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Detection, Hepatotoxicity, and Tumorigenicity of Pyrrolizidine Alkaloids in Chinese Herbal Plants and Herbal Dietary Supplements

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ABSTRACT

Since the U.S. Congress passed the Dietary Supplement Health and Education Act (DSHEA) in 1994, herbal products, including herbal dietary supplements, represent the fastest growing segment of the vitamin, mineral supplements, and herbal products industry. To ensure consumer health protection, the quality and safety of raw herbal plants used for dietary supplement preparations have to be determined. To date, safety issues concerning the hepatotoxic and tumorigenic ingredients in many raw herbs and herbal dietary products are quite limited. Pyrrolizidine alkaloids are a class of hepatotoxic and tumorigenic phytochemicals present in more than 6000 plants and have been detected in herbal plants and dietary supplements. In this review, the human exposure, metabolic activation leading to hepatotoxicity and tumorigenicity of the pyrrolizidine alkaloid-containing Chinese herbal plants, and analytical methods used to identify and quantify pyrrolizidine alkaloids in herbal plants and commercial samples are discussed. Suggestions for future research are provided.

Key words: pyrrolizidine alkaloid, herbal products, herbal dietary supplement, carcinogenicity

INTRODUCTION

Traditional Chinese medicine, predominantly being derived from herbal plants, has been used in China for treating illness and improving health for more than 2000 years⁽¹⁾. During the last two decades, the use of herbal plants as natural remedies, functional foods (mixing functional herbs in conventional food), and dietary supplements in Europe, Australia, and North America for health care has been dramatically increasing⁽²⁾. However, to date, safety issues concerning the hepatotoxic and genotoxic, particularly tumorigenic, ingredients in many raw herbal plants and herbal products are limited.

Pyrrolizidine alkaloids are a class of genotoxic and tumorigenic phytochemicals present in more than 6000 plants⁽²⁻⁶⁾. The International Programme on Chemical Safety (IPCS) determined that pyrrolizidine alkaloids present in food are a threat to human health and safety^(7,8). Because toxic and tumorigenic pyrrolizidine alkaloids were found in herbal plants, regulatory decisions have been made in several Western countries. For example, in 1992 the Federal Health Department of Germany restricted the manufactures and use of phar-

maceuticals which contain pyrrolizidine alkaloids with an unsaturated necine base. The herbal plants "may be sold and used only if daily external exposure to no more than 100 µg pyrrolizidine alkaloids and internal exposure to no more than 1 µg per day for no more than six weeks a year"^(2,6). In 1994, the U.S. Congress passed the Dietary Supplement Health and Education Act (DSHEA) that amended the U.S. Federal Food, Drug, and Cosmetic Act (FFDCA) and created a new regulatory category, safety standards, and other rules for the U.S. Food and Drug Administration (FDA) to regulate dietary supplements. Since then the use of dietary supplements and functional foods has grown rapidly in the United States and many other countries. Under such a circumstance, it is important to ensure the therapeutic efficacy and safety of commercial herbal products. In this review, the origin, identification, human exposure, and mechanisms leading to hepatotoxicity and tumorigenicity of pyrrolizidine alkaloids in Chinese herbal plants and herbal dietary supplements are described.

SOURCES OF PYRROLIZIDINE ALKALOID-CONTAINING PLANTS

Pyrrolizidine alkaloids contain a necine base with a characteristic bicyclic nitrogen-containing heterocyclic

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ring^(2,3). Pyrrolizidine alkaloids are phytochemicals that are not involved in the normal growth, development, or reproduction of plants. Instead, pyrrolizidine alkaloids are constitutively produced by plants as secondary metabolites for exerting a defense mechanism against insect herbivores and are deterrent and toxic to most vertebrates⁽⁹⁻¹⁴⁾. The production of pyrrolizidine alkaloids by the plants is mediated through the chemical ecological interactions between the plant and insect herbivores. As such, generalist insect herbivores potentially play an important role in the evolution and maintenance of the diversity of pyrrolizidine alkaloids⁽¹⁵⁾. Thus, it is essential that pyrrolizidine alkaloids are common constituents of hundreds of plant species of different unrelated botanical families distributed in many geographical regions in the world^(2,5,8,16-19). To date, more than 660 pyrrolizidine alkaloids and their corresponding pyrrolizidine alkaloid N-oxides have been identified in over 6,000 plants and about half of them exhibit toxic activities^(2,3,20). It has been reported that about 3% of the world's flowering plants contain toxic pyrrolizidine alkaloids⁽¹⁷⁾. Pyrrolizidine alkaloids are found in more than twelve higher plant families of the Angiosperms, among which three families, *Compositae* (*Asteraceae*), *Boraginaceae*, and *Legumionaceae* (*Fabaceae*), contain the most toxic pyrrolizidine alkaloids. Toxic pyrrolizidine alkaloid-containing plants grow in South Africa, Central Africa, West Indies, China, Jamaica, Canada, Europe, New Zealand, Australia, and the United States^(20,21). Because toxic pyrrolizidine alkaloid-containing plants are widely distributed in the world, the risk to human health posed by exposure to these compounds has been a concern.

PYRROLIZIDINE ALKALOIDS IN CHINESE HERBAL PLANTS

Pyrrolizidine alkaloids have long been found as contaminants in many human food sources, such as wheat, milk, honey, and herbal teas^(4,22-29). Since the early 1960s, pyrrolizidine alkaloids have been found in Chinese herbal plants⁽³⁰⁻³²⁾. In 1992, Edgar *et al.*⁽³³⁾ estimated that there are more than 50 Chinese herbal plants containing pyrrolizidine alkaloids. In 2000, Roeder⁽²⁾ found about 90 pyrrolizidine alkaloids from 38 traditional Chinese medicinal herb plants, which is in agreement with that reported by Zhao *et al.* in 1998⁽³¹⁾. The recent study by Lin *et al.*⁽³⁰⁾ in Hong Kong and Wang *et al.*⁽³⁰⁾ in China identified additional 11 pyrrolizidine alkaloid-containing Chinese herbal plants that all belong to *Ligularia* genus of the *Asteraceae* (*Compositae*) family. Thus, a total of 49 species of pyrrolizidine alkaloids-containing Chinese herbal plants have been identified. All these plants are listed as traditional Chinese medicine (TCM) herbs in Traditional Chinese Dictionary published in 1979⁽³²⁾.

Among the more than 90 pyrrolizidine alkaloids identified as herbal plants in China, 15 pyrrolizidine

alkaloids and one pyrrolizidine alkaloid N-oxide have been found to be capable of inducing tumors in experimental animals. The names and structures of these 15 compounds are shown in Figure 1. These tumorigenic pyrrolizidine alkaloids are present in 19 Chinese herbal plants. The names of these herbal plants and their medicinal usefulness are listed in Table 1.

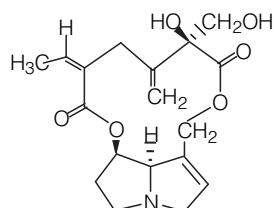
The detection and identification of pyrrolizidine alkaloids in herbal plants have not been extensively studied, and only a few pyrrolizidine alkaloids have been examined for their tumorigenicity. Consequently, it is highly probable that there are many more Chinese herbal plants containing pyrrolizidine alkaloids that have yet to be characterized. This is supported by study of Lin and co-workers that 11 pyrrolizidine alkaloid-containing Chinese herbal plants were identified in the *Ligularia* genus of the *Asteraceae* (*Compositae*) family⁽³⁰⁾. *Compositae* (*Asteraceae*) is the largest family of plants in the world, including in China. The *Compositae* family consists of more than 900 genera and 20,000 species, among which the *Ligularia* genus is of the *Asteraceae* (*Compositae*) family. The finding by Lin *et al.* implicates that there should be many more pyrrolizidine alkaloid-containing Chinese herbal plants grown in China. Lin *et al.*⁽³⁴⁾ determined that clivorine, a tumorigen in rats, is among the pyrrolizidine alkaloids identified in these 11 species from *Ligularia*. Since the identification of tumorigenic pyrrolizidine alkaloids in Chinese herbal plants in China has never been intensively pursued, the human health risk posed by consumption of pyrrolizidine alkaloid containing Chinese herbal plants and the products (dietary supplements and functional foods) made from these Chinese herbal plants is a serious concern.

Pyrrolizidine alkaloid-containing herbal plants, including comfrey, coltsfoot, and borage, have been sold as dietary supplements^(2,5,16,24-26). Comfrey and coltsfoot are Chinese herbal medicine and produced in many countries including China, and borage is produced in Chile, Mexico, France, Spain, Turkey, and USA. It is not known whether pyrrolizidine alkaloids are present in any of the other commercial dietary supplements. Since the use of dietary supplements and functional foods has increased rapidly in the world, the risk of human exposure to toxic pyrrolizidine alkaloids by taking dietary supplements needs to be determined.

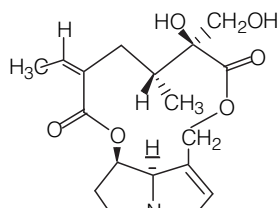
TOXICITY OF PYRROLIZIDINE ALKALOIDS

Livestock and human poisoning upon consuming pyrrolizidine alkaloid-containing plants have been reported in many areas of the world. The poisoned animal species include horses, cattle, sheep, goats, swine, chickens, quails, and doves^(35,36). Acute poisoning causes massive hepatotoxicity with hemorrhagic necrosis. Chronic poisoning takes place mainly in liver, lungs, and blood vessels, and in some instances kidneys, pancreas, gastro-

