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RELATIVE TOXICITY OF SELECT DEHYDROPYRROLIZIDINE ALKALOIDS  
AND EVALUATION OF A HETEROZYGOUS P53 KNOCKOUT MOUSE MODEL  
FOR DEHYDROPYRROLIZIDINE ALKALOID INDUCED CARCINOGENESIS

by

Ammon W. Brown

A dissertation submitted in partial fulfillment  
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Toxicology

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Logan, UT

2015

## ABSTRACT

Relative Toxicity of Select Dehydropyrrolizidine Alkaloids and Evaluation of a  
Heterozygous P53 Knockout Mouse Model for Dehydropyrrolizidine Alkaloid Induced  
Carcinogenesis

by

Ammon W. Brown, Doctor of Philosophy

Utah State University, 2015

Major Professor: Jeffery O. Hall  
Program: Toxicology

Dehydropyrrolizidine alkaloids (DHPAs) are a large group of globally important plant-derived pro-toxins that can contaminate or are naturally present in animal feed and the human food supply as well as herbal supplements. Their bioactive metabolites are potentially hepatotoxic, pneumotoxic, genotoxic and carcinogenic. Due to the difficulty in obtaining sufficient quantities of purified DHPAs, toxicity studies have largely relied on single intraperitoneal injections in rodent models, and carcinogenicity studies have been limited to a small handful of the hundreds of isolated DHPAs. To assess the relative toxicity of structurally diverse DHPAs in a more biologically relevant manner, male California White chicks were dosed orally with 0.01, 0.04, 0.13, or 0.26 mmol of seven different DHPAs and three DHPA N-oxides  $\text{kg}^{-1}$  bodyweight for 7 days. DHPAs were grouped in relation to their toxicity based on clinical, serum biochemical, and

histopathological evaluations as well as tissue adduct accumulation rates. Using the same model, a reduced extract from comfrey, a commonly used DHPA containing herb, was compared to its two major constituent DHPAs, intermedine and lycopsamine. Based on the same parameters, the comfrey extract was more toxic than pure lycopsamine or intermedine. Addressing the need for a more sensitive carcinogenicity model, male heterozygous p53 knockout mice were treated with riddelliine 5, 15 or 45 mg kg<sup>-1</sup> bodyweight day<sup>-1</sup> by oral gavage for 14 days, or given a long-term treatment of riddelliine 1 mg kg<sup>-1</sup> bodyweight day<sup>-1</sup> in pelleted feed for 12 months. Exposure to riddelliine increased the odds of tumor development in a dose-responsive manner (odds ratio 2.05 and Wald 95% confidence limits between 1.2 and 3.4). The most common neoplasm was hepatic hemangiosarcoma, which is consistent with previously published lifetime rodent studies.

The results of this research demonstrate that the California White chick model is sensitive for comparison of DHPA toxicity, and data obtained from this research can be used to validate previous DHPA toxicity research. It also demonstrates that comfrey toxicity may have been previously underestimated. The heterozygous p53 knockout mouse model is beneficial for further investigation of comparative carcinogenesis of structurally and toxicologically different DHPAs and their N-oxides.

## PUBLIC ABSTRACT

Relative Toxicity of Select Dehydropyrrolizidine Alkaloids and Evaluation of a Heterozygous P53 Knockout Mouse Model for Dehydropyrrolizidine Alkaloid Induced Carcinogenesis

Ammon W. Brown

Dehydropyrrolizidine alkaloids (DHPAs) are toxins produced by approximately 3% of the world's flowering plants that can be present naturally or as contaminants in animal feed and the human food supply. Many of these compounds have been determined to cause cancer in animals and probably also cause cancer in humans. Due to the difficulty in obtaining sufficient amounts of pure DHPAs most toxicity research has been done via injection of a small amount into the abdomen of a rodent, although natural exposure is exclusively oral. For the same reason, cancer research is limited to a handful of the hundreds of known DHPAs. The present study evaluated more sensitive animal models for both toxicity and cancer research. Toxicity of 10 different DHPA compounds was tested using male, California White chicks. Through the evaluation of blood tests, tissue tests, and microscopic analysis of organs, the selected DHPAs were grouped according to toxicity. In this model, some compounds such as heliotrine were found to be more toxic than expected based on previous research. Using this same chick model, an extract from comfrey, a commonly used herb, was found to be more toxic than either of the two major DHPAs that it contains. Because plants often contain multiple DHPAs, this suggests that the toxicity of plants, such as comfrey, may be higher than previously estimated. A

mouse strain that is genetically susceptible to cancer (heterozygous p53 knockout) was tested and compared to previous studies using the same carcinogenic DHPA (riddelliine). The most common type of cancer was hemangiosarcoma (cancer of blood vessels) of the liver. These results were similar to previous studies using substantially less pure DHPA, indicating the benefit of this model to test the cancer causing potential of other DHPAs. Additionally, it was also determined that exposure to DHPAs for only two weeks (compared to years) also increased the odds of developing cancer.

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First I must acknowledge the United States Army for allowing me to pursue this degree and paying my wages and tuition. This has been an excellent opportunity, and I will strive to use what I have learned as I return and continue my career.

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## CHAPTER I

### Introduction

Pyrrolizidine alkaloids are a group of chemical compounds composed of two five-membered rings, which share a nitrogen at the four position. They are produced by a wide variety of plants and have presumably evolved to protect the plants from herbivory. It has been estimated that pyrrolizidine alkaloid containing plant species make up approximately 3% of the world's flowering plants (Smith and Culvenor 1981). A subset of pyrrolizidine alkaloids that contain a critical double bond between C1 and C2 are potentially toxic. This group of potentially toxic dehydropyrrolizidine alkaloids (DHPAs) consists of approximately 500 structural variants of the monoesters, open chain diesters or macrocyclic diesters of the necine bases retronecine, heliotridine and otonecine (Wiedenfeld, *et al.* 2008). The DHPAs and their co-occurring (usually the major components) N-oxides are pro-toxins requiring activation *in vivo* by the cytochrome P450 monooxygenases that results in hepatotoxicity (Campbell 1956, Gilruth 1904, Johnson, *et al.* 1985, Vardiman 1952), pneumotoxicity (Stalker and Hayes 2007) genotoxicity (Mei, *et al.* 2004, Mei, *et al.* 2005, Mei and Chen 2007), and carcinogenicity (Allen, *et al.* 1975, Schoental 1975, Schoental and Cavanagh 1972, Schoental, *et al.* 1970, Shumaker, *et al.* 1976, Yuzo, *et al.* 1977).

DHPAs are likely the most common poisonous plant problem worldwide (Stegelmeier, *et al.* 1999). Animal exposure to DHPAs results in direct loss to cattle, swine, horse and poultry producers in the form of animal deaths and reduced

productivity. Outbreaks in Africa and Middle Eastern countries have resulted in thousands of human deaths (Chauvin, *et al.* 1994; Mayer and Lüthy 1993; Mohabbat, *et al.* 1976; Tandon, *et al.* 1976; Tandon, *et al.* 1978) not to mention non-lethal effects.

As a result of the health hazards posed by DHPAs, health organizations and food and drug safety agencies in several countries have developed regulations and recommendations regarding the sale and use of DHPAs. The US Food and Drug Administration sent an advisory letter in 2001 to manufacturers of dietary supplements requesting that they remove all comfrey products intended for consumption from the market (FDA 2001). In 2008, the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment released a statement on DHPAs in food, limiting daily oral exposures to less than  $0.007 \mu\text{g DHPAs kg}^{-1}$  bodyweight BW (Committee on Toxicity of Chemicals in Food 2008). In Germany, the Federal Institute for Risk Assessment (BfR) conducted a risk assessment for DHPAs and concluded that exposure should be kept as low as possible while also limiting tolerable daily intake to  $0.007 \mu\text{g}$  of unsaturated pyrrolizidine alkaloids  $\text{kg}^{-1}$  (BW) (BfR 2011). The European Food Safety Authority, World Health Organization, and Dutch National Institute for Public Health and the Environment have all conducted similar reviews with similar concerns and recommendations (EFSA 2011; RIVM 2005; WHO 2011). The Food Standards Australia New Zealand authority, basing their recommendation solely on hepatotoxicity as opposed to potential carcinogenicity, recommends a somewhat higher tolerable exposure of  $1 \mu\text{g DHPAs kg}^{-1}$  BW per day (FSANZ 2001).

A potential relationship to common chronic human diseases has been postulated (Edgar, *et al.* 2014; Gyorik and Stricker 2009), which if substantiated would undoubtedly lead to further regulatory constraints and requirements for testing a wide variety of products. Indirect human exposure from sources such as meat, milk, honey and pollen (BfR 2011; Committee on Toxicity of Chemicals in Food 2008; FSANZ 2001; WHO 2011) are likely and the ability of the associated industries to control this exposure is limited.

Not only are DHPAs toxic, they have also been found to be carcinogenic in multiple animal species. One DHPA, riddelliine, has been listed as reasonably anticipated to be a human carcinogen by the National Toxicology Program (NTP 2011), and two DHPAs (riddelliine and lasiocarpine) have been listed by the International Agency for Research on Cancer (IARC) as class 2b carcinogens (IARC 1987; 2002). More research is needed to determine if these two are human carcinogens as well as the relative potential for carcinogenicity of other DHPAs.

The mechanisms for toxicity and carcinogenicity of DHPAs are thought to be the same. DHPAs are bioactivated by cytochrome P450 enzymes primarily in the liver to reactive intermediates. These reactive metabolites have the ability to bind with protein and DNA, and form cross-links (Coulombe, *et al.* 1999; Hincks, *et al.* 1991; Kim, *et al.* 1995; Petry, *et al.* 1984). Because of the common mechanism, The Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) recommends using LD50 riddelliine equivalents to extrapolate carcinogenicity risks (Committee on Toxicity of Chemicals in Food 2008).



Plants containing DHPAs although common (to the extent that in some cases they invade pastures, fields, and ranges becoming the dominant species), do not tend to grow in large monocultures that allow for mechanical harvesting, rather they tend to be scattered among other plants. The highest concentration of DHPA is often found in the roots of the plants making it labor intensive to harvest large quantities. Furthermore, most DHPA containing plants have a number of different DHPAs along with their N-oxides and other derivatives, thus making it necessary to separate very similar compounds in order to determine the impact of individual DHPAs.

As a result of the cost in time, resources and money to obtain sufficiently large quantities of DHPA to perform studies, the vast majority of toxicity studies have been done using intraperitoneal (IP) injections rather than oral exposure. Such studies avoid loss of valuable DHPA due to incomplete absorption. Natural exposure however, is almost exclusively oral. Because of the inherent differences that result from route of exposure, it is necessary to compare the large bank of information derived via IP exposure in a more biologically relevant model.

Where oral exposure studies do exist, they are usually done with plants rather than a single DHPA, and therefore contain a mixture of DHPAs. The results of these studies often do not correlate well with the IP exposure studies. For example, based on IP exposure studies, the anticipated toxicity of *Senecio douglasii* var. *longilobus* would be substantially higher than that of *Cynoglossum officinale* because the *Senecio* species contain primarily macrocyclic diesters and *Cynoglossum officinale* contains a mixture of monoesters and open chain diesters. If comparisons are made using oral exposure studies

however, the two plants are very similar in toxicity (Stegelmeier, *et al.* 1996, Baker, *et al.* 1989, Johnson and Molyneux 1984).

A model must meet a number of minimum requirements to be biologically relevant. First, it must be able to be repeatedly exposed orally, and with minimal risk to the animals as well as researchers. It must be a sensitive species that requires the least amount of purified alkaloid possible to detect a toxic effect. Ideally a good model is also small enough that it does not require large amounts of laboratory space and specialized facilities.

A number of studies have compared the relative susceptibilities of various species to DHPAs (Bull, *et al.* 1968, Cheeke 1998, Culvenor, *et al.* 1976, Mattocks 1972). The most sensitive domestic species appears to be swine (Hooper 1978) followed by chickens. Young animals have been found to be more sensitive than adults, and in many species males have been shown to be more sensitive than females (Mattocks 1986). Due to the large size difference between poultry and swine, the total amount of DHPA required to produce a toxic effect in chickens is far less than that required for swine. Furthermore, due to ease of restraint and differences in behavior, oral dosing of chicks is much easier and less traumatic than oral dosing of piglets.

In order to compare results of previous studies to this more biologically relevant model, it is necessary to compare the toxicity of multiple different chemical structures of DHPAs, preferably different bases as well as different R group structures.

Carcinogenicity studies require chronic exposure, and consequently larger quantities of DHPA. In order to study the relative carcinogenicity of DHPAs a more

sensitive model requiring less purified alkaloid is necessary. The last phase of this research will therefore focus on development of a small animal model that will require less of the pure alkaloid, less time, and hence less funding to test the carcinogenic potential of DHPAs in a manner relevant to natural exposure in both humans and animals.

## References

- Allen JR, Hsu IC, Carstens LA. 1975. Dehydroretronecine-induced rhabdomyosarcomas in rats. *Cancer Res.* 35:997-1002.
- Baker DC, Smart RA, Ralphs M, Molyneux RJ. 1989. Hound's-tongue (*Cynoglossum officinale*) poisoning in a calf. *J Am Vet Med Assoc.* 194:929-930.
- BfR. 2011. Chemical analysis and toxicity of pyrrolizidine alkaloids and assessment of the health risk posed by their occurrence in honey, BfR opinion no. 038/2011 11.
- Bull, L. F., C. C. J. Culvenor and A. T. Dick (1968). *The Pyrrolizidine Alkaloids: Their Chemistry-Pathogenicity and Other Biological Properties.* Amsterdam, North Holland Publications.
- Campbell J. 1956. VI. An Investigation of the Hepatotoxic Effects in the Fowl of Ragwort (*Senecio jacobæa*), with special reference to the Induction of Liver Tumours with Seneciphylline. *Proceedings of the Royal Society of Edinburgh. Section B. Biology* 66:111-130.
- Chauvin P, Dillon J, Moren A. 1994. An outbreak of Heliotrope food poisoning, Tadjikistan, November 1992-March 1993. *Santé (Montrouge, France)* 4:263.
- Cheeke PR. 1998. *Natural Toxicants in Feeds, Forages, and Poisonous Plants.* Interstate Publishers Inc.: Danville, IL.
- Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT). 2008. COT statement on pyrrolizidine alkaloids in food. Available: <http://cot.food.gov.uk/pdfs/cotstatementpa200806.pdf> [Accessed 6 June 2015].
- Coulombe RA, Jr., Drew GL, Stermitz FR. 1999. Pyrrolizidine alkaloids crosslink DNA with actin. *Toxicol Appl Pharmacol.* 154:198-202.
- Culvenor CC, Edgar JA, Jago MV, Qutteridge A, Peterson JE, Smith LW. 1976. Hepato- and pneumotoxicity of pyrrolizidine alkaloids and derivatives in relation to molecular structure. *Chem Biol Interact.* 12:299-324.
- Edgar JA, Molyneux RJ, Colegate SM. 2014. Pyrrolizidine Alkaloids: Potential Role in the Etiology of Cancers, Pulmonary Hypertension, Congenital Anomalies and Liver Disease. *Chem Res Toxicol* 28: 4-20.
- EFSA. 2011. EFSA Panel on Contaminants in the Food Chain (CONTAM). Scientific Opinion on Pyrrolizidine alkaloids in food and feed. *EFSA Journal* 9:1-134.

- FDA. 2001. FDA advises dietary supplement manufacturers to remove comfrey products from the market. U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition. Available: <http://www.cfsan.fda.gov/~dms/dspltr06.html> [Accessed 6 July 2001].
- FSANZ. 2001. Pyrrolizidine alkaloids in food. A toxicological review and risk assessment. In Food Standards Australia New Zealand Tech. Report series No. 2. Canberra, Australia.
- Gilruth JA. 1904. Hepatic cirrhosis due to ragwort (*Senecio jacobea*). Div Vet Sci NZ Dept Agric Bull. 9:252–254.
- Gyorik S, Stricker H. 2009. Severe pulmonary hypertension possibly due to pyrrolizidine alkaloids in polyphytotherapy. Swiss Med. Wkly 139:210-211.
- Hincks JR, Kim HY, Segall HJ, Molyneux RJ, Stermitz FR, Coulombe RA, Jr. 1991. DNA cross-linking in mammalian cells by pyrrolizidine alkaloids: structure-activity relationships. Toxicol Appl Pharmacol. 111:90-98.
- Hooper P. 1978. Pyrrolizidine alkaloid poisoning. Pathology with particular reference to differences in animal and plant species. In Joint United States-Australian Symposium on Poisonous Plants, Logan, Utah (USA), 19-24 Jun 1977. Academic Press.
- IARC. 1987. IARC monographs on the evaluation of carcinogenic risks to humans. Lyon, France.
- IARC. 2002. IARC monographs on the evaluation of carcinogenic risks to humans. Lyon, France.
- Johnson AE, Molyneux RJ. 1984. Toxicity of threadleaf groundsel (*Senecio douglasii* var *longilobus*) to cattle. Am J Vet Res. 45:26-31.
- Johnson AE, Molyneux RJ, Stuart LD. 1985. Toxicity of Riddell's groundsel (*Senecio riddellii*) to cattle. Am J Vet Res. 46:577-582.
- Kim HY, Stermitz FR, Coulombe RA, Jr. 1995. Pyrrolizidine alkaloid-induced DNA-protein cross-links. Carcinogenesis. 16:2691-2697.
- Mattocks A. 1986. Chemistry and toxicology of pyrrolizidine alkaloids. Academic Press London.
- Mattocks AR. 1972. Acute hepatotoxicity and pyrrolic metabolites in rats dosed with pyrrolizidine alkaloids. Chem Biol Interact. 5:227-242.

- Mayer F, Lüthy J. 1993. Heliotrope poisoning in Tadjikistan. *Lancet*. 342:246-247.
- Mei N, Chou MW, Fu PP, Heflich RH, Chen T. 2004. Differential mutagenicity of riddelliine in liver endothelial and parenchymal cells of transgenic big blue rats. *Cancer Lett*. 215:151-158. DOI: 10.1016/j.canlet.2004.06.013.
- Mei N, Guo L, Fu PP, Heflich RH, Chen T. 2005. Mutagenicity of comfrey (*Symphytum officinale*) in rat liver. *Br J Cancer*. 92:873-875.
- Mei X, Chen T. 2007. The mutant frequencies and types of mutations induced by comfrey in the lungs of transgenic big blue rats. *J Food Drug Anal*. 15:458.
- Mohabbat O, Shafiq Younos M, Merzad A, Srivastava R, Ghaos Sediq G, Aram G. 1976. An outbreak of hepatic veno-occlusive disease in north-western Afghanistan. *Lancet*. 308:269-271.
- NTP. 2011. Report on Carcinogens. DIANE Publishing.
- Petry TW, Bowden GT, Huxtable RJ, Sipes IG. 1984. Characterization of hepatic DNA damage induced in rats by the pyrrolizidine alkaloid monocrotaline. *Cancer Res*. 44:1505-1509.
- RIVM. 2005. Advisory Report on Pyrrolizidine Alkaloids in Herb Preparations. Available:[http://www.rivm.nl/en/Documents\\_and\\_publications/Scientific/Reports/2015/april/Pyrrolizidine\\_alkaloids\\_in\\_herbal\\_preparations](http://www.rivm.nl/en/Documents_and_publications/Scientific/Reports/2015/april/Pyrrolizidine_alkaloids_in_herbal_preparations) [Accessed 8 April 2015].
- Schoental R. 1975. Pancreatic islet-cell and other tumors in rats given heliotrine, a monoester pyrrolizidine alkaloid, and nicotinamide. *Cancer Res*. 35:2020-2024.
- Schoental R, Cavanagh J. 1972. Brain and spinal cord tumors in rats treated with pyrrolizidine alkaloids. *J Natl Cancer I*. 49:665-671.
- Schoental R, Fowler M, 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.
- Shumaker RC, Robertson KA, Hsu IC, Allen JR. 1976. Neoplastic transformation in tissues of rats exposed to monocrotaline or dehydroretronecine. *J Natl Cancer I*. 56:787-790.
- Smith LW, Culvenor CC. 1981. Plant sources of hepatotoxic pyrrolizidine alkaloids. *J Nat Prod*. 44:129-152.

- Stalker M, Hayes M. 2007. Liver and biliary system. Jubb, Kennedy, and Palmer's Pathology of Domestic Animals 2:297-388.
- Stegelmeier BL, Edgar JA, Colegate SM, Gardner DR, Schoch TK, Coulombe RA, Molyneux RJ. 1999. Pyrrolizidine alkaloid plants, metabolism and toxicity. J Nat Toxins. 8:95-116.
- Stegelmeier BL, Gardner DR, James LF, Molyneux RJ. 1996. Pyrrole detection and the pathologic progression of *Cynoglossum officinale* (houndstongue) poisoning in horses. J Vet Diagn Invest. 8:81-90.
- Tandon BN, Tandon H, Tandon R, Narndranathan M, Joshi Y. 1976. An epidemic of veno-occlusive disease of liver in central India. Lancet. 308:271-272.
- Tandon H, Tandon B, Mattocks A. 1978. An epidemic of veno-occlusive disease of the liver in Afghanistan. Pathologic features. Am J Gastroenterol. 70:607-613.
- Vardiman P. 1952. Experimental feeding of *Senecio* Silage to calves. J Am Vet Med Assoc. 121:397.
- WHO. 2011. Discussion paper on pyrrolizidine alkaloids, Joint FAO/WHO food standards programme, Codex committee on contaminants in foods. The Hague, The Netherlands.
- Wiedenfeld H, Roeder E, Bourauel T, Edgar J. 2008. Pyrrolizidine Alkaloids Structure and Toxicity. Bonn University Press: Gottingen.
- Yuzo H, Mariko S, Hiro K. 1977. Experimental insuloma in rats after a single administration of monocrotaline. Toxicol Lett. 1:41-44.

## CHAPTER II

### PYRROLIZIDINE ALKALOID LITERATURE REVIEW

#### Introduction

Pyrrolizidine alkaloids are a large group of plant-derived pro-toxins. To date, more than 500 pyrrolizidine alkaloids have been structurally identified. Combined with their N-oxides, there are more than 950 different compounds (Wiedenfeld, Roeder *et al.* 2008). Plant sources of these pro-toxins can be found around the globe. Pyrrolizidine alkaloid-containing plant species make up an estimated 3% of the world's flowering plants (Smith and Culvenor 1981). Not all pyrrolizidine alkaloids are potentially toxic. Pyrrolizidine alkaloids which consist of a saturated ring structure lack the necessary double bond that is required for them to exert their toxic effects. In this review, only the dehydropyrrolizidine alkaloids (DHPAs) that contain the double bond that allows them to be bioactivated will be discussed. This review will focus on sources, chemistry, toxicology, factors influencing toxicology, as well as human exposure and disease.

#### Sources

The original sources of all DHPAs are plants, and as a result this section will focus primarily on those plants known to produce hepatotoxic DHPAs. There are however, secondary sources of exposure to DHPAs such as honey, pollen, and milk that will be discussed more thoroughly later.



Plants containing DHPAs are primarily from three plant families (*Compositae*, *Leguminosae*, and *Boragnaceae*) (Smith and Culvenor 1981, Stegelmeier, Edgar *et al.* 1999). The most common plant genera involved in domestic animal poisonings include: *Amsinkia*, *Crotalaria*, *Cynoglossum*, *Heliotropium*, *Senecio*, and *Trichodesma* (Stalker and Hayes 2007). DHPA containing plants typically have a mixture of different DHPAs, and concentrations of individual DHPAs between plants can vary significantly (Stegelmeier, Edgar *et al.* 1999).

### **Pyrrolizidine Alkaloid Containing Plants**

*Senecio spp.*

Of the more than 3000 species of *Senecio* located throughout the world many contain toxic DHPAs (Stegelmeier, *et al.* 1999). More than 30 of these have been linked to livestock and human poisonings (Johnson, *et al.* 1989; Mattocks 1986; Stegelmeier, *et al.* 1999). In the western United States, three species of *Senecio* are responsible for the majority of DHPA associated cattle losses: Tansy ragwort (*Senecio jacobea*), threadleaf or woolly groundsel (*Senecio douglasii* var. *longilobus*), and Riddell's groundsel (*Senecio riddellii*) (Johnson, *et al.* 1989).

*Senecio jacobea* or Tansy ragwort is an invasive weed originating in Western Europe (Johnson, *et al.* 1989; Stegelmeier 2011) that has invaded Eastern Europe, North America, Australia, New Zealand, and South Africa (Stegelmeier 2011). In the United States, it has invaded millions of acres of private and public land in the Pacific Northwest (Whitson, *et al.* 1996). *S. jacobea* is a tall (0.3 – 2 m.) biennial or short lived perennial with a taproot. It flowers from July to September producing many heads of yellow

flowers, and its leaves are two to eight inches long and deeply lobed (Whitson, *et al.* 1996). Because Tansy ragwort is not particularly palatable, poisoning by this plant tends to be the result of seeds contaminating feeds, lack of other forage, or when animals cannot differentiate early rosettes from other plants (Stegelmeier 2011).

*S. jacobea* tends to have a lower concentration of DHPA than *S. douglasii* var. *longilobus* or *S. riddellii*, but its invasive nature and palatability tend to make it more potently toxic (Johnson, *et al.* 1989). A dose of 2.5 mg kg<sup>-1</sup> for 18 days is toxic for cattle. Higher doses result in acute hepatocellular necrosis and liver failure, but these poisonings are rare due to the unpalatable nature of the plant (Stegelmeier 2011).

*S. douglasii* var. *longilobus* (threadleaf or wooly groundsel) is a small perennial shrub that grows primarily in abused or degraded rangelands of the Southwestern United States in loamy to clay soils (Johnson, Molyneux *et al.* 1989). It grows from 0.3 to 1 meter tall and has numerous arching and branching stems (Whitson, Burrill *et al.* 1996). Threadleaf groundsel has long, thin leaves (less than 3/16 in x 3 in) that are covered with fine hairs giving it a gray appearance (Whitson, Burrill *et al.* 1996). Approximately 750 g of green plant for 15 days is lethal for cattle (Stegelmeier 2011).

*S. riddellii* or Riddell's groundsel is similar in appearance to *S. douglasii* var. *longilobus* except that the leaves do not have hairs (Whitson, *et al.* 1996). It grows primarily in the Midwestern and Southwestern United States, and tends to have a higher DHPA concentration than the previously discussed *Senecio* spp (Johnson, Molyneux *et al.* 1989). *S. riddellii* has been found to contain up to 18% riddelliine on a dry matter basis (Johnson, *et al.* 1989), although typically concentrations tend to be significantly

lower. It is unique from most other DHPA containing plants in that it contains primarily one DHPA, riddelliine (Stegelmeier 2011).

*S. vulgaris* or common groundsel, another *Senecio spp.* plant that has been involved in poisonings of animals is also used in some herbal medicines. Unlike the previously discussed *Senecio spp.* plants, *S. vulgaris* is an annual, or sometimes a biennial (Whitson, *et al.* 1996). *S. vulgaris* has wider (1 to 3 cm) leaves that are irregularly notched or toothed that range from one to four inches long. Its stems are 8 to 40 cm tall and the leaves can be haired or hairless. The approximate lethal dose for cattle is 250 g of green plant for 15 days (Stegelmeier 2011).

*S. douglasii* var. *longilobus* (threadleaf or wooly groundsel) is a small perennial shrub that grows primarily in abused or degraded rangelands of the Southwestern United States in loamy to clay soils (Johnson, *et al.* 1989). It grows from 12 to 40 inches tall, and has numerous arching and branching stems (Whitson, *et al.* 1996). It has long, thin leaves (less than 3/16 in x 3 in ) that are covered with fine hairs giving it a gray appearance (Whitson, *et al.* 1996). Approximately 750 g of green plant for 15 days is lethal for cattle (Stegelmeier 2011).

#### *Crotalaria spp.*

In an effort to improve soils, *Crotalaria spp.* were introduced into North America because of their ability to fix nitrogen (Stegelmeier 2011; Stegelmeier, *et al.* 1999). Initially used as a cover crop, several have escaped cultivation and grow as weeds along fence lines and ditch banks where they often invade pastures and fields (Stegelmeier 2011). They have a common name of “rattle pod” which comes from the long seed pods

containing 2-3 mm long seeds that tend to become detached as they mature and rattle (Stegelmeier, *et al.* 1999). *Crotalaria spp.* have been involved in poisonings of horses (Stegelmeier, *et al.* 1999), and seeds have contaminated grains resulting in poisoning of livestock and poultry (Stegelmeier 2011).

Fletcher *et al.* found most DHPAs in *Crotalaria spp.* to be present in the N-oxide form with some exceptions (Fletcher, *et al.* 2009).

Table 2-1 contains a list of plants containing known toxic DHPAs as well as the DHPAs that have been isolated from these plants, and the portion of the plant containing DHPAs.

Studies on DHPA biosynthesis (Molyneux, *et al.* 2011) demonstrated that DHPAs are formed in the roots and transported to other parts of the plant such as leaves and reproductive organs where they accumulate as tertiary bases or N-oxides. Johnson *et al.* (1985) compared different *Senecio*, *Amsinkia*, and *Crotalaria* species, and found that the ratio of N-oxides and tertiary bases varies with species, growth stage, and location within the plant. They also determined that the maximum total DHPA content was present at the early bud or preflower stage of most plants (Johnson, *et al.* 1985).

**Table 2-1. Plants containing hepatotoxic pyrrolizidine alkaloids**

<b>Plant</b>	<b>Constituent Alkaloids</b>	<b>Plant Part</b>
APOCYNACEAE		
<i>Parsonsia eucalyptophylla</i>	lycopsamine	AG
<i>P. heterophylla</i>	parsonsine heterophylline	WP
<i>P. spiralis</i>	spiraline spiranine spiracine	Lf
<i>P. straminea</i>	lycopsamine	AG

<b>Plant</b>	<b>Constituent Alkaloids</b>	<b>Plant Part</b>
<i>Urechites karwinsky</i>	loroquin	Rt
<b>BORAGINACEAE</b>		
<i>Amsinckia hispida</i>	intermediate lycopsamine echiumine	WP
<i>A. intermedia</i>	intermediate lycopsamine echiumine sincamidine	WP
<i>A. lycosoides</i>	intermediate lycopsamine echiumine	WP
<i>Anchusa arvensis</i>	lycopsamine 7-acetyllycopsamine (or diastereoisomer)	WP
<i>A. officinalis</i>	lycopsamine 7-acetyllycopsamine (or diastereoisomer)	WP
<i>Asperugo procumbens</i>	amabiline (or diastereoisomer)	WP
<i>Cynoglossum amabile</i>	amabiline echinatine 7-acetylechinatine	WP
<i>C. austral</i>	cynaustine cynaustaline	WP
<i>C. creticum</i>	echinatine heliosupine	AG
<i>C. glochidiatum</i>	amabiline	WP
<i>C. lanceolatum</i>	cynaustaline cynaustine	WP
<i>C. latifolium</i>	latifoline 7-angelylretronecine	AG
<i>C. officinale</i>	heliosupine echinatine acetylheliosupine 7-angelylheliotridine	AG Rt, AG AG
<i>C. pictum</i>	heliosupine echinatine pictumine	Rt, AG AG
<i>C. viridiflorum</i>	viridiflorine heliosupine	Rt
<i>Echium italicum</i>	echimidine	WP
<i>E. plantagineum</i>	echiumine echimidine	AG

<b>Plant</b>	<b>Constituent Alkaloids</b>	<b>Plant Part</b>
<i>E. vulgare</i>	heliosupine asperumine lycopsamine (or diastereoisomer)	AG WP
<i>Heliotropium acutiflourm</i>	heliotrine	AG
<i>H. amplexicaule</i>	indicine	AG
<i>H. arbainense</i>	europine lasiocarpine heliotrine	AG
<i>H. arborescens</i>	lasiocarpine	AG
<i>H. arguzioides</i>	heliotrine trichodesmine	AG AG, Rt
<i>H. curassavicum</i>	heliotrine lasiocarpine angelylheliotridine curassavine coromandaline heliovicine trachelanthamidine	WP AG
<i>H. dasycarpum</i>	heliotrine	AG, Rt, Sd
<i>H. eichwaldi</i>	heliotrine lasiocarpine 7-angelylheliotrine	WP AG
<i>H. europaeum</i>	heliotrine lasiocarpine europine supinine heleurine N-(dihydropyrrolizino-methyl)-heliotrine chloride acetylasiocarpine	WP
<i>H. indicum</i>	indicine acetylindicine indicinine echinatine supinine heleurine lasiocarpine heliotrine	AG
<i>H. lasiocarpum</i>	heliotrine lasiocarpine	AG
<i>H. maris mortui</i>	Europine Lasiocarpine	AG

<b>Plant</b>	<b>Constituent Alkaloids</b>	<b>Plant Part</b>
<i>H. olgae</i>	Heliotrine incanine	AG, Rt
<i>H. popovii</i>	heliotrine	Sd
<i>H. ramosissimum</i>	heliotrine	AG
<i>H. rotundifolium</i>	europine	AG
<i>H. steudneri</i>	lycopsamine	Lf
<i>H. supinum</i>	supinine heliosupine echinatine 7-angelylheliotridine trachelanthic and viridifloric esters of 7-Angelylheliotridine	Rt  WP
<i>H. transoxanum</i>	heliotrine	AG
<i>Lappula glochidiata</i>	echinatine	AG
<i>L. intermedia</i>	lasiocarpine	AG
<i>Lindelofia angustifolia</i>	echinatine amabiline	AG
<i>L. spectabilis</i>	echinatine 7-acetylechinatine monocrotaline	AG
<i>L. stylosa</i>	viridiflorine echinatine lindelofine	AG Sd
<i>L. tschimganica</i>	carategine echinatine viridiflorine	AG
<i>Myosotis sylvatica</i>	viridiflorine 9-angelylretronecine heliosupine acetylheliosupine	WP
<i>ParaCynoglossum imeritinum</i>	heliosupine echinatine	AG, Rt
<i>Rindera austroechinata</i>	echinatine	Lf, St, Sd, Rt
<i>R. baldschuanica</i>	rinderine echinatine trachelanthamine turkestanine	AG
<i>R. cyclodonta</i>	echinatine	AG
<i>R. echinata</i>	echinatine trachelanthamine	AG
<i>R. oblongifolia</i>	carategine echinatine turkestanine	AG
<i>Solenanthus circinnatus</i>	echinatine	Sd, AG, Rt
<i>S. coronatus</i>	echinatine	AG

<b>Plant</b>	<b>Constituent Alkaloids</b>	<b>Plant Part</b>
<i>Skarategenius</i>	carategine echinatine	AG
<i>S. turkestanicus</i>	rinderine turkestanine	AG
<i>Symphytum asperum</i>	asperumine echinatine heliosupine 7-acetylechimidine (or diastereoisomer)	WP
<i>S. caucasicum</i>	lasiocarpine asperumine echinatine echimidine	AG, Rt
<i>S. officinale</i>	symphytine echimidine lasiocarpine heliosupine viridiflorine echinatine 7-acetyllycopsamine (or diastereoisomer) 7-angelylretronecine viridiflorate (or diastereoisomer) acetylechimidine (or diastereoisomer)	Rt  AG, Rt  Rt  WP
<i>S. orientale</i>	anadoline symphytine echimidine	WP
<i>S. tuberosum</i>	echimidine anadoline	WP
<i>S. xuplandicum</i>	symphytine symlandine echimidine 7-acetyllycopsamine 7-acetylintermedine uplandicine lycopsamine intermedine	AG
<i>Tournefortia sarmentosa</i>	supinine	Lf, St
<i>Trichodesma Africana</i>	europine intermedine	WP
<i>T. incanum</i>	trichodesmine incanine	AG Sd, AG, Rt
<i>T. zeylanicum</i>	supinine	Sd
<i>Ulegbekia tschimganica</i>	uluganine	
COMPOSITAE		



<b>Plant</b>	<b>Constituent Alkaloids</b>	<b>Plant Part</b>
<i>Adenostyles alliariae</i>	platyphylline seneciphylline	Rt
<i>A. rhombifolia</i>	platyphylline seneciphylline	AG
<i>Brachyglottis repanda</i>	senecionine senkirkine brachyglottine	AG
<i>Cacalia floridaiia</i>	otosenine florosenine floridanine floricaline	AG
<i>C. hastate</i>	Integerrimine	Rt
<i>c. yatabei</i>	yamataimine	Rt
<i>Doronicum macrophyllum</i>	otosenine floridanine doronine	Rt
<i>Emilia flammea</i>	otosenine emiline	AG, Rt
<i>E. sonchifolia</i>	senecionine	AG
<i>Erechtites hieracifolia</i>	senecionine seneciphylline	AG
<i>Eupatorium cannabinum</i>	echinatine supinine	AG
<i>E. maculatum</i>	echinatine trachelanthamidine	Rt
<i>E. purpureum</i>	probably echinatine	AG
<i>E. serotinum</i>	supinine rinderine	AG
<i>E. stoechadosmum</i>	lindelofine supinine	Rt
<i>Farfugium japonicum</i>	senkirkine	Rt, Lf
<i>Ligularia brachyphylla</i>	clivorine ligularine ligudentine	AG
<i>L. clivorum</i>	clivorine	AG
<i>L. dentata</i>	clivorine ligularine ligudentine ligularidine	AG  Rt, AG
<i>L. elegans</i>	clivorine ligularine ligudentine	AG
<i>Petasites hybridus</i>	senecionine	Lf

<b>Plant</b>	<b>Constituent Alkaloids</b>	<b>Plant Part</b>
<i>P. japonicas</i>	fukinotoxin neopetasitenine senkirkine petasinine petasinoside	AG  St AG
<i>P. laevigalus</i>	platyphylline senkirkine senecionine	AG
<i>Senecio aegypticus</i>	senecionine otosenine riddelliine	WP
<i>S. alpines</i>	seneciphylline jacozine	AG
<i>S. ambrosioides</i>	seneciphylline senecionine retrorsine riddelliine	WP
<i>S. ampullaceus</i>	senecionine seneciphylline retrorsine	WP
<i>S. antieuphorbium</i>	integerrimine senkirkine senaetnin isosenaetin	AG
<i>S. aquaticus</i>	seneciphylline	AG
<i>S. auresu</i>	senecionine	AG
<i>S. auricola</i>	neosenkirkine	AG
<i>S. bipinnatisectus</i>	retrorsine	AG, Rt
<i>S. borysthenicus</i>	seneciphylline	AG, Rt
<i>S. brasiliensis</i>	senecionine seneciphylline jacobine integerrimine retrorsine	Lf   AG
<i>S. bupleuroides</i>	retrorsine	AG
<i>S. cannabifolius</i>	seneciphylline	AG
<i>S. carthamoides</i>	senecionine seneciphylline	WP
<i>S. chrysanthemoides</i>	seneciphylline	

<b>Plant</b>	<b>Constituent Alkaloids</b>	<b>Plant Part</b>
<i>S. cineraria</i>	jacobine senecionine seneciphylline otosenine retrorsine	AG Sd AG
<i>S. cruentus</i>	cruentine A cruentine B	AG
<i>S. cymbalarioides</i>	senecionine	
<i>S. desfontainei</i>	senecionine otosenine riddelliine seneciphylline	AG
<i>S. discolor</i>	retrorsine senecionine	Lf, AG
<i>S. doronicum</i>	doronine bulgarsenine	WP
<i>S. douglasii</i>	retrorsine riddelliine seneciphylline senecionine	WP
<i>S. durieui</i>	integerrimine	WP
<i>S. eremophilus</i>	senecionine seneciphylline retrorsine riddelliine	WP
<i>S. erraticus</i>	senecionine otosenine erucifoline seneciphylline integerrimine	AG  Lf AG
<i>S. erucifolius</i>	senecionine seneciphylline erucifoline (base S-C) retrorsine	AG
<i>S. fluviatilis</i>	seneciphylline otosenine florosenine	AG
<i>S. formosus</i>	integerrimine retrorsine	AG
<i>S. fremonti</i>	senecionine seneciphylline	WP
<i>S. glabellus</i>	senecionine	WP
<i>S. glaberrimus</i>	retrorsine	AG

<b>Plant</b>	<b>Constituent Alkaloids</b>	<b>Plant Part</b>
<i>S. graminifolius</i>	retrorsine graminifoline	AG
<i>S. grandifolia</i>	platyphylline seneciphylline	Rt, Lf, St
<i>S. griesebachii</i>	retrorsine	AG
<i>S. ilicifolius</i>	senecionine seneciphylline retrorsine	AG
<i>S. incanus</i>	seneciphylline	AG
<i>S. integerrimus</i>	integerrimine senecionine platyphylline neoplatyphylline	AG
<i>S. isatideus</i>	retrorsine	AG
<i>S. jacobea</i>	seneciphylline senecionine jacobine jaconine jacoline jacozine otosenine senkirkine retrorsine	AG
<i>S. kirkii</i>	senkirkine O-acetylsenkirkine	Bk, Lf Lf
<i>S. kleinia</i>	integerrimine senkirkine	St
<i>S. krylovii</i>	seneciphylline	AG
<i>S. kubensis</i>	seneciphylline	AG
<i>S. lampsanoides</i>	seneciphylline	AG, Rt
<i>S. latifolius</i>	retrorsine seneciphylline platyphylline	AG
<i>S. lautus</i>	senecionine	WP
<i>S. longilobus</i>	seneciphylline retrorsine riddelliine	WP
<i>S. magnificus</i>	senecionine integerrimine	AG
<i>S. minimus</i>	seneciphylline	AG
<i>S. morrisonensis</i>	integerrimine	WP
<i>S. nebrodensis</i>	integerrimine senecionine	WP

<b>Plant</b>	<b>Constituent Alkaloids</b>	<b>Plant Part</b>
<i>S. nemorensis</i> var. <i>bulgaricus</i>	bulgarsenine retroisosenine nemorensine	Lf
<i>S. nemorensis</i> var. <i>subdecurrens</i>	nemorensine retroisosenine bulgarsenine	AG Rt
<i>S. nemorensis</i> ssp. <i>fuchsia</i>	fuchsisenecionine senecionine	AG
<i>S. othonnae</i>	otosenine onetine seneciphylline floridanine doronine	AG Rt AG, Rt AG
<i>S. othonniformis</i>	bisline Isoline	AG
<i>S. palmatus</i>	seneciphylline	Rt
<i>S. paludosus</i>	seneciphylline jacobine	Rt, AG
<i>S. paeanus</i>	senecionine	AG
<i>S. paucicalyculatus</i>	paucicaline retrorsine	WP
<i>S. paucifolius</i>	seneciphylline	
<i>S. petasitis</i>	senecionine probably bisline	AG
<i>S. platyphylloides</i>	platyphylline seneciphylline	Rt
<i>S. platyphyllus</i>	platyphylline seneciphylline neoplatyphylline sarracine	Rt, AG Lf Rt Rt
<i>S. pojarkovae</i>	sarracine seneciphylline	Rt
<i>S. procerus</i> var. <i>procerus</i>	senkirkine procerine	AG, Rt
<i>S. propinquus</i>	seneciphylline	AG, Rt
<i>S. pseudo-arnica</i>	senecionine	AG
<i>S. pterophorus</i>	senecionine seneciphylline setrorsine rosmarinine acetyl-seneciphylline pterophorin	AG

<b>Plant</b>	<b>Constituent Alkaloids</b>	<b>Plant Part</b>
<i>S. quadridentatus</i>	senecionine seneciphylline retrorsine	AG
<i>S. racemosus</i>	seneciphylline	Rt
<i>S. renardii</i>	seneciphylline senkirkine otosenine	AG
<i>S. retrorsus</i>	retrorsine	AG
<i>S. rhombifolius</i>	sarracine platyphylline neoplatyphylline seneciphylline	Rt Ag, Rt
<i>S. riddellii</i>	riddelliine	WP
<i>S. riddellii</i> var. <i>parksii</i>	retrorsine riddelliine	AG
<i>S. rivularis</i>	7-angelyheliotridine	AG
<i>S. ruderalis</i>	retrorsine	AG
<i>S. ruwenzoriensis</i>	ruwenine ruzorine	WP
<i>S. scandens</i>	senecionine seneciphylline	WP
<i>S. sceleratus</i>	retrorsine sceleratine chlorodeoxyscleratine	AG
<i>S. spartioides</i>	seneciphylline spartioidine senecionine retrorsine riddelliine	AG
<i>S. spathulatus</i>	senecionine integerrimine seneciphylline	AG, Rt
<i>S. squalidus</i>	senecionine integerrimine	AG
<i>S. stenocephalus</i>	seneciphylline	AG
<i>S. subalpinus</i>	senecionine seneciphylline	Lf AG
<i>S. swaziensis</i>	retrorsine swazine	AG
<i>S. tomentosus</i>	senecionine otosenine	AG
<i>S. triangularis</i>	senecionine	AG

<b>Plant</b>	<b>Constituent Alkaloids</b>	<b>Plant Part</b>
<i>S. uintahensis</i>	senecionine senkirkine	AG
<i>S. venosus</i>	retrorsine	AG
<i>S. vernalis</i>	senecionine senkirkine retrorsine senecivernine	
<i>S. viscosus</i>	senecionine integerrimine	Ag
<i>S. vulgaris</i>	senecionine seneciphylline retrorsine riddelliine	AG
<i>S. werneriaefolius</i>	senecionine retrorsine	
<i>Syneilesis palmata</i>	syneilesine acetylsyneilesine senecionine	AG, Rt
<i>Tussilago farara</i>	senkirkine	Fl, Lf
<b>LEGUMINOSAE</b>		
<i>Crotalaria aegyptica</i>	monocrotaline crosemperine 7 $\beta$ -hydroxy-1-methylene-8 $\beta$ -pyrrolizidine	AG
<i>C. agatiflora</i>	madurensine anacrotine 7-acetylmadurensine 6-acetylanacrotine 7-acetyl-cis-madurensine 6-acetyl-trans-anacrotine crotaflorine 6-angelyl-trans-anacrotine	AG
<i>C. anagyroides</i>	1-methylenepyrrolizidine senecionine anacrotine	Sd
<i>C. assamica</i>	monocrotaline	Sd
<i>C. axillaris</i>	axillarine	Sd
<i>C. barbata</i>	crobarbatine	Sd
<i>C. brevifolia</i>	integerrimine usaramine	Sd
<i>C. burhia</i>	orotalarine monocrotaline	AG

<b>Plant</b>	<b>Constituent Alkaloids</b>	<b>Plant Part</b>
<i>C. crispata</i>	monocrotaline fulvine crispatine	WP
<i>C. dura</i>	dicrotaline	AG
<i>C. fulva</i>	fulvine	AG
<i>C. globifera</i>	dicrotaline	AG
<i>C. grahamiana</i>	monocrotaline grahamine monocrotalinine	Sd WP
<i>C. grantiana</i>	grantianine grantaline 1-hydroxymethyl-1 $\beta$ -2 $\beta$ -epoxypyrrolizidine	Sd Sd
<i>C. incana</i>	integerrimine anacrotine usaramine	Sd Ag Sd
<i>C. intermedia</i>	integerrimine usaramine	Sd
<i>C. juncea</i>	senecionine seneciphylline riddelline trichodesmine junceine	Sd
<i>C. laburnifolia</i>	anacrotine 1-methylenepyrrolizidine	Sd
<i>C. laburnifolia</i> subsp. <i>eldomae</i>	madurensine anacrotine senkirkine hydroxysenkirkine crotafoline	AG
<i>C. lechnaultii</i>	monocrotaline crispatine	Sd
<i>C. leiloba</i>	monocrotaline	Sd
<i>C. madurensis</i>	madurensine crispatine fulvine cromadurine isocromadurine	Sd AG Sd Sd
<i>C. mitchellii</i>	monocrotaline retusamine	WP
<i>C. mysorensis</i>	monocrotaline	WP
<i>C. novae-hollandiae</i> subsp. <i>Lasiophylla</i>	monocrotaline retusamine	WP



<b>Plant</b>	<b>Constituent Alkaloids</b>	<b>Plant Part</b>
<i>C. novaehollandiae</i> subsp. <i>Novae-hollandiae</i>	retusamine	Sd
<i>C. pallida</i>	usaramine integerrimine mucronatine (usaramine?) nilgirine crotastratine	Sd Sd Sd
<i>C. paniculata</i>	fulvine	Sd
<i>C. quinquefolia</i>	monocrotaline	Sd
<i>C. recta</i>	monocrotaline trichodesmine	AG
<i>C. retusa</i>	monocrotaline retusine retusamine retronecine	Sd Sd, AG
<i>C. rubiginosa</i>	junceine trichodesmine	Sd
<i>C. sagittalis</i>	monocrotaline	Sd
<i>C. semperflorens</i>	crosemperine	Sd
<i>C. sericea</i>	monocrotaline spectabiline	Sd
<i>C. spartioides</i>	retrorsine	AG
<i>C. spectabilis</i>	monocrotaline spectabiline retusine	Sd Sd, WP Sd
<i>C. stipularia</i>	monocrotaline	Sd
<i>C. tetragona</i>	integerrimine trichodesmine	Sd
<i>C. usaramoensis</i>	integerrimine usaramine senