detoxification pathway may be species and tissue dependent (Williams et al., 1989b).

Chung et al. (1995) determined that CYP2B isoform is the major bioactivation enzyme of senecionine in the guinea pig. It was found that CYP3A is the major enzyme mediating metabolic activation and detoxification of senecionine in sheep and in hamster species, whereas CYP2B enzyme is less efficient (Huan et al., 1998a). CYP3A4 is the major enzyme catalyzing the bioactivation (DHP formation) and detoxification (senecionine *N*-oxide formation) of senecionine in the human liver (Miranda et al., 1991a).

Rats displayed a marked sex difference in the oxidation of senecionine, especially in the formation of senecionine *N*-oxides (Williams et al., 1989a). The marked sex difference in senecionine *N*-oxidation was believed to be due to the specificity of CYP2C11and CYP3A isozymes (Williams et al., 1989a).

The substrate specificity of the two guinea pig liver microsomal carboxylesterases GPL1 and GPH1 to a mixture of senecionine and integerrimine was determined (Dueker et al., 1992b). GPH1 was able to hydrolyze these two pyrrolizidine alkaloids. This may explain the resistance of guinea pig to pyrrolizidine alkaloid intoxication. Excretion and blood radioactivity levels following intravenous administration of [^{14}C]senecionine administration in the rat were studied (Estep et al., 1990b). The total radioactivity levels excreted in the bile and urine were nearly equal. Senecionine *N*-oxide was identified as the major metabolite in bile and urine (Estep et al., 1990b).

Species differences in metabolism of senecionine to the pyrrolic metabolites and senecionine *N*-oxide by liver microsomes of eight animal species, including sheep, cattle, gerbils, rabbits, hamsters, Japanese quail, chickens, and rats, were studied (Huan et al., 1998b). It was found that the production of pyrrolic metabolites was not correlated with susceptibility of pyrrolizidine alkaloid-induced toxicity to the animals (Huan et al., 1998b).

A comparison of the metabolism of senecionine in vitro by hepatic microsomes of rat, guinea pig, cow, horse, and sheep was conducted (Winter et al., 1988b). The level of DHP formed was higher in the guinea pig than in other species.

The binding of the reactive pyrrolic metabolites of senecionine to glutathione was extensively studied (Estep et al., 1990a; Reed et al., 1992). The *N*-acetylcysteine conjugate of DHP was identified in the urine of rats that were administered senecionine (Estep et al., 1990a).

The compound *trans*-4-hydroxy-2-hexenal was formed from hepatic microsomal metabolism of senecionine (Griffin and Segall, 1986, 1987a,b, 1989; Segall et al., 1985; Winter et al., 1986). This compound caused hepatic necrosis in vivo and exerted positive cytotoxic response in primary cultures of rat hepatocytes (Griffin and Segall, 1986). It reacted with deoxyguanosine to form two pairs of diastereomeric adducts (Winter et al., 1986). Both senecionine and *trans*-4-hydroxy-2-hexenal induced lipid peroxidation in isolated rat hepatocytes, although lipids peroxidation is not entirely

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responsible for the cellular damage (Griffin and Segall, 1987a). Apparently, the mechanisms by which senecionine induces liver tumors in rodents were not exclusively elucidated.

Monocrotaline

Structurally, there are two distinct differences between monocrotaline and the above-described retronecine-type pyrrolizidine alkaloids, e.g., riddelliine, retrorsine, and senecionine. First, monocrotaline is an 11-membered macrocyclic diester pyrrolizidine alkaloid, not a 12-membered macrocyclic diester compound. Second, it does not have an α,β-unsaturated double bond linked to the ester group at the C7 position of the necine base. As such, chemically and biologically, monocrotaline is less active than riddelliine and retrorsine. The structure–activity studies confirmed that monocrotaline exhibits toxicities lower than riddelliine, retrorsine, and several other macrocyclic diester pyrrolizidine alkaloids (Mattocks, 1968).

Monocrotaline is mutagenic, clastogenic, and tumorigenic (Muller et al., 1992). Monocrotaline has been a model chemical for studying pulmonary hypertension. It causes a pulmonary vascular syndrome in rats characterized by proliferative pulmonary vasculitis, and pulmonary hypertension, with the pulmonary vascular endothelium likely being the early target of the reactive monocrotaline pyrrole metabolite (Bruner et al., 1986; Roth and Reindel, 1991; Wilson et al., 1992). Monocrotaline induced micronucleus formation in human lymphocytes and in the human hepatoma cell line Hep-G2 (Kevekordes et al., 2001).

Metabolism of monocrotaline has been most studied among pyrrolizidine alkaloids (Araya and Fuentealba, 1990; Arzt and Mount, 1999; Bah et al., 1994; Baker et al., 1991; Chan et al., 1994; Chung and Buhler, 1995; Curran et al., 1996; Hill et al., 1997; Ingolfsdottir and Hylands, 1990; Mattocks, 1968, 1971a,b; McLean, 1970; Newberne and Rogers, 1973; Phillipson, 1971; Seaman, 1978, 1987; Schoental et al., 1954; Svoboda and Reddy, 1972; W.H.O. (WHO), 1988). Metabolism to the reactive pyrrolic metabolites is required to produce pneumotoxicity. Monocrotaline causes a syndrome in rats that has been utilized as a animal model for the study of human primary pulmonary hypertension (Reid et al., 1998). Monocrotaline is metabolically activated by liver cytochrome P450 enzymes to the pyrrolic metabolites, which caused injury of the lung endothelium and resulted in the development of pulmonary hypertension in rats (Kasahara et al., 1997).

The CYP3A was found to be the major isozyme for metabolic activation of monocrotaline to the reactive pyrrolic metabolites in rat liver (Kasahara et al., 1997; Reid et al., 1998). The metabolism and covalent binding of [^{14}C]monocrotaline in SD rat liver microsomes in the presence of the inducers dexamethasone, clotrimazole, pregnenolone-16 α-carbonitrile, and phenobarbital, respectively, were studied (Reid et al., 1998).

Monocrotaline inhibited the liver drug-metabolizing enzymes in rats (Dalvi, 1987). In adult male SD rats given 20 mg/kg of monocrotaline intraperitoneally, significant changes in microsomal content of cytochrome P450 and activities of benzphetamine *N*-demethylase and aniline hydroxylase were not observed. However, at a dose of 80 mg/kg, there was a significant reduction of cytochrome P450 activity and a marked elevation of serum sorbitol dehydrogenase and glutamic pyruvic transaminase activities.

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Metabolism of [^{14}C]monocrotaline by isolated perfused rat liver resulted in the formation of monocrotalic acid as the major acidic metabolite (Lame et al., 1991). 1-Formyl-7-hydroxy-6,7-dihydro-5H-pyrrolizine, DHP, and 1-hydroxymethyl-7-oxo-6,7-dihydro-5H-pyrrolizine were formed in trace amounts (Lame et al., 1991). However, retronecine was not formed. These results suggest that formation of pyrrolic metabolites, which subsequently react with cellular nucleophiles, is the major pathway (Lame et al., 1991).

Strain differences in the response of Fischer 344 and SD rats to monocrotaline-induced pulmonary vascular disease were found (Pan et al., 1993). The metabolisms of [^{14}C]monocrotaline by rat and guinea pig hepatic microsomes were compared (Dueker et al., 1992a). The involvement of carboxylesterases and cytochrome P450 was determined by metabolism of this compound in the presence of triorthocresylphosphate and carbon monoxide, respectively. While enzymatic hydrolysis was predominant in the metabolism in the guinea pig; the rat exhibited no esterase activity. These results may explain the guinea pig's resistance to pyrrolizidine alkaloid toxicity. Dehydromonocrotaline was found to directly cause immunotoxicity in C57BL/6 mice in vivo, with the activity much more potent than monocrotaline (Deyo et al., 1994). However, a role for the dehydromonocrotaline metabolite in monocrotaline immunotoxicity in vitro has not yet been demonstrated.

Study of the kinetics of monocrotaline metabolism in the rat found that red blood cells may act as the carriers of metabolites from the liver to the lung and may play a significant role in pulmonary toxicity (Estep et al., 1991).

Binding of the reactive pyrrolic metabolites of monocrotaline to glutathione was extensively studied (Estep et al., 1990a; Glowaz et al., 1992; Huxtable et al., 1991; Lame et al., 1990, 1995; Mattocks and Jukes, 1992a; Mattocks et al., 1991; Yan and Huxtable, 1995a,b, 1996b, 1998). The *N*-acetylcysteine conjugate of DHP was identified in the urine of rats that were administered monocrotaline (Estep et al., 1990a). Pyrrolic metabolites in biliary excretion of monocrotaline-treated rat were identified (Lame et al., 1990, 1995; Mattocks and Jukes, 1992a; Mattocks et al., 1991). The identified metabolites included glutathione and cysteinyl–glycine conjugates of DHP and 1-formyl-7-hydroxy-6,7-dihydro-5H-pyrrolizine. Also found was 7-glutathionyl-dehydroretronecine (Glowaz et al., 1992; Huxtable et al., 1991; Mattocks et al., 1991).

The binding of monocrotaline to DNA in different enzymatic activation systems was found (Hoorn et al., 1993; Thomas et al., 1996, 1998). Cultured rat pulmonary endothelium treated with dehydromonocrotaline results in delayed and progressive pneumotoxicity, formation of DNA cross-linking, covalent binding to DNA, cell cycle arrest, and delayed but progressive cell death (Hoorn et al., 1993; Thomas et al., 1998). Monocrotaline induced DNA–DNA interstrand cross-links in a dose-dependent manner (Petry and Sipes, 1987). A significant increase in the number of micronuclei was found in the cell line Hep-G2 incubated with monocrotaline in the presence of S9-mix (Kevekordes et al., 2001).

Monocrotaline-mediated DNA adduct formation in vitro and in vivo was studied in our laboratory (unpublished data). The female F344 rats treated with monocrotaline produced the same eight DHP-derived DNA adducts as those from the metabolism of riddelliine in vitro and in vivo. Similar results were obtained when there was incubation

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of monocrotaline by rat liver microsomes in the presence of calf thymus DNA. These results provide evidence that DHP-derived DNA adduct formation is involved in the metabolic activation of retronecine-type pyrrolizidine alkaloids. These results combined with those described previously suggest that these DHP-derived DNA adducts are potential biomarkers of pyrrolizidine alkaloid exposure and tumorigenicity.

Metabolism of monocrotaline *N*-oxide by rat and human liver microsomes was studied (Wang et al., unpublished data). Both monocrotaline and DHP were formed as the major metabolites, suggesting that monocrotaline *N*-oxide may be tumorigenic.

Heliotridine-Type Pyrrolizidine Alkaloids

Lasiocarpine

Lasiocarpine is the prototype of heliotridine-type pyrrolizidine alkaloid. It induced liver tumors in F344 rats as assayed by the National Toxicology Program (1990). Lasiocarpine induced mutation, chromosomal aberrations, and cytoplasmic vacuolization, and caused cellular and nuclear enlargement in V79 Chinese hamster cells (Takanashi et al., 1980). It also induced *Drosophila* mutagenicity (Yoon et al., 1985).

Rats treated with lasiocarpine showed chronic and progressive lesions in the liver, forming a long-lasting block in the cell cycle, megalocytosis, fibrosis, cirrhosis, and malignant neoplasma (Laconi et al., 1995). Laconi et al. (1995) reported that transplantation of normal hepatocytes is able to modulate the development of lasiocarpine-induced chronic lesions in male Fischer 344 rat liver and suggested that this may be relevant to the pathogenesis of progressive liver diseases such as neoplasia and cirrhosis (Laconi et al., 1995).

Heliotropium dolosum seeds contains about 0.1% pyrrolizidine alkaloids, of which lasiocarpine is the predominant component ($\sim$ 80%), while heliosupine accounts for 12%, and echimidine and heliotrine are present in small amounts (Eroksuz et al., 2001). Swiss mice fed diets containing different doses of *Heliotropium dolosum* seeds showed hepatomegalocytosis, renal tubular megalocytosis, and animal deaths in a dose-dependent manner (Eroksuz et al., 2001).

Rats given a single injection of several hepatotoxic pyrrolizidine alkaloids including monoesters (heliotrine, indicine), a diester (lasiocarpine), and macrocyclic diesters (retrorsine and senecionine), respectively, resulted in the formation of sulfur-bound pyrrolic metabolites identified in the blood and liver tissue. The proximal pyrrolic metabolites lead to *S*-binding by attacking at the C9 position in dehydroheliotrine and dehydroindicine, and at the C7 position in dehydroanacrotine (Mattocks and Jukes, 1992b).

Herbal medication has been recognized in recent years with regard to both treatment options and health hazards. Comfrey, used for inflammatory disorders including arthritis, thrombophlebitis and gout, contains lasiocarpine, symphytine, and other pyrrolizidine alkaloids. The use of comfrey leaves was recently recognized as a substantial health hazard, with hepatic toxicity shown in humans and carcinogenic potential shown for rodents (Stickel and Seitz, 2000).

Metabolism of lasiocarpine by F344 rat liver microsomes resulted in the formation of DHP (Xia et al., unpublished data). The levels of DHP formed from lasiocarpine

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metabolism by liver microsomes of male and female rats were 0.35 ± 0.02 and 0.17 ± 0.01 nmol/min/mg protein, respectively. When incubating lasiocarpine in the presence of calf thymus DNA, the same eight DHP-derived DNA adducts as those from metabolizing of riddelliine in vivo and in vitro were produced. These results provide evidence that DHP-derived DNA adduct formation is involved in the metabolic activation of heliotridine-type pyrrolizidine alkaloids. Support that these eight DHP-derived DNA adducts are potential biomarkers of pyrrolizidine alkaloid exposure and tumorigenicity.

Heliotrine

Heliotrine is mutagenic tested in *Salmonella typhimurium* TA100 with S9 (Yamanaka et al., 1979) and induces somatic mutation and teratogenic effect in *Drosophila* (Brink, 1982; Sivlingham and Brink, 1988). In the micronucleus assays, heliotrine exhibited clastogenic activity higher than benzidine, monocrotaline, and urethane (Sanderson and Clark, 1993). Tested with V79 Chinese hamster cells, heliotrine induced chromosomal aberrations, interchromosomal exchanges, and 8-azaguanine-resistant mutation (Takanashi et al., 1980). Swiss mice fed *Heliotropium dolosum* seeds containing heliotrine were dose-dependently led to death, hepatomegalocytosis, and renal tubular megalocytosis in all test animals (Eroksuz et al., 2001).

Rats injected with heliotrine formed metabolites conjugated to hemoglobin thiol groups. The metabolites were identified as pyrrolic monoethyl ethers by testing blood samples using the ethanolic silver nitrate treatment method developed by Mattocks and Jukes, (1992b). This result indicated that the proximal pyrrolic metabolites led to *S*-binding through attacking at the C9 position in dehydroheliotrine and dehydroindicine and at the C7 position in dehydroanacrotine (Mattocks and Jukes, 1992b).

Mattocks and Jukes (1992a) developed a simple procedure for detecting sulfur-conjugated pyrrolic metabolites, including monoesters (heliotrine and indicine), a diester (lasiocarpine), and macrocyclic diesters (retrorsine and senecionine), in blood and fresh or fixed liver tissue obtained from rats that were given a variety of toxic pyrrolizidine alkaloids. This method is applicable to the diagnosis of pyrrolizidine alkaloid exposure in livestock by using fresh or dried blood or fresh or preserved liver samples (Mattocks and Jukes, 1992a).

From the human study, patients in India ingesting heliotrine-containing herbal medicine were found to have veno-occlusive disease and decompensated liver cirrhosis (Datta et al., 1978). It is suspected that the use of herbal medicines containing pyrrolizidine alkaloids may be partly responsible for causing acute and chronic liver disease in India.

Savin et al. (1983) found that heliotrine significantly altered the rat liver microsomal oxidation system, resulting in a decreased liver microsomal cytochrome P450 concentration and an increased rate of inactivation of the reduced form of such enzyme.

Xia et al. (unpublished data) reported that metabolism of heliotridine by F344 rat liver microsomes resulted in the formation of dehydroheliotridine. Similar to lasiocarpine, incubation of heliotridine in the presence of calf thymus DNA resulted in the formation of the eight DHP-derived DNA adducts.

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Otonecine-Type Pyrrolizidine Alkaloids

Clivorine

So far, clivorine is the only otonecine-type pyrrolizidine alkaloid for which metabolism and metabolism-induced toxicities have been studied. This compound was identified as a natural hepatotoxin from the plants in several *Ligularia* species, including a traditional Chinese medicinal herb *L. hodgsonii Hook* (Klásek et al., 1967; Zhao et al., 1998). Clivorine was reported to be genotoxic in rats and mice (Mori et al., 1985), carcinogenic in ACI rats (Kuhara et al., 1980), and mutagenic in *Salmonella typhimuriun* in the presence of a mammalian microsomal enzyme system (Yamanaka et al., 1979). Owing to the unique structure of the otonecine-type necine base, clivorine exists in either a lipophilic nonionized form or a hydrophilic ionized form dependent upon the matrix, and this unique dual solubility may influence its toxicity by enhancing the liquid solubility in the herbal decoction and also by favoring absorption and distribution through the lipid membranes (Lin et al., 2000b).

The in vitro metabolisms of clivorine by rat, guinea pig, and human liver microsomes were reported (Fig. 10) (Cui, 1999; Lin et al., 1998a, 2000a, 2001, 2002, 2003; Xia et al., 2003b). In the liver microsomes of male SD rats (Lin et al., 1998a, 2000a, 2003) and F344 rats of both sexes (Xia et al., 2003b), metabolism of clivorine produces pyrrolic ester (dehydroclivorine) via oxidative *N*-demethylation of the necine base followed by ring closure and dehydration. This reactive pyrrolic ester can 1) be hydrolyzed to form the DHP; 2) react with glutathione to form the nontoxic glutathione conjugates; and 3) bind to protein, leading to the tissue-bound pyrroles responsible for the induction of hepatotoxicity. In addition, clivoric acid, the metabolite produced from the acid moiety, is concurrently formed during these three biotransformations.

Metabolism of clivorine by F344 rat liver microsomes in the presence of calf thymus DNA resulted in the formation of eight DHP-derived DNA adducts (Xia et al., 2003b). DHP and DHP-derived DNA adducts were also obtained when microsomal incubations were conducted with extracts of *Ligularia hodgsonnii* Hook (Xia et al., 2003b). This is the first report found that describes that DHP-derived DNA adducts are formed from the metabolic activation of otonecine-type pyrrolizidine alkaloid. The formation of these DHP-derived DNA adducts from clivorine and from the retronecine- and heliotridine-type pyrrolizidine alkaloids, as previously described, suggests that these DNA adducts are potential biomarkers of pyrrolizidine alkaloid exposure and pyrrolizidine alkaloid-induced tumorigenicity. There are two possible pathways that lead to DHP-derived DNA adduct formation: 1) metabolism of clivorine generates dehydroclivorine that binds to DNA, followed by hydrolysis; and 2) dehydroclivorine hydrolyzes to DHP, and then DHP binds to DNA. It is currently not known which pathway is predominant.

In the case of the female SD rat (Lin et al., 2003), an additional direct hydrolysis pathway was observed. The structure of the hydrolyzed metabolite was not definitely identified but was reported to be different from the hydrolyzed metabolite produced in the guinea pig microsomes. Furthermore, the direct hydrolysis pathway predominates, whereas the metabolic activation accounts only for a minor pathway in the female rat, although the overall metabolic rate of clivorine was found to be similar in rats of both sexes (Lin et al., 2003). The human metabolic profile of clivorine was identified as the

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same as that observed in the male rat (Cui, 1999; Lin et al., 2001). In the case of microsomal metabolism of clivorine in guinea pig, the same metabolic profile was observed in both sexes. In addition to the metabolic activation pathway, a direct hydrolysis pathway leading to clivoric acid was found as the major metabolic pathway (Lin et al., 2002). In comparison with rat and human microsomal metabolism, the metabolic rate of clivorine in guinea pig is significantly higher.

These results provide evidence that the principal metabolic activation pathway of clivorine leading to toxicity includes formation of the unstable dehydroclivorine through oxidative *N*-demethylation of the necine base, followed by ring closure and dehydration; and binding of the pyrrolic ester to the hepatic nucleophilic constitutes to form bound pyrroles leading to hepatotoxicity, or to the DNA, leading to DNA adduct formation and tumor initiation. The proposed metabolic detoxification pathways to form glutathione conjugates and the hydrolyzed metabolites were reported by Cui (1999) and Lin et al. (2000a, 2002, 2003).

The enzymes mediating the in vitro microsomal metabolism of clivorine were investigated (Cui, 1999; Lin et al., 2000a, 2002, 2003). In the specific enzyme induction and inhibition studies, CYP3A1 and CYP3A2 isoforms are found to be the primary enzymes responsible for the oxidative *N*-demethylation of clivorine in rat (Lin et al., 2003). These two isozymes are male rat specific, but their levels are significantly low in the female rat, and both isozymes are inducible in both sexes (Imaoka et al., 1991; Mahnke et al., 1997; Ribeiro and Lechner, 1992; Waxman et al., 1985). High expression of these two isozymes is responsible for catalyzing the metabolic activation of clivorine to generate the unstable pyrrolic ester leading to toxicities in the male rat. In the normal female rat, the rate of such metabolic activation of clivorine is significantly low due to the lower activity of these two isozymes in the liver (Lin et al., 2000a, 2003). When the female rats were pretreated with CYP3A1 and CYP3A2 inducer dexamethasone, the rates of metabolic activation became comparable to that found in the male rats (Lin et al., 2003). Moreover, both CYP3A1 and CYP3A2 were further confirmed to be the specific CYP isozymes mediating the metabolic activation of clivorine in rat liver by using different cDNA expressed CYPs (Lin et al., 2003). In the human liver microsomal metabolism of clivorine, CYP3A4 was revealed as the key enzyme mediating the formation of the toxic pyrrolic ester (Cui, 1999; Lin et al., 2001), which was also confirmed by studies using various cDNA expressed human CYP isozymes (Lin, unpublished data; Lin et al., 2001). In addition, the CYP3A subfamily was also evidenced to be responsible for the metabolic activation of clivorine, leading to pyrrolic ester metabolite in guinea pig, however, the specific CYP3A isozyme that plays the major role on the metabolic activation was not determined (Lin et al., 2002).

The female rat liver microsomal carboxylesterases that catalyzed the metabolic hydrolysis of clivorine were identified (Lin, unpublished data; Lin et al., 2003). It was determined that both cytosolic and microsomal carboxylesterases were involved in the hydrolysis of clivorine in guinea pigs of both sexes (Lin et al., 2002). However, the esterase isoforms that catalyze the hydrolysis of clivorine in different animal models were not determined.

Senkirkine

Senkirkine is a 12-membered macrocyclic diester pyrrolizidine alkaloid with an α,β-unsaturated double bond linked to the ester group at the C7 position of the

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otonecine-type necine base. It is mainly present in the plants of the genera of *Tussilago*, *Senecio*, *Farfugium*, *Emilia*, and *Petasites*, of which various plant materials are utilized as the plant sources for herbal medicines (Fu et al., 2002a,b; Huxtable, 1989; Mattocks, 1986; Prakash et al., 1999; Roeder, 1995, 2000; Stegelmeier et al., 1999). Isolated from *T. farfara*, the crystal structure of senkirkine was elucidated in 1974 (Birnbaum, 1974). An intramolecular N. . .C═O interaction in the necine base was revealed by x-ray crystallography (Birnbaum, 1974) and ^{13}C-NMR spectroscopy (Morel et al., 1989), which indicates that, similar to clivorine, this otonecine-type pyrrolizidine alkaloid also exists in nonionized and ionized forms and, thus, possesses a dual solubility.

Senkirkine was reported to be hepatotoxic (Huxtable, 1989; Mattocks, 1986; Roeder, 1995, 2000), genotoxic (Frei et al., 1992; Mori et al., 1985), carcinogenic (Hirono et al., 1979b; Kapadia et al., 1990), and mutagenic (Candrian et al., 1984; Yamanaka et al., 1979). In 20 male rats fed with 10% of the LD_{50} dose of senkirkine twice weekly for 4 weeks, nine rats developed liver tumors (Hirono et al., 1979b). In a separate study of senkirkine-containing plants in cattle, fed with either fresh (1 kg/day for 8 days) or dried (equivalent to about 400 g of the fresh material/day for 10 days) plant materials, no obvious liver damages were observed (Skaanild et al., 2001). However, as the absolute contents of senkirkine and other pyrrolizidine alkaloids present in the plant were not described in the study, it is possible that the dosage regimens used in the study were not adequate, causing toxicity. Senkirkine produces genotoxicity in mouse and hamster hepatocytes (Mori et al., 1985). It also induces mutagenicity in *Salmonella typhimuriun* in the presence of mammalian microsomes (Yamanaka et al., 1979). The genotoxicity of senkirkine and other toxic pyrrolizidine alkaloids in the wing spot test of *Drosophila* was investigated, and the results demonstrated a good correlation between hepatotoxicity of the pyrrolizidine alkaloids tested in rodents and genotoxicity in *Drosophila*, suggesting that pyrrolizidine alkaloids are similarly bioactivated in the mammalian liver and in the somatic cells of *Drosophila* (Frei et al., 1992).

PERSPECTIVES

Pyrrolizidine alkaloids are probably the most common poisonous plant constituents that poison livestock, wildlife, and humans, worldwide. Consequently, it is important to determine the sources of human exposure, assess human health risk posed by these compounds, and reduce exposure to these compounds. Currently, regulatory restriction on the use of pyrrolizidine alkaloid-containing plants and plant-derived products was made in several countries. For example, the comfrey dietary supplements were recently withdrawn from the market in the United States. It is necessary that more efforts be continuously exerted worldwide to achieve the goals.

The assessment of human health risk posed by exposure to pyrrolizidine alkaloids has to be justified based on toxicological mechanisms. Only those intervention strategies that are based on the mechanistic understanding are expected to be the most effective. Thus, understanding the biotransformations by which pyrrolizidine alkaloids exert toxic activities, including tumorigenicity, is important not only for the advance of the science of this field but also for the development of effective strategies on prevention.

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The dogged pursuit of mechanistic understanding of the carcinogenesis induced by pyrrolizidine alkaloids has continued over the past several decades. However, it was not satisfied until recently that our mechanistic study determined that riddelliine induced liver tumors through a genotoxic mechanism mediated with DHP-derived DNA adduct formation. These DNA adducts were also formed from several other pyrrolizidine alkaloids of different types in vivo or in vitro, indicating that formation of these DNA adducts is a general mechanism of tumor induction. Therefore, this finding sheds light on the hope that more detailed understanding on the mechanisms of tumor induction by pyrrolizidine alkaloids can be made in the near future.

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