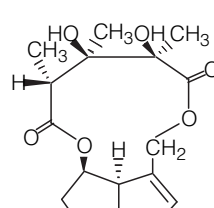
1. Retronecine-type



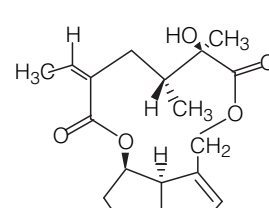
Riddelliine



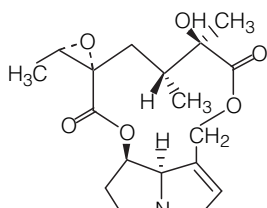
Retrorsine



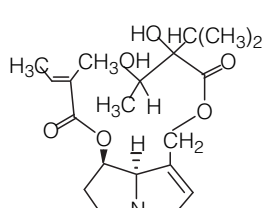
Monocrotaline



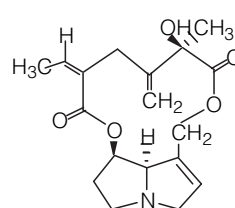
Senecionine



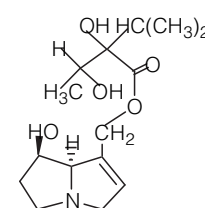
Jacobine



Symphytine

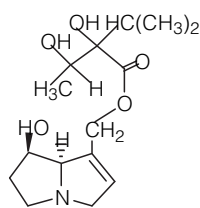


Seneciophylline

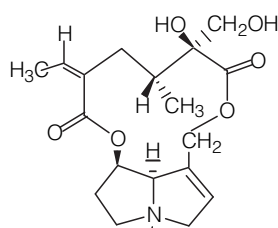


Intermedine

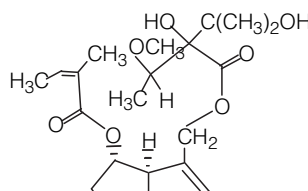
2. Heliotridine-type



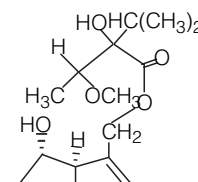
Lycopsamine



Retrorsine N-oxide

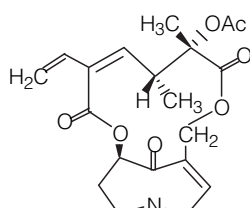


Lasiocarpine

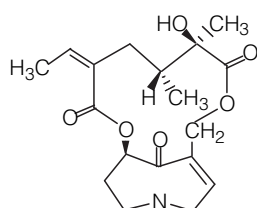


Heliotrine

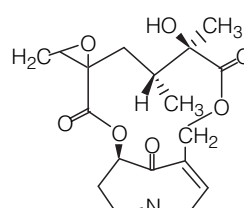
3. Otonecine-type



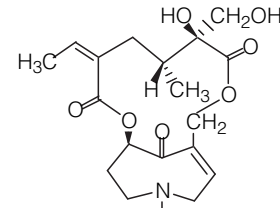
Clivorine



senkirkine



Petasitenine



Hydroxysenkirkine

Figure 1. Names and structures of tumorigenic pyrrolizidine alkaloid identified in Chinese herbal plants in China.

intestinal tract, bone marrow, and brain. Exposure over a longer period of time causes cell enlargement (megalocytosis), veno-occlusion in liver and lungs, fatty degeneration, nuclei enlargement with increasing nuclear chromatin, loss of metabolic function, inhibition of mitosis, proliferation of biliary tract epithelium, liver cirrhosis, nodular hyperplasia, and adenomas or carcinomas^(6,37).

Pyrrolizidine alkaloids themselves are not toxic, and require metabolic activation to the "pyrrolic" metabolites to exert acute, chronic toxicity, and genotoxicity, includ-

ing DNA binding, DNA cross-linking, DNA-protein cross-linking, sister chromatid exchange, chromosomal aberrations, mutagenicity, and carcinogenicity^(4,5,18,22,38-62). Some pyrrolizidine alkaloids, including clivorine, heliotrine, lasiocarpine, retrorsine, senkirkine, seneciophylline, and riddelliine, have been found to be mutagenic in *Salmonella typhimurium* TA100 in the presence of hepatic S9 activation enzyme system⁽⁶³⁻⁶⁶⁾. Pyrrolizidine alkaloids are among the first naturally occurring carcinogens identified in plants⁽⁵⁾.

Table 1. List of the tumorigenic pyrrolizidine alkaloid-containing Chinese herbal plants grown in China^a

Plant	Herb name in Chinese	Medicinal purpose	Tumorigenic pyrrolizidine alkaloids	References
1. Family compositae (Asteraceae)				
<i>Ageratum conyzoides</i> L.	Sheng hong ji	Common colds, fever, malaria	Lycopsamine	(67)
<i>Chromolaena odorata</i> R. M. King & H. Rob.	Fei ji cao	Hemostatic	Intermidine	(5)
<i>Eupatorium cannabinum</i> L.	Pei lan	Influenza, cerebral stroke	Lycopsamine, intermidine	(5,67)
<i>Eupatorium japonicum</i> Thunb.	Hua zhe lan and Cheng gan cao	Measles, rheumatic bone pains and colds	Lycopsamine, intermidine	(5,67)
<i>Crassocephalum crepidioides</i> S. Moore	Jia tong hao	Cold, dysentery, gastroenteritis, urinary infection	Jacobine	(2)
<i>Emilia sonchifolia</i> DC	Yang ti cao, Yi dian hong	Antipyretic, diarrhea, hemoptysis	Senkirkine	(2)
<i>Farfugium japonicum</i> Kitam	Lian peng cao	Colds and flu	Petasitenine, senkirkine	(2)
<i>Gynura bicolor</i> DC	Guan yin xian	Dysmenorrhea, tuberculous hemoptysis	Retrorsine	(35)
<i>Gynura segetum</i> Merr.	Ju shan qi, Tu san chii	Hemoptysis, peripheral blood circulation disorder	Senecionine, seneciphylline	(2,68)
<i>Ligularia hodgsonii</i> Hook		Antitussive	Clivorine	(69)
<i>Senecio argunensis</i> Turcz.	Yu yie qian li guang, Zhan long cao	Folk medicine, dysentery	Senecionine, seneciphylline	(2)
<i>Senecio chrysanthemoides</i> DC	Chien li kuang, Tsang tu san chi	Traumatic injury, breast abscesses	Seneciphylline	(2)
<i>Senecio nemorensis</i>	Huana wan	Enteritis, hepatitis, boils	Senecionine	(2)
<i>Senecio scandens</i>	Quian li guang, Chiu li ming	Oral and pharyngeal infection	Senecionine, seneciphylline	(2)
<i>Tussilago farfara</i> L.	Kuan dong hua, Chien hua	Chronic bronchitis, asthma, influenza	Senecionine, senkirkine	(2)
2. Family Boraginaceae				
<i>Heliotropium indicum</i> L.	Da wei yao	Ulcer, wounds and local inflammations	Heliotrine, lasiocarpine	(2)
<i>Lappula intermedia</i> M. Popov.	He shi	Ascariasis, oxyuriasis, infantile malnutrition	Lasiocarpine	(2)
<i>Lithospermum erythrorhizon</i> Sieb. et Zucc.	Zi cao	Antipyretic and antiphlogistic	Intermidine	(5)
3. Family Legumionsae (Fabaceae)				
<i>Crotalaria assamica</i> Benth	Zi xiao rong (Nung gi li)	Folk remedy	Monocrotaline	(70)
<i>Crotalaria mucronata</i>	Zhu zi tou	Folk medicine	Monocrotaline, retrorsine	(1)
<i>Crotalaria sessiliflora</i> L. or <i>Crotalaria assamica</i> Benth	Ye bai he	Folk medicine	Monocrotaline	(70)

^aMost of the data obtained from⁽²⁾. In most cases, the major tumor type is hepatocarcinoma.

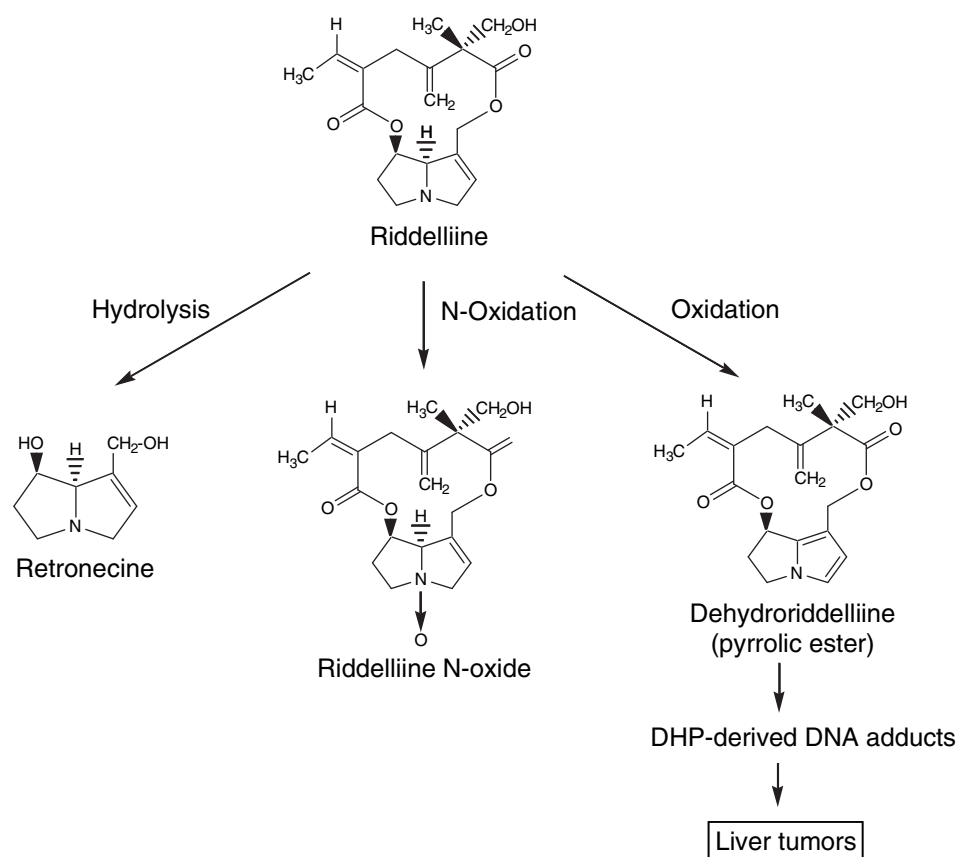


Figure 2. Three major metabolic pathways of a representative retronecine-type pyrrolizidine alkaloid, riddelliine

METABOLIC ACTIVATION OF PYRROLIZIDINE ALKALOIDS LEADING TO HEPATOTOXICITY

It has been found that most of the pyrrolizidine alkaloids that exhibit toxic effects are derived from esters of basic alcohols, the necine bases, and with a double bond between the C1 and C2. The retronecine-, heliotridine-, and otonecine-type pyrrolizidine alkaloids that have a double bond at the C1 and C2 positions of the necine base exhibit high levels of toxicity, including hepatotoxicity and carcinogenicity (Figure 1). Consequently, studying on toxicity and tumorigenicity, the most attention has been focused on these three types of toxic pyrrolizidine alkaloids^(2,4,6,16,22,25).

Metabolic activation of pyrrolizidine alkaloids leading to hepatotoxicity and genotoxicities, including tumorigenicity has been extensively studied^(3,5,16,22,34,37,58,71-95). The retronecine-type and heliotridine-type pyrrolizidine alkaloids, which are enantiomers at the C7 position, exhibit three principal metabolism pathways. Using riddelliine as an example⁽³⁷⁾, the three pathways include: (i) hydrolysis of the ester functional groups to form the necine bases and acidic metabolites; (ii) N-oxidation of the necine bases to the corresponding

N-oxides; and (iii) formation of the corresponding dehydropyrrolizidine (pyrrolic) derivatives through hydroxylation at the C-3 or C-8 position of the necine base to form 3- or 8-hydroxynecine derivatives followed by dehydration (Figure 2). As to otonecine-type pyrrolizidine alkaloids, the principal metabolic pathways of involve: (i) hydrolysis of the ester functional groups to form the corresponding necine bases and acids; and (ii) formation of the corresponding dehydropyrrolizidine (pyrrolic) derivatives through oxidative N-demethylation of the necine base followed by ring closure and dehydration (Figure 3)^(34,82,84).

Formation of dehydropyrrolizidine (pyrrolic) metabolites is generally recognized as the metabolic activation pathway responsible for the genotoxic and tumorigenic activities of pyrrolizidine alkaloids. This is evidenced by the fact that dehydropyrrolizidine (pyrrolic) compounds can bind with DNA leading to DNA cross-linking, DNA-protein cross-linking, and DNA adduct formation^(5,8,16,51,52,86,96). The pyrrolic metabolites also bind with glutathione, catalyzed by glutathione S-transferase, to produce glutathione conjugates. This enzymatic reaction is possibly a major detoxification pathway^(34,82,97-99). Lin and co-workers studied rat liver microsomal metabolism of clivorine^(34,82) and identified dehydroclivorine (the pyrrolic

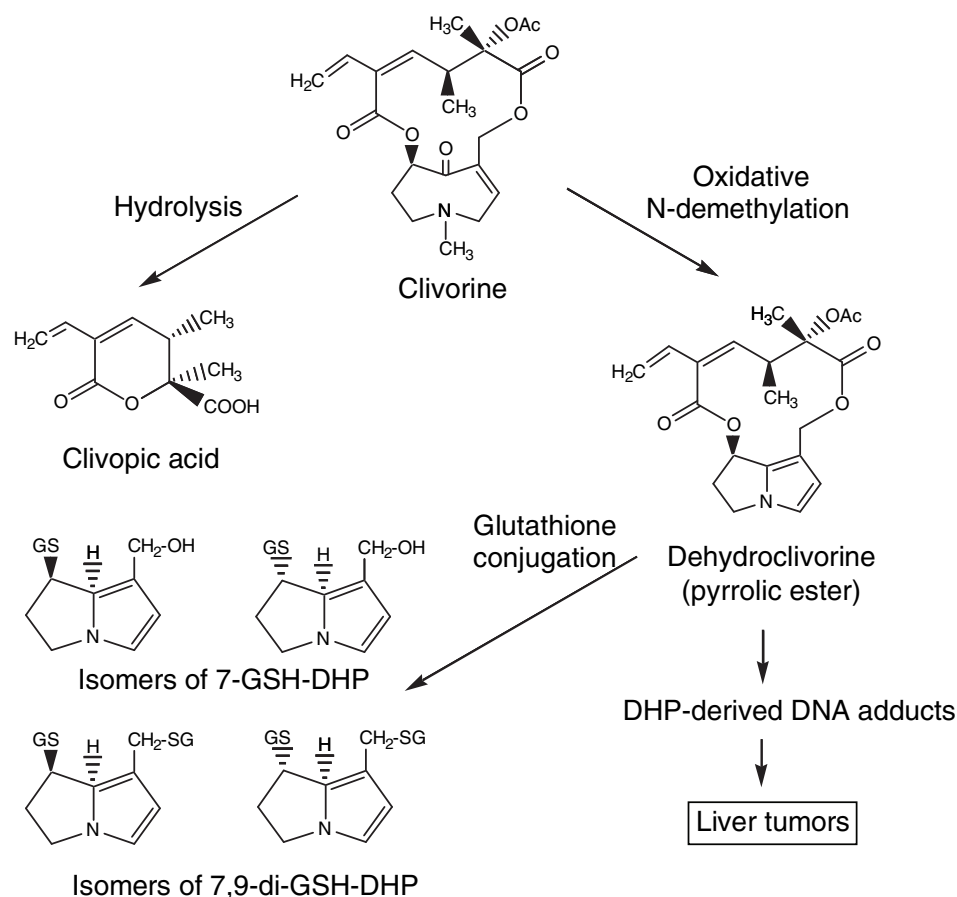


Figure 3. Two major metabolic pathways of a representative otonecine-type pyrrolizidine alkaloid, clivorine, and glutathione conjugations of toxic intermediate dehydroclivorine as detoxification pathways

ester) as a reactive metabolite that covalently bound to the tissue constituents in the liver and induced liver damage. This metabolite can further react with glutathione to form four glutathione conjugates and this biotransformation is considered as a detoxification process (Figure 3). Thus, factors that can modulate the metabolic rates of pyrrolic ester and glutathione conjugations formation play the key roles in clivorine genotoxicity^(83,84,100).

Metabolism of pyrrolizidine alkaloids of all three toxic types to form dehydropyrrolizidines is mainly catalyzed by cytochrome P-450 monooxygenases, specifically the CYP3A and CPY2B6 isoforms^(34,71,73-77,83,91,100,101). Metabolism of pyrrolizidine alkaloids of the retronecine- and heliotridine-type pyrrolizidine alkaloids to produce the corresponding pyrrolizidine alkaloid *N*-oxides is catalyzed by both cytochrome P-450 and flavin-containing monooxygenases^(72,76,101,102). Hydrolysis of the ester groups to form the corresponding necine base and acidic metabolites is mainly mediated by liver microsomal carboxylesterases^(71,73-77,91,100).

Species difference in susceptibility to the pyrrolizidine alkaloid toxicities is mainly due to the variations in the balance between the formation of the toxic pyrro-

lic metabolites and the detoxification pathways to generate hydrolyzed metabolites and/or non-toxic *N*-oxides^(5,103). The species difference in susceptibility to clivorine induced hepatotoxicity was studied by Lin *et al.*⁽⁸⁴⁾. The *in vitro* metabolic activation of clivorine in both male rat and human was similar but was different from that in guinea pig. The higher activation rates for the reactive pyrrolic ester formation followed by forming the toxic tissue-bound pyrroles contribute to the high susceptibility of human and male rat to clivorine hepatotoxicity. The higher metabolic rates for the hydrolysis in combination with a lower rate for the formation of toxic tissue-bound pyrroles play a key role in guinea pig resistance to clivorine intoxication.

MECHANISMS LEADING TO TUMORIGENICITY

Although it has been known for more than half a century^(16,37) that pyrrolizidine alkaloids can induce tumors in experimental animals, the mechanism of tumor induction was not known until our recent finding that riddelliine induces liver tumors through a geno-

toxic mechanism mediated by 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine (DHP)-derived DNA adduct formation published in 2001^(86,87,89). It was determined that riddelliine is metabolized by male and female rat liver microsomes to form DHP, which leads to the formation of a set of eight DHP-derived DNA adducts^(16,86). The levels of DNA adduct formation correlated closely with the tumorigenic potencies of the mice fed with different doses of riddelliine^(86,89,104).

Metabolism of riddelliine by human liver microsomes was reported⁽¹⁰⁵⁾. The kinetic parameters, V_{\max} and K_m , from human liver microsomal metabolism are comparable to those from rat liver microsomal metabolism. The metabolic study in the presence of a human CYP3A4 inhibitor strongly suggests that the formation of DHP and riddelliine *N*-oxide is principally catalyzed by the CYP3A4 isozyme. These results strongly indicate that the *in vivo* and *in vitro* mechanistic studies with experimental rodents⁽⁸⁶⁾ are highly relevant to humans. Since riddelliine induces liver tumors in male and female rats and male mice⁽¹⁰⁴⁾ and the DHP-derived DNA adducts are responsible for liver tumor induction, these results suggest that riddelliine can be highly genotoxic to humans and the genotoxic mechanism is mediated by DHP-derived DNA adduct formation.

To examine the relationship between DNA adduct levels and the incidence of hemangiosarcomas, DHR-derived DNA adduct levels in purified rat and mouse liver endothelial cells, the cells of origin for the hemangiosarcomas, were determined⁽⁸⁹⁾. Riddelliine was orally administered to F344 rats at 1.0 mg/kg/day and B6C3F1 mice at 3.0 mg/kg/day 5 days per week for 2 weeks. At day 1, 3, 7, and 28 after the last dose, the quantities of DHR-derived DNA adducts in liver parenchymal and endothelial cell fractions were determined by ³²P-post-labeling/HPLC. DHR-derived DNA adduct levels in the endothelial cells were significantly higher than in the parenchymal cells, and the DNA adduct levels in rat endothelial cells were greater than in the mouse endothelial cells. These results indicate that the levels of riddelliine-induced DNA adducts in specific populations of liver cells correlate with the preferential induction of liver hemangiosarcomas by riddelliine⁽⁸⁹⁾.

A subsequent metabolism study on the tumorigenic pyrrolizidine alkaloids, retrorsine, monocrotaline, clivorine, and lasiocarpine, under similar conditions generated the same set of DHP-derived DNA adducts⁽¹⁰⁶⁻¹⁰⁹⁾. Riddelliine, retrorsine, and monocrotaline are retronecine-type tumorigenic pyrrolizidine alkaloids. Clivorine and lasiocarpine are otonecine- and heliotridine-type tumorigenic pyrrolizidine alkaloids, respectively. As mentioned earlier, all the tumorigenic pyrrolizidine alkaloids so far examined belong to these three types of pyrrolizidine alkaloids. Thus, formation of the same set of DHP-derived DNA adducts from metabolism of these tumorigenic pyrrolizidine alkaloids clearly indicate that DHP-derived DNA adducts are potential biomarkers of

pyrrolizidine alkaloid tumorigenicity as well as pyrrolizidine alkaloids exposure. The proposed general metabolic activation of all the three types of pyrrolizidine alkaloids leading to DNA adduct formation and then the liver tumor induction is shown in Figure 4.

TOXICITY AND TUMORICITY OF PYRROLIZIDINE ALKALOID N-OXIDES

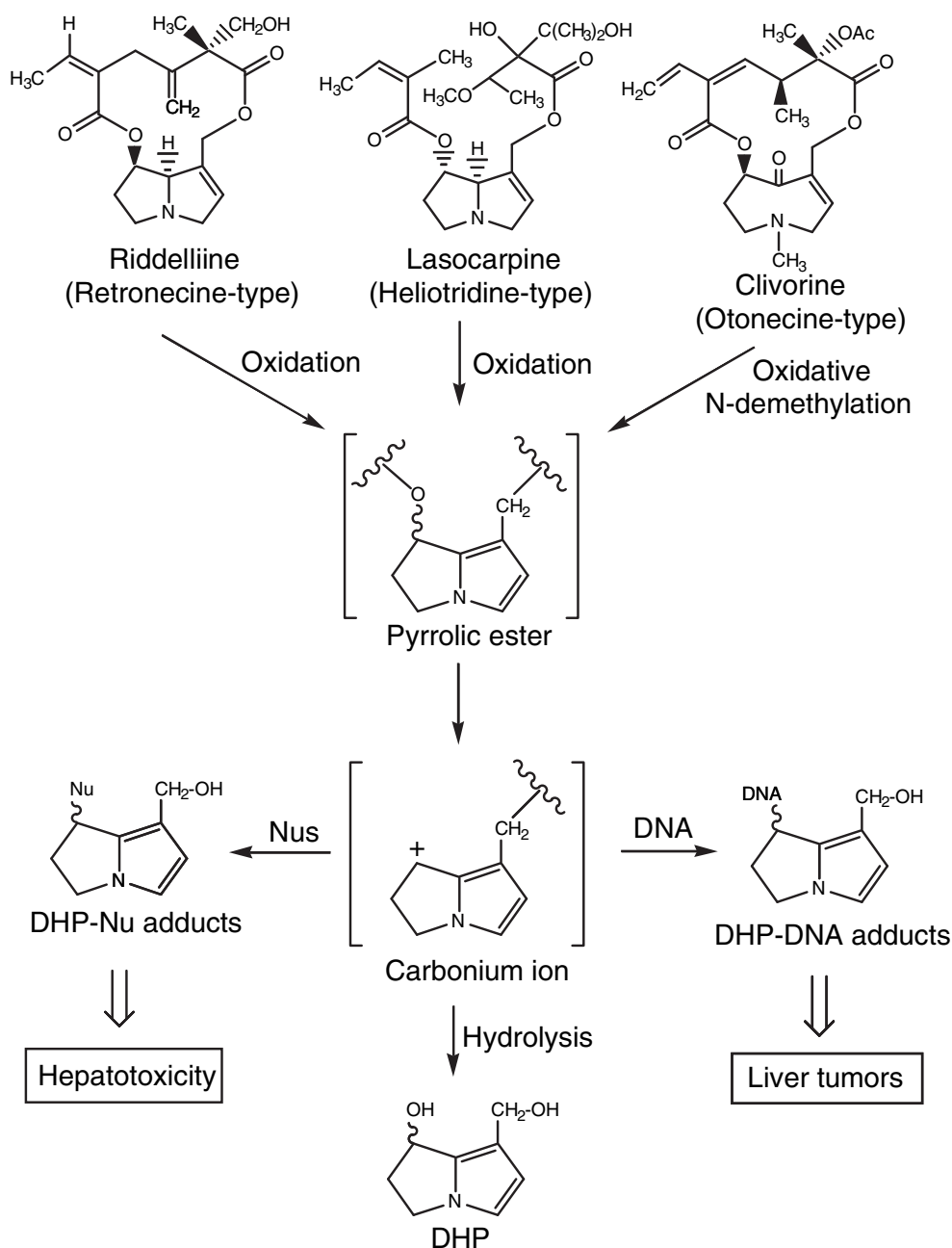
In the plants, the quantities of pyrrolizidine alkaloid *N*-oxides in many cases are greater than the parent pyrrolizidine alkaloids and in some cases may be exclusively the *N*-oxide⁽⁵⁾. Pyrrolizidine alkaloid *N*-oxides are also the major metabolites of the retronecine- and heliotridine-type pyrrolizidine alkaloids, and were generally regarded as detoxification products. However, Chou *et al.*⁽⁸⁸⁾ have recently determined that rat liver microsomal metabolism of riddelliine *N*-oxide formed DHP, the genotoxic metabolite. When metabolism was conducted in the presence of calf thymus DNA, the same set of DHP-derived DNA adducts formed from metabolism of riddelliine *in vivo* was produced. These DHP-derived DNA adducts were also formed in liver DNA of F344 rats fed riddelliine *N*-oxide⁽⁸⁸⁾. These results indicate that riddelliine *N*-oxide, through its conversion to riddelliine, is also a potential genotoxic hepatocarcinogen.

IDENTIFICATION AND QUANTITATION OF TOXIC PYRROLIZIDINE ALKALOIDS IN HERBAL PLANTS, HERBAL DIETARY SUPPLEMENTS, AND BIOLOGICAL SAMPLES

For human health protection, it is important to insure that the use of herbal medicines, herbal teas (as beverage and medicine), and herbal dietary supplements (as functional food) is safe. The widespread distribution of toxic and tumorigenic pyrrolizidine alkaloids in the environment suggests that they can contaminate herbal plants, eventually resulting in human health damage. Consequently, a number of extraction and analytical methodologies have been developed to identify and quantify pyrrolizidine alkaloids in herbal plants and commercial herbal plant products, such as herbal dietary supplements^(5,6,24,25,30,31,69,110-123). The established methods are used to identify (i) toxic pyrrolizidine alkaloids in herbal plants, herbal dietary supplements, and food; (ii) their active pyrrolic metabolites, such as DHR and pyrrolic pyrrolizidine alkaloids; and (iii) the pyrrolizidine alkaloid derived DNA adducts *in vitro* and *in vivo*. These analytical methods are briefly described below:

I. Thin Layer Chromatographic (TLC) and Column Chromatographic Methods

Thin-layer chromatography (TLC) coupled with the



Nu: Nucleophilic biological macromolecules, e.g. proteins

Figure 4. Metabolic activation of retronecine-, heliotridine-, and otonecine-type pyrrolizidine alkaloids leading to hepatotoxicity and tumorigenicity

Ehrlich reagent is a photometric method. It was originally developed by Mattocks in 1967 and has been used to detect pyrrolizidine alkaloids and pyrrolizidine alkaloids *N*-oxides in plants⁽¹¹⁾. This method was subsequently modified by Roeder's group to detect otonecine-type pyrrolizidine alkaloids, including senkirkine, a reference pyrrolizidine alkaloid used for gas chromatographic

determination of pyrrolizidine alkaloids in plants and other sources⁽¹²⁴⁾. This method is highly sensitive, and able to detect 1 ppm of pyrrolizidine alkaloids present in plants. However, the drawback of this method is that the overall reaction yield is not known and the pyrrolizidine alkaloids cannot be quantified.

Yu *et al.*⁽¹²⁵⁾ reported the development of a simple

and rapid micellar electrokinetic chromatography method for the separation and determination of four toxic pyrrolizidine alkaloids, senkirkine, senecionine, retrorsine, and seneciphylline in two traditional Chinese herbal medicines, Qian liguang and Kuan donghua. A buffer consisting of 20 mM borate, 30 mM SDS, and 20% methanol at pH 9.1 was used for the separation. Yu *et al.*⁽¹²⁵⁾ claimed that this separation method could potentially be an effective alternative tool for quantitative analysis of herbal medicines in pharmaceutical industry. Another hyphenated online dynamic pH junction-sweeping CE (capillary electrophoresis)-UV method was reported to analyze the same four toxic pyrrolizidine alkaloids in Kuan donghua by the same research group⁽¹²⁶⁾.

II. Gas chromatography/Mass Spectroscopy (GC/MS) Method

An on-line GC/MS method has been an effective technique for the detection of pyrrolizidine alkaloids in food items, such as honey and milk^(5,24,25,123). GC/MS is also a convenient analytical method for detection and quantitation of pyrrolizidine alkaloids and pyrrolizidine alkaloid *N*-oxides present in herbal plants and herbal dietary supplements^(24,123,127). For example, GC/MS was employed to analyze pyrrolizidine alkaloids in the three species of the Boraginaceae family⁽¹²⁸⁾.

Betz *et al.*⁽²⁴⁾ developed an analytical method for detection and quantitation of hepatotoxic pyrrolizidine alkaloids and their *N*-oxides in comfrey-containing products (as capsules, bulk tea, tea bags, bags, tablets, bulk powder, and dried leaf) which were purchased in the Washington, D.C. area. This analytical method involved extraction, solid-phase concentration, and capillary gas chromatographic analysis of the samples. Nine out of the eleven products contained pyrrolizidine alkaloids in the range of 0.1 to 400 ppm. The levels of pyrrolizidine alkaloids in bulk comfrey root are higher than in bulk comfrey leaf. The identified pyrrolizidine alkaloids in comfrey include intermedine, lycopsamine, 7-acetylintermedine, and 7-acetyllycopsamine, among which intermedine and lycopsamine are tumorigenic (Figure 1).

III. Liquid Chromatography/mass Spectroscopic (LC/MS) Method

Liquid chromatography/mass spectroscopy (LC/MS) is the most powerful and convenient method for detection and quantitation of pyrrolizidine alkaloids and their *N*-oxides both in biological samples as well in herbal plants and commercial products^(5,24,69,119,122,123,129,130). Similar to the GC/MS method, prior to LC/MS analysis, an efficient extraction is required to isolate pyrrolizidine alkaloids and their *N*-oxides from the samples. Thus, a number of convenient extraction methods have been developed for pyrrolizidine alkaloid isolation. For instance, Gray *et al.*⁽¹³¹⁾ reported a rapid cleanup method for the isolation and concentration of pyrrolizidine alka-

loids in comfrey root. The powdered comfrey root was first extracted by sonication and shaking with basic chloroform, followed by a solid-phase extraction method employing an Ergosil cleanup column that specifically binds the pyrrolizidine alkaloids. Mroczek *et al.*⁽¹³²⁾ reported that pyrrolizidine alkaloid in dried plant material was extracted with boiling 1% tartaric acid in methanol on an electric basket followed by extraction with cation-exchange solid phase extraction. The semi-purified extracts containing pyrrolizidine alkaloids were separated on Zorbax SB RP18 stationary phase in gradient of 0.1% formic acid in methanol.

Pyrrolizidine alkaloids and their *N*-oxides can be extracted from the dried plant material using dilute aqueous acid. The subsequent integration of strong-cation-exchange, solid-phase extraction of pyrrolizidine alkaloids and their *N*-oxides from herbal products is a common extraction method⁽¹³³⁻¹³⁵⁾. Since this extraction method isolates both pyrrolizidine alkaloids and their *N*-oxides from the samples, the *N*-oxides in the mixture are reduced to the parent pyrrolizidine alkaloids and then the total pyrrolizidine alkaloids are determined⁽¹³⁴⁻¹³⁷⁾.

Schaneberg *et al.*⁽¹³⁸⁾ developed a reverse-phase HPLC method utilizing evaporative light scattering detection (ELSD) for the simultaneous detection of hepatotoxic pyrrolizidine alkaloids with and without chromophores. Thus, riddelliine, senecionine, seneciphylline, retrorsine, integerrimine, lasiocarpine, heliotrine, riddelliine *N*-oxide, and senecionine *N*-oxide contained in five different plant species (*Senecio spartioides*, *S. douglasii* var. *longilobus*, *S. jacobaea*, *S. intergerrimus* var. *exaltatus* and *Symphytum officinale*) can be detected simultaneously.

Besides using different experimental conditions for HPLC separation, different modes of mass spectrometry are also used, including electrospray ionization mass spectrometry^(136,137,139), thermabeam electron impact mass spectrometry⁽¹³²⁾, atmospheric pressure chemical ionization mass spectrometry^(112,134,135), and ion trap MS method with atmospheric pressure chemical ionization (APCI) interface⁽¹³²⁾. Wuilloud *et al.*⁽¹⁴⁰⁾ employed LC-MS/MS for identification of pyrrolizidine alkaloids and their *N*-oxides. This method can identify a number of pyrrolizidine alkaloids present in each sample, including those that were not completely resolved chromatographically.

Lin *et al.*⁽¹³⁰⁾ identified different types of hepatotoxic pyrrolizidine alkaloids in plants and biological samples using on-line high performance liquid chromatography-mass spectrometry with an electrospray interface. The developed method has an advantage using either in-source or in the collision cell CID (collision induced dissociation) to obtain a full scan mass spectrum or tandem MS/MS spectrum. As illustrated in Figure 5, based on the specific diagnostic fragment ions obtained from the CID spectrum, three types of toxic pyrrolizidine alkaloids can be identified unequivocally. Further, using this method, the non-toxic pyrrolizidine alkaloids can also be definitively distinguished from all three toxic types.

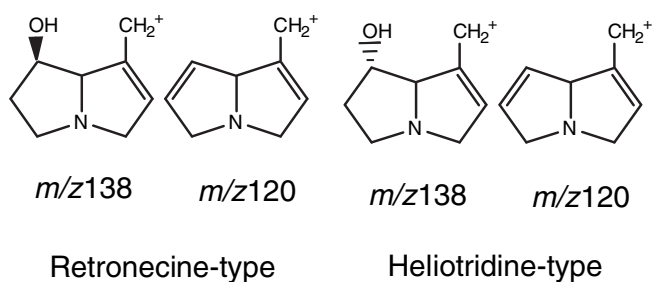


Figure 5. Specific mass spectrometric diagnostic fragment ions of different types of pyrrolizidine alkaloids. All ions were obtained by CID-electrospray ionization in a positive ion mode.

IV. Competitive Inhibition Enzyme-linked Immunosorbent Assays (ELISA)

For detection of pyrrolizidine alkaloids in biological samples, competitive inhibition enzyme-linked immunosorbent assays (ELISA) have been developed as an effective methodology for detection of poisoned livestock^(113,114,124). These simple, rapid, and sensitive methods can accurately detect pyrrolizidine alkaloids and pyrrolizidine alkaloid *N*-oxides in biological samples. For example, in 2001, Lee *et al.* utilized the developed enzyme-linked immunosorbent assays for detection of the hepatotoxic alkaloids riddelliine and riddelliine *N*-oxide⁽¹¹⁴⁾.

V. Identification and Quantification of DHR-derived DNA Adducts in Biological Samples

(I) Detection in Blood of Rats Treated with Riddelliine

The measurement of DNA adducts *in vivo* is considered a biomarker for assessing the risk of chemically induced carcinogenesis. As described earlier, the DHP-derived DNA adducts formed *in vivo* are potential biomarkers of pyrrolizidine alkaloid tumorigenicity as well as pyrrolizidine alkaloids exposure. Yan *et al.*⁽⁹⁵⁾ reported the formation of these DHP-derived DNA adducts in blood DNA of rats fed riddelliine. Male and female F344 rats were administered riddelliine by gavage at a single dose of 10.0 mg/kg body weight in 0.1 M phosphate buffer. The levels of DHP-derived DNA adducts in blood and liver were determined by ³²P-postlabeling/HPLC. During 2 to 7 day time-period, the DHP-derived adducts were detected in the blood, with the levels in female rat blood 4-fold greater than those in male rats. In the dose-response experiment, female rats were gavaged 0.1 and 1.0 mg/kg doses of riddelliine for three consecutive days. The levels of the DHP-derived DNA adducts in blood of rats receiving 0.1 and 1.0 mg/kg doses were 12.9 and 51.8 adducts/10⁷ nucleotides. These results suggest that: (i) leucocyte DNA can bind with DHP to form the same set of DHP-derived DNA adducts generated in liver,

although at a lower level; (ii) since these DNA adducts are responsible for liver tumor formation, riddelliine may be able to induce leukemia in rats; and (iii) DHP-derived DNA adducts in blood can serve as a potential non-invasive biomarkers for assessing the exposure as well as the risk that riddelliine poses⁽⁹⁵⁾.

(II) Detection from Dietary Supplements and Chinese Herbal Plant Extracts Containing Carcinogenic Pyrrolizidine Alkaloids *In Vivo*

Comfrey and coltsfoot are two naturally occurring herbs sold as dietary supplements worldwide. Comfrey has been used for centuries as a wound healer and cough suppressant, and dried coltsfoot has been used for cough remedy^(141,142). Comfrey contains the hepatotoxic and tumorigenic pyrrolizidine alkaloids, intermediate, symphytine, and lycopsamine, and coltsfoot root extract and flos farfara root extract contain the tumorigenic senkirkine and senecionine^(23,27,143). Chou and Fu⁽¹⁴⁴⁾ determined that these DHP-derived DNA adducts are formed in the livers of rats fed these dietary supplements and the herbal plant extracts described above. These results suggest that these exogenous DHP-derived DNA adducts can potentially be employed to detect genotoxic and tumorigenic pyrrolizidine alkaloids in dietary supplements and herbal medicinal plants, and are potential biomarkers of pyrrolizidine alkaloid tumorigenicity and exposure⁽¹⁴⁴⁾.

VI. Mutation Frequency Pattern

Mei *et al.*⁽⁶²⁾ studied mutagenicity of riddelliine in female transgenic Big Blue rats gavaged with 0.1, 0.3, and 1.0 mg riddelliine per kg body weight 5 days a week for 12 weeks and sacrificed one day after the last treatment. The types of mutant frequency in the transgenic cII gene of liver DNA was characterized by sequencing the mutants. Molecular analysis of the mutants indicated that a significant dose-dependent increase in mutant frequency and that there was a significant difference between the mutational spectra between the riddelliine-treated and the control rats. A G:C → T:A transversion (35%) was the major type of mutation in rats treated with riddelliine, whereas a G:C → A:T transition (55%) was the predominant mutation in the controls. In addition, mutations from the riddelliine-treated rats included an eight percent of tandem base substitutions of GG → TT and GG → AT which was an unusually high frequency. These results support that riddelliine is a genotoxic carcinogen in rat liver and that the types of mutations induced by riddelliine are consistent with riddelliine adducts involving G:C base pairs⁽⁶²⁾. These results also suggest that the unusually high frequency of tandem base substitutions of GG → TT and GG → AT may be a signature mutation pattern of riddelliine as well as the other tumorigenic pyrrolizidine alkaloids.

PERSPECTIVES

Since the U.S. Congress passed the Dietary Supplement Health and Education Act (DSHEA) in 1994, herbal products represent the fastest growing segment of the VMH (Vitamin, Mineral supplements, and herbal products) industry. To ensure consumer health protection, the quality and safety of raw herbal plants used for dietary supplement preparations have to be determined. To date, safety issues concerning potential side-effects and toxic contamination of herbal products have not been addressed adequately and toxicological data on the identification of genotoxic and tumorigenic ingredients in many raw herbs are lacking.

Pyrrolizidine alkaloids are the leading hepatotoxic and tumorigenic phytochemicals associated with human and animal diseases. Although it has been known since the early 1950s that some pyrrolizidine alkaloids are tumorigenic^(38,145), the mechanisms by which these compounds induce tumors are not known until our findings reported in 2001⁽⁸⁶⁾. We determined that riddelliine induces liver tumors through a genotoxic mechanism mediated by DHP-derived DNA adduct formation. The formation of these DHR-derived DNA adducts *in vivo* has been found to be a general biomarker of pyrrolizidine alkaloid-induced tumorigenicity^(37,107). Since pyrrolizidine alkaloid-containing plants are widespread in the world and many of these compounds are hepatotoxic and tumorigenic, for human health protection, it is highly important to determine whether or not the commercial herbal plants and herbal dietary supplements contain toxic pyrrolizidine alkaloids, and to assess the risk posed by consumption of these commercial products.

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REFERENCES

- Huang, K.C. 1998. The pharmacology of Chinese herbs. Boca Raton, FL: CRC Press LLC.
- Roeder, E. 2000. Medicinal plants in China containing pyrrolizidine alkaloids. *Pharmazie*. 55: 711-726.
- Fu, P. P., Yang, Y.-C., Xia, Q., Chou, M. W., Cui, Y. and Lin, G. 2002. Pyrrolizidine alkaloids - tumorigenic components in Chinese herbal medicines and dietary supplements. *J. Food Drug Anal.* 10: 198-211.
- (IARC), I.A.f.R.i.C., editor. 1976. Pyrrolizidine alkaloids. Lyon, France: International Agency for Research in Cancer.
- Mattocks, A. R. 1986. Chemistry and toxicology of pyrrolizidine alkaloids. London, N Y: Academic Press.
- Roeder, E. 1995. Medicinal plants in Europe containing pyrrolizidine alkaloids. *Pharmazie* 50: 83-98.
- (IPCS), I.P.o.C.S. 1988. Pyrrolizidine alkaloids. In *Environmental Health Criteria* 80. WHO: Geneva.
- (IPCS), I.P.o.C.S. 1989. Pyrrolizidine Alkaloids Health and Safety Guides. In *Health and Safety Criteria Guides* 26. WHO: Geneva.
- Anke, S., Niemuller, D., Moll, S., Hansch, R. and Ober, D. 2004. Polyphyletic origin of pyrrolizidine alkaloids within the Asteraceae. Evidence from differential tissue expression of homospermidine synthase. *Plant Physiol.* 136: 4037-4047.
- Beuerle, T., Theuring, C., Klewer, N., Schulz, S. and Hartmann, T. 2007. Absolute configuration of the cretonotines and callimorphines, two classes of arctiid-specific pyrrolizidine alkaloids. *Insect. Biochem. Mol. Biol.* 37: 80-89.
- Hartmann, T. 2004. Plant-derived secondary metabolites as defensive chemicals in herbivorous insects: a case study in chemical ecology. *Planta* 219: 1-4.
- Kirk, H., Macel, M., Klinkhamer, P. G. and Vrieling, K. 2004. Natural hybridization between *Senecio jacobaea* and *Senecio aquaticus*: molecular and chemical evidence. *Mol. Ecol.* 13: 2267-2274.
- Pelser, P. B., de Vos, H., Theuring, C., Beuerle, T., Vrieling, K. and Hartmann, T. 2005. Frequent gain and loss of pyrrolizidine alkaloids in the evolution of *Senecio* section *Jacobaea* (Asteraceae). *Phytochemistry* 66: 1285-1295.
- Reimann, A., Nurhayati, N., Backenkohler, A. and Ober, D. 2004. Repeated evolution of the pyrrolizidine alkaloid-mediated defense system in separate angiosperm lineages. *Plant Cell* 16: 2772-2784.
- Macel, M., Bruinsma, M., Dijkstra, S. M., Ooijendijk, T., Niemeyer, H. M. and Klinkhamer, P.G. 2005. Differences in effects of pyrrolizidine alkaloids on five generalist insect herbivore species. *J. Chem. Ecol.* 31: 1493-1508.
- Fu, P. P., Chou, M. W., Xia, Q., Yang, Y. C., Yan, J., Doerge, D.R. and Chan, P. C. 2001. Genotoxic pyrrolizidine alkaloids and pyrrolizidine alkaloid N-oxides - mechanisms leading to DNA adduct formation and tumorigenicity. *Environ. Carcinogen. Ecotoxicol. Rev.* 19: 353-386.
- Smith, L. W. and Culvenor, C. C. 1981. Plant sources of hepatotoxic pyrrolizidine alkaloids. *J. Nat. Prod.* 44: 129-152.
- Schoental, R. (ed.) 1976. Carcinogens in plants and microorganisms. American Chemical Society. Washington, DC. U. S. A.
- Bull, L. B., Culvenor, C. C. and Dick, A. J. 1968. The pyrrolizidine alkaloids. Their chemistry, pathogenicity and other biological properties. North-Holland, Amsterdam.
- Stegelmeier, B. L., Edgar, J. A., Colegate, S. M., Gardner, D. R., Schoch, T. K., Coulombe, R.A. and Molyneux, R. J. 1999. Pyrrolizidine alkaloid plants,

- metabolism and toxicity. *J. Nat. Toxins*. 8: 95-116.
21. Steenkamp, V., Stewart, M. J. and Zuckerman, M. 2000. Clinical and analytical aspects of pyrrolizidine poisoning caused by South African traditional medicines. *Ther. Drug Monit.* 22: 302-306.
 22. Mattocks, A.R. 1968. Toxicity of pyrrolizidine alkaloids. *Nature* 217: 723-728.
 23. Rosberger, D. F., Resch, J. F. and Meinwald, J. 1981. The occurrence of senecionine in *Tussilago farfara*. *Mitt. Gebiete Letensm. Hyg.* 72: 432-436.
 24. Betz, J. M., Eppley, R. M., Taylor, W. C. and Andrzejewski, D. 1994. Determination of pyrrolizidine alkaloids in commercial comfrey products (*Symphytum* sp.). *J. Pharm. Sci.* 83: 649-653.
 25. Edgar, J. A., Roeder, E. and Molyneux, R. J. 2002. Honey from plants containing pyrrolizidine alkaloids: a potential threat to health. *J. Agric. Food Chem.* 50: 2719-2730.
 26. Huxtable, R. J. 1989. Human embryotoxicity of pyrrolizidine-containing drugs. *Hepatology* 9: 510-511.
 27. Winship, K. A. 1991. Toxicity of comfrey. *Adverse Drug React Toxicol Rev.* 10: 47-59.
 28. Byron, J. 1998. Pyrrolizidine alkaloids in eggs; new alkaloid found in potatoes. *Food Chem. News.* 14: 6-7.
 29. Prakash, A. S., Pereira, T. N., Reilly, P. E. and Seawright, A. A. 1999. Pyrrolizidine alkaloids in human diet. *Mutat Res.* 443: 53-67.
 30. Zhao, X. G., Zhang, M., Li, Z. M., Wang, Z. T., Xu, L. S., Xu, G. J., Yu, G. D. and Lin, G. 2000. Investigation into the Chinese traditional drug, *Zi Wan*. pp. 134-189.
 31. Zhao, X. G., Wang, Z. T., Lin, G., Cui, Y. Y., Zhang, M., Xu, L. S., Xu, G. J. and Damani, L. A. 1998. Hepatotoxic pyrrolizidine alkaloids and transitional Chinese medicines. *Chin. Tradit. Herb. Drugs* 29: 115-119.
 32. Traditional, Chinese, and Dictionary, editors. 1979. Shanghai: Shanghai Science and Technology.
 33. Edgar, J. A., Lin, H. J., Kumana, C. R. and Ng, M. M. 1992. Pyrrolizidine alkaloid composition of three Chinese medicinal herbs, *Eupatorium cannabinum*, *E. japonicum* and *Crotalaria assamica*. *Am. J. Chin. Med.* 20: 281-288.
 34. Lin, G., Cui, Y. Y. and Hawes, E. M. 2000a. Characterization of rat liver microsomal metabolites of clivorine, an hepatotoxic otonecine-type pyrrolizidine alkaloid. *Drug Metab. Dispos.* 28: 1475-1483.
 35. Buckmaster, G. W., Cheeke, P. R., Arcsott, G. H., Dickinson, E. O., Pierson, M. L. and Shull, L. R. 1977. Response of Japanese quail to dietary and injected pyrrolizidine (*Senecio*) alkaloid. *J. Anim. Sci.* 45: 1322-1325.
 36. de Lanux-Van Gorder, V. 2000. Tansy ragwort poisoning in a horse in southern Ontario. *Can. Vet. J.* 41: 409-410.
 37. Fu, P. P., Xia, Q., Lin, G. and Chou, M. W. 2004. Pyrrolizidine alkaloids--genotoxicity, metabolism enzymes, metabolic activation, and mechanisms. *Drug Metab. Rev.* 36: 1-55.
 38. Schoental, R., head, M. A. and Peacock, P. R. 1954. *Senecio* alkaloids: Primary liver tumours in rats as a result of treatment with (1) a mixture of alkaloids from *S. jacobaea* lin.; (2) retrorsine; (3) isatidine. *Br. J. Cancer* 8: 458-465.
 39. Schoental, R., Fowler, M.E. and Coady, A. 1970. Islet cell tumors of the pancreas found in rats given pyrrolizidine alkaloids from *Amsinckia intermedia* Fisch and Mey and from *Heliotropium supinum* L. *Cancer Res.* 30: 2127-2131.
 40. Schoental, R. and Cavanagh, J. B. 1972. Brain and spinal cord tumors in rats treated with pyrrolizidine alkaloids. *J. Natl. Cancer Inst.* 49: 665-671.
 41. Schoental, R. 1975. Pancreatic islet-cell and other tumors in rats given heliotrine, a monoester pyrrolizidine alkaloid, and nicotinamide. *Cancer Res.* 35: 2020-2024.
 42. Svoboda, D. J. and Reddy, J. K. 1972. Malignant tumors in rats given lasiocarpine. *Cancer Res.* 32: 908-913.
 43. Kuhara, K., Takanashi, H., Hirono, I., Furuya, T. and Asada, Y. 1980. Carcinogenic activity of clivorine, a pyrrolizidine alkaloid isolated from *Ligularia dentata*. *Cancer Lett.* 10: 117-122.
 44. Rao, M. S. and Reddy, J. K. 1978. Malignant neoplasms in rats fed lasiocarpine. *Br. J. Cancer* 37: 289-293.
 45. Mattocks, A. R. and Cabral, J. R. 1982. Carcinogenicity of some pyrrolic pyrrolizidine alkaloid metabolites and analogues. *Cancer Lett.* 17: 61-66.
 46. Eastman, D. F. and Segall, H. J. 1982. A new pyrrolizidine alkaloid metabolite, 19-hydroxysenecionine isolated from mouse hepatic microsomes *in vitro*. *Drug Metab. Dispos.* 10: 696-699.
 47. Galloway, S. M., Armstrong, M. J., Reuben, C., Colman, S., Brown, B., Cannon, C., Bloom, A. D., Nakamura, F., Ahmed, M. and Duk, S. 1987. Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: evaluations of 108 chemicals. *Environ. Mol. Mutagen.* 10 Suppl 10: 1-175.
 48. Mirsalis, J. C., Steinmetz, K. L., Blazak, W. F. and Spalding, J. W. 1993. Evaluation of the potential of riddelliine to induce unscheduled DNA synthesis, S-phase synthesis, or micronuclei following *in vivo* treatment with multiple doses. *Environ. Mol. Mutagen.* 21: 265-271.
 49. Kim, H. Y., Stermitz, F. R., Molyneux, R. J., Wilson, D. W., Taylor, D. and Coulombe, R. A. 1993. Structural influences on pyrrolizidine alkaloid-induced cytopathology. *Toxicol. Appl. Pharmacol.* 122: 61-69.
 50. MacGregor, J. T., Wehr, C. M., Henika, P. R. and Shelby, M. D. 1990. The *in vivo* erythrocyte micronucleus test: measurement at steady state increases assay efficiency and permits integration with toxicity studies. *Fundam. Appl. Toxicol.* 14: 513-522.

51. Kim, H. Y., Stermitz, F. R. and Coulombe, R. A. 1995. Pyrrolizidine alkaloid-induced DNA-protein cross-links. *Carcinogenesis* 16: 2691-2697.
52. Kim, H. Y., Stermitz, F. R., Li, J. K. and Coulombe, R. A. 1999. Comparative DNA cross-linking by activated pyrrolizidine alkaloids. *Food Chem. Toxicol.* 37: 619-625.
53. Griffin, D. S. and Segall, H. J. 1986. Genotoxicity and cytotoxicity of selected pyrrolizidine alkaloids, a possible alkenal metabolite of the alkaloids, and related alkenals. *Toxicol. Appl. Pharmacol.* 86: 227-234.
54. Petry, T. W., Bowden, G. T., Buhler, D. R., Sipes, I.G. and Sipes, K. G. 1986. Genotoxicity of the pyrrolizidine alkaloid jacobine in rats. *Toxicol. Lett.* 32: 275-281.
55. Hincks, J. R., Kim, H. Y., Segall, H. J., Molyneux, R. J., Stermitz, F. R. and Coulombe, R. A. 1991. DNA cross-linking in mammalian cells by pyrrolizidine alkaloids: structure-activity relationships. *Toxicol. Appl. Pharmacol.* 111: 90-98.
56. Coulombe, R. A., Jr., Drew, G. L. and Stermitz, F. R. 1999. Pyrrolizidine alkaloids crosslink DNA with actin. *Toxicol. Appl. Pharmacol.* 154: 198-202.
57. Chan, P. C., Mahler, J., Bucher, J. R., Travlos, G. S. and Reid, J. B. 1994. Toxicity and carcinogenicity of riddelliine following 13 weeks of treatment to rats and mice. *Toxicol.* 32: 891-908.
58. Chan, P. C., Haseman, J. K., Prejean, J. D. and Nyska, A. 2003. Toxicity and carcinogenicity of riddelliine in rats and mice. *Toxicol. Lett.* 144: 295-311.
59. Hirono, I., Ueno, I., Aiso, S., Yamaji, T. and Haga, M. 1983. Carcinogenic activity of *Farfugium japonicum* and *Senecio cannabifolius*. *Cancer Lett.* 20: 191-198.
60. Mei, N., Chou, M. W., Fu, P. P., Heflich, R. H. and Chen, T. 2004. Differential mutagenicity of riddelliine in liver endothelial and parenchymal cells of transgenic big blue rats. *Cancer Lett.* 215: 151-158.
61. Mei, N., Guo, L., Fu, P. P., Heflich, R. H. and Chen, T. 2005. Mutagenicity of comfrey (*Symphytum Officinale*) in rat liver. *Br. J. Cancer* 92: 873-875.
62. Mei, N., Heflich, R. H., Chou, M. W. and Chen, T. 2004. Mutations induced by the carcinogenic pyrrolizidine alkaloid riddelliine in the liver cII gene of transgenic big blue rats. *Chem. Res. Toxicol.* 17: 814-818.
63. Wehner, F.C., Thiel, P.G. and van Rensburg, S. J. 1979. Mutagenicity of alkaloids in the Salmonella/microsome system. *Mutat. Res.* 66: 187-190.
64. Yamanaka, H., Nagao, M., Sugimura, T., Furuya, T., Shirai, A. and Matsushima, T. 1979. Mutagenicity of pyrrolizidine alkaloids in the Salmonella/mammalian-microsome test. *Mutat. Res.* 68: 211-216.
65. Zeiger, E., Anderson, B., Haworth, S., Lawlor, T. and Mortelmans, K. 1988. Salmonella mutagenicity tests: IV. Results from the testing of 300 chemicals. *Environ. Mol. Mutagen.* 11 Suppl 12: 1-157.
66. Rubiolo, P., Pieters, L., Calomme, M., Bicchi, C., Vlietinck, A. and Vanden Berghe, D. 1992. Mutagenicity of pyrrolizidine alkaloids in the Salmonella typhimurium/mammalian microsome system. *Mutat. Res.* 281: 143-147.
67. McLean, E. K. 1970. The toxic actions of pyrrolizidine (senecio) alkaloids. *Pharmacol. Rev.* 22: 429-483.
68. Man'ko, I. V. 1972. Alkaloids of *Cynoglossum amabile* and *C. viridiflorum*. *Rast. Resur.* 8: 243-246.
69. Lin, G., Rose, P., Chatson, K. B., Hawes, E. M., Zhao, X. G., and Wang, Z. T. 2000b. Characterization of two structural forms of otonecine-type pyrrolizidine alkaloids from *Ligularia hodgsonii* by NMR spectroscopy. *J. Nat. Prod.* 63: 857-860.
70. Man'ko, I. V. 1959. Alkaloids of *Cynoglossum officinale*. *Ukrain. Khim. Zhur.* 25: 627-630.
71. Williams, D. E., Reed, R.L., Kedzierski, B., Dannan, G. A., Guengerich, F. P. and Buhler, D. R. 1989. Bioactivation and detoxication of the pyrrolizidine alkaloid senecionine by cytochrome P-450 enzymes in rat liver. *Drug Metab. Dispos.* 17: 387-392.
72. Miranda, C. L., Reed, R. L., Guengerich, F. P. and Buhler, D. R. 1991. Role of cytochrome P450III A4 in the metabolism of the pyrrolizidine alkaloid senecionine in human liver. *Carcinogenesis* 12: 515-519.
73. Chung, W. G. and Buhler, D. R. 1994. The effect of spironolactone treatment on the cytochrome P450-mediated metabolism of the pyrrolizidine alkaloid senecionine by hepatic microsomes from rats and guinea pigs. *Toxicol. Appl. Pharmacol.* 127: 314-319.
74. Reid, M. J., Lame, M. W., Morin, D., Wilson, D. W. and Segall, H. J. 1998. Involvement of cytochrome P450 3A in the metabolism and covalent binding of ¹⁴C-monocrotaline in rat liver microsomes. *J. Biochem. Mol. Toxicol.* 12: 157-166.
75. Kasahara, Y., Kiyatake, K., Tatsumi, K., Sugito, K., Kakusaka, I., Yamagata, S., Ohmori, S., Kitada, M. and Kuriyama, T. 1997. Bioactivation of monocrotaline by P-450 3A in rat liver. *J. Cardiovasc Pharmacol.* 30: 124-129.
76. Chung, W. G., and Buhler, D. R. 1995. Major factors for the susceptibility of guinea pig to the pyrrolizidine alkaloid jacobine. *Drug Metab. Dispos.* 23: 1263-1267.
77. Buhler, D. R. and Kedzierski, B. 1986. Biological reactive intermediates of pyrrolizidine alkaloids. *Adv. Exp. Med. Biol.* 197: 611-620.
78. Dueker, S. R., Lame, M. W., Morin, D., Wilson, D. W. and Segall, H. J. 1992. Guinea pig and rat hepatic microsomal metabolism of monocrotaline. *Drug Metab. Dispos.* 20: 275-280.
79. Lame, M. W., Jones, A. D., Morin, D. and Segall, H. J. 1991. Metabolism of [¹⁴C]monocrotaline by isolated perfused rat liver. *Drug Metab. Dispos.* 19: 516-524.
80. Kedzierski, B. and Buhler, D. R. 1985. Configuration of necine pyrroles--toxic metabolites of pyrrolizidine alkaloids. *Toxicol. Lett.* 25: 115-119.

81. Kedzierski, B. and Buhler, D. R. 1986. The formation of 6,7-dihydro-7-hydroxy-1-hydroxy-methyl-5H-pyrrolizine, a metabolite of pyrrolizidine alkaloids. *Chem. Biol. Interact.* 57: 217-222.
82. Lin, G., Cui, Y. Y. and Hawes, E. M. 1998a. Microsomal formation of a pyrrolic alcohol glutathione conjugate of clivorine. Firm evidence for the formation of a pyrrolic metabolite of an otonecine-type pyrrolizidine alkaloid. *Drug Metab. Dispos.* 26: 181-184.
83. Lin, G., Cui, Y. Y. and Liu, X. Q. 2003. Gender differences in microsomal metabolic activation of hepatotoxic clivorine in rat. *Chem. Res. Toxicol.* 16: 768-774.
84. Lin, G., Cui, Y. Y., Liu, X. Q. and Wang, Z. T. 2002. Species differences in the *in vitro* metabolic activation of the hepatotoxic pyrrolizidine alkaloid clivorine. *Chem. Res. Toxicol.* 15: 1421-1428.
85. Yang, Y., Yan, J., Churchwell, M., Beger, R., Chan, P., Doerge, D. R., Fu, P. P. and Chou, M. W. 2001. Development of a (32)P-postlabeling/HPLC method for detection of dehydroretronecine-derived DNA adducts *in vivo* and *in vitro*. *Chem. Res. Toxicol.* 14: 91-100.
86. Yang, Y. C., Yan, J., Doerge, D. R., Chan, P. C., Fu, P. P. and Chou, M. W. 2001. Metabolic activation of the tumorigenic pyrrolizidine alkaloid, riddelliine, leading to DNA adduct formation *in vivo*. *Chem. Res. Toxicol.* 14: 101-109.
87. Chou, M. W., Jian, Y., Williams, L. D., Xia, Q., Churchwell, M., Doerge, D. R. and Fu, P. P. 2003. Identification of DNA adducts derived from riddelliine, a carcinogenic pyrrolizidine alkaloid. *Chem. Res. Toxicol.* 16: 1130-1137.
88. Chou, M. W., Wang, Y. P., Yan, J., Yang, Y. C., Beger, R. D., Williams, L. D., Doerge, D.R. and Fu, P. P. 2003. Riddelliine N-oxide is a phytochemical and mammalian metabolite with genotoxic activity that is comparable to the parent pyrrolizidine alkaloid riddelliine. *Toxicol. Lett.* 145: 239-247.
89. Chou, M. W., Yan, J., Nichols, J., Xia, Q., Beland, F. A., Chan, P. C. and Fu, P. P. 2003. Correlation of DNA adduct formation and riddelliine-induced liver tumorigenesis in F344 rats and B6C3F(1) mice. *Cancer Lett.* 193: 119-125.
90. Jago, M. V., Edgar, J. A., Smith, L. W. and Culvenor, C. C. 1970. Metabolic conversion of heliotridine-based pyrrolizidine alkaloids to dehydroheliotridine. *Mol. Pharmacol.* 6: 402-406.
91. Eastman, D. F. and Segall, H. J. 1981. Effects of the pyrrolizidine alkaloids senecionine, retrorsine and seneciphylline on aminopyrine N-demethylase activity on the rat liver S- 10 fraction. *Toxicol. Lett.* 8: 217-222.
92. Mattocks, A. R. and White, I. N. 1971. The conversion of pyrrolizidine alkaloids to N-oxides and to dihydropyrrolizine derivatives by rat-liver microsomes *in vitro*. *Chem. Biol. Interact.* 3: 383-396.
93. Miranda, C. L., Reed, R. L., Cheeke, P. R. and Buhler, D. R. 1981. Protective effects of butylated hydroxyanisole against the acute toxicity of monocrotaline in mice. *Toxicol. Appl. Pharmacol.* 59: 424-430.
94. Segall, H. J., Wilson, D. W., Dallas, J. L. and Haddon, W. F. 1985. trans-4-Hydroxy-2-hexenal: a reactive metabolite from the macrocyclic pyrrolizidine alkaloid senecionine. *Science* 229: 472-475.
95. Yan, J., Nichols, J., Yang, Y. C., Fu, P. P. and Chou, M. W. 2002. Detection of riddelliine-derived DNA adducts in blood of rats fed riddelliine. *Intl. J. Mol. Sci.* 3: 1019-1026.
96. Robertson, K. A. 1982. Alkylation of N2 in deoxyguanosine by dehydroretronecine, a carcinogenic metabolite of the pyrrolizidine alkaloid monocrotaline. *Cancer Res.* 42: 8-14.
97. Yan, C. C. and Huxtable, R. J. 1995. The effect of the pyrrolizidine alkaloids, monocrotaline and trichodesmine, on tissue pyrrole binding and glutathione metabolism in the rat. *Toxicol.* 33: 627-634.
98. Yan, C. C. and Huxtable, R. J. 1995. Relationship between glutathione concentration and metabolism of the pyrrolizidine alkaloid, monocrotaline, in the isolated, perfused liver. *Toxicol. Appl. Pharmacol.* 130: 132-139.
99. White, I. N. 1976. The role of liver glutathione in the acute toxicity of retrorsine to rats. *Chem. Biol. Interact.* 13: 333-342.
100. Lin, G., Tang, J., Liu, X. Q., Jiang, Y. and Zheng, J. 2007. Deacetylclivorine: a gender-selective metabolite of clivorine formed in female Sprague-Dawley rat liver microsomes. *Drug Metab. Dispos.* 35: 607-613.
101. Miranda, C. L., Chung, W., Reed, R. E., Zhao, X., Henderson, M. C., Wang, J. L., Williams, D. E. and Buhler, D. R. 1991. Flavin-containing monooxygenase: a major detoxifying enzyme for the pyrrolizidine alkaloid senecionine in guinea pig tissues. *Biochem. Biophys. Res. Commun.* 178: 546-552.
102. Williams, D. E., Reed, R. L., Kedzierski, B., Ziegler, D. M. and Buhler, D. R. 1989. The role of flavin-containing monooxygenase in the N-oxidation of the pyrrolizidine alkaloid senecionine. *Drug Metab. Dispos.* 17: 380-386.
103. White, I. N., Mattocks, A. R. and Butler, W. H. 1973. The conversion of the pyrrolizidine alkaloid retrorsine to pyrrolic derivatives *in vivo* and *in vitro* and its acute toxicity to various animal species. *Chem. Biol. Interact.* 6: 207-218.
104. Chan, P. C. 2001. Toxicology and carcinogenesis studies of riddelliine in F344/N rats and B6C3F1 mice. In "NTP Technical Report (CAS No. 23246-96-0)". NTP TR 508.
105. Xia, Q., Chou, M. W., Kadlubar, F. F., Chan, P. C. and Fu, P. P. 2003. Human liver microsomal metabolism and DNA adduct formation of the tumorigenic pyrrolizidine alkaloid, riddelliine. *Chem. Res. Toxicol.* 16: 66-73.
106. Xia, Q., Chou, M. W., Lin, G. and Fu, P. P. 2004. Metabolic formation of DHP-derived DNA adducts

- from a representative otonecine type pyrrolizidine alkaloid clivorine and the extract of *Ligularia hodgsonii* hook. *Chem. Res. Toxicol.* 17: 702-708.
107. Xia, Q., Chou, M. W., Edgar, J. A., Doerge, D. R. and Fu, P. P. 2006. Formation of DHP-derived DNA adducts from metabolic activation of the prototype heliotridine-type pyrrolizidine alkaloid, lasiocarpine. *Cancer Lett.* 231: 138-145.
 108. Wang, Y. P., Fu, P. P. and Chou, M. W. 2005. Metabolic activation of the tumorigenic pyrrolizidine alkaloid, retrorsine, leading to DNA adduct formation *in vivo*. *Int. J. Environ. Res. Public Health* 2: 74-79.
 109. Wang, Y. P., Yan, J., Beger, R. D., Fu, P. P. and Chou, M. W. 2005. Metabolic activation of the tumorigenic pyrrolizidine alkaloid, monocrotaline, leading to DNA adduct formation *in vivo*. *Cancer Lett.* 226: 27-35.
 110. Deinzer, M. L., Thomson, P. A., Burgett, D. M., and Isaacson, D. L. 1977. Pyrrolizidine alkaloids: their occurrence in honey from tansy ragwort (*Senecio jacobaea* L.). *Science* 195: 497-499.
 111. Mattocks, A. R. 1967. Detection of pyrrolizidine alkaloids on thin-layer chromatograms. *J. Chromatogr.* 27: 505-508.
 112. Crews, C., Startin, J. R. and Clarke, P. A. 1997. Determination of pyrrolizidine alkaloids in honey from selected sites by solid phase extraction and HPLC-MS. *Food Addit. Contam.* 14: 419-428.
 113. Roseman, D. M., Wu, X. and Kurth, M. J. 1996. Enzyme-linked immunosorbent assay detection of pyrrolizidine alkaloids: immunogens based on quaternary pyrrolizidinium salts. *Bioconjug. Chem.* 7: 187-195.
 114. Lee, S. T., Schoch, T. K., Stegelmeier, B. L., Gardner, D. R., Than, K. A. and Molyneux, R. J. 2001. Development of enzyme-linked immunosorbent assays for the hepatotoxic alkaloids riddelliine and riddelliine N-oxide. *J. Agric. Food Chem.* 49: 4144-4151.
 115. Mattocks, A. R. and Jukes, R. 1990. Trapping and measurement of short-lived alkylating agents in a recirculating flow system. *Chem. Biol. Interact.* 76: 19-30.
 116. Wiedenfeld, H., Montes, C., Tawil, B., Contin, A. and Wynsma, R. 2006. Pyrrolizidine alkaloid level in *Senecio bicolor* (Willd.) Tod, ssp. *cineraria* (DC.) from middle Europe. *Pharmazie* 61: 559-561.
 117. Wiedenfeld, H., Pietrosiuk, A., Furmanowa, M. and Roeder, E. 2003. Pyrrolizidine alkaloids from *Lithospermum canescens* Lehm. *Z Naturforsch [C]* 58: 173-176.
 118. Vrieling, K. and Derridj, S. 2003. Pyrrolizidine alkaloids in and on the leaf surface of *Senecio jacobaea* L. *Phytochemistry* 64: 1223-1228.
 119. Brown, M. S., Molyneux, R. J. and Roitman, J. N. 1994. A general method for high performance liquid chromatographic analysis of pyrrolizidine alkaloid free bases and N-oxides. *Phytochem. Anal.* 5: 251-255.
 120. Bartkowski, J. B., Wiedenfeld, H. and Roeder, E. 1997. Quantitative photometric determination of senkirkine in *Farfarae folium*. *Phytochem. Anal.* 8: 1-4.
 121. Roeder, E. 1999. Analysis of pyrrolizidine alkaloids. *Curr. Org. Chem.* 3: 557-576.
 122. Wiedenfeld, H., Lebada, R. and Kopp, B. 1995. Pyrrolizidine alkaloids in coltsfoot. *Dtsch Apoth Ztg.* 135: 1037-1046.
 123. Witte, L., Rubiolo, P., Bicchi, C. and Hartmann, T. 1993. Comparative analysis of pyrrolizidine alkaloids from natural sources by gas chromatography-mass spectrometry. *Phytochemistry* 32: 187-196.
 124. Roeder, E. and Pflueger, T. 1995. Analysis of pyrrolizidine alkaloids: a competitive enzyme-linked immunoassay (ELISA) for the quantitative determination of some toxic pyrrolizidine alkaloids. *Nat. Toxins.* 3: 305-309.
 125. Yu, L., Xu, Y., Feng, H. and Li, S. F. 2005. Separation and determination of toxic pyrrolizidine alkaloids in traditional Chinese herbal medicines by micellar electrokinetic chromatography with organic modifier. *Electrophoresis* 26: 3397-3404.
 126. Yu, L. and Li, S. F. 2005. Dynamic pH junction-sweeping capillary electrophoresis for online preconcentration of toxic pyrrolizidine alkaloids in Chinese herbal medicine. *Electrophoresis* 26: 4360-4367.
 127. Conradie, J., Stewart, M. J. and Steenkamp, V. 2005. GC/MS identification of toxic pyrrolizidine alkaloids in traditional remedies given to two sets of twins. *Ann. Clin. Biochem.* 42: 141-144.
 128. Frolich, C., Ober, D. and Hartmann, T. 2007. Tissue distribution, core biosynthesis and diversification of pyrrolizidine alkaloids of the lycopsamine type in three Boraginaceae species. *Phytochemistry* 68: 1026-1037.
 129. Pu, S. B., Xu, D. R., Zhang, M., Zhou, H. H., Wang, Z. T. and Yu, G. D. 2004. [Detection of hepatotoxic pyrrolizidine alkaloids in *Ligularia Cass.* with LC/MSn]. *Yao Xue Xue Bao* 39: 831-835.
 130. Lin, G., Zhou, K. Y., Zhao, X. G., Wang, Z. T. and But, P. P. 1998b. Determination of hepatotoxic pyrrolizidine alkaloids by on-line high performance liquid chromatography mass spectrometry with an electrospray interface. *Rapid Commun Mass Spectrom.* 12: 1445-1456.
 131. Gray, D. E., Porter, A., O'Neill, T., Harris, R. K. and Rottinghaus, G. E. 2004. A rapid cleanup method for the isolation and concentration of pyrrolizidine alkaloids in comfrey root. *J. AOAC Int.* 87: 1049-1057.
 132. Mroczek, T., Baj, S., Chrobok, A. and Glowniak, K. 2004a. Screening for pyrrolizidine alkaloids in plant materials by electron ionization RP-HPLC-MS with thermabeam interface. *Biomed. Chromatogr.* 18: 745-751.
 133. Mroczek, T., Ndjoko, K., Glowniak, K. and Hostettmann, K. 2004b. On-line structure characterization of pyrrolizidine alkaloids in *Onosma stellatum* and *Emilia coccinea* by liquid chromatography-ion-trap

- mass spectrometry. *J. Chromatogr. A* 1056: 91-97.
134. Beales, K.A., Betteridge, K., Colegate, S. M. and Edgar, J. A. 2004. Solid-phase extraction and LC-MS analysis of pyrrolizidine alkaloids in honeys. *J. Agric. Food Chem.* 52: 6664-6672.
135. Betteridge, K., Cao, Y. and Colegate, S. M. 2005. Improved method for extraction and LC-MS analysis of pyrrolizidine alkaloids and their N-oxides in honey: application to *Echium vulgare* honeys. *J. Agric. Food Chem.* 53: 1894-1902.
136. Altamirano, J. C., Gratz, S. R. and Wolnik, K. A. 2005. Investigation of pyrrolizidine alkaloids and their N-oxides in commercial comfrey-containing products and botanical materials by liquid chromatography electrospray ionization mass spectrometry. *J. AOAC Int.* 88: 406-412.
137. Colegate, S. M., Edgar, J. A., Knill, A. M. and Lee, S. T. 2005. Solid-phase extraction and HPLC-MS profiling of pyrrolizidine alkaloids and their N-oxides: a case study of *Echium plantagineum*. *Phytochem. Anal.* 16: 108-119.
138. Schaneberg, B. T., Molyneux, R. J. and Khan, I. A. 2004. Evaporative light scattering detection of pyrrolizidine alkaloids. *Phytochem. Anal.* 15: 36-39.
139. Siciliano, T., Leo, M. D., Bader, A., Tommasi, N. D., Vrieling, K., Braca, A. and Morelli, I. 2005. Pyrrolizidine alkaloids from *Anchusa strigosa* and their anti-feedant activity. *Phytochemistry* 66: 1593-1600.
140. Wuilloud, J. C., Gratz, S. R., Gamble, B. M. and Wolnik, K. A. 2004. Simultaneous analysis of hepatotoxic pyrrolizidine alkaloids and N-oxides in comfrey root by LC-ion trap mass spectrometry. *Analyst* 129: 150-156.
141. Culvenor, C. C., Edgar, J. A., Smith, L. W. and Hirono, J. H. 1976. The occurrence of senkirkine in *Tussilago farfara*. *Aust. J. Chem.* 29: 229-233.
142. Awang, D. V. 1987. Comfrey. *Can. Pharm. J.* 125: 100-104.
143. Hirono, I., Mori, H. and Culvenor, C. C. 1976. Carcinogenic activity of coltsfoot, *Tussilago farfara* L. *Gann.* 67: 125-129.
144. Chou, M. W. and Fu, P. P. 2006. Formation of DHP-derived DNA adducts *in vivo* from dietary supplements and chinese herbal plant extracts containing carcinogenic pyrrolizidine alkaloids. *Toxicol. Ind. Health* 22: 321-327.
145. Cook, J. W., Duffy, E. and Schoental, R. 1950. Primary liver tumours in rats following feeding with alkaloids of *Senecio jacobaea*. *Br. J. Cancer* 4: 405-410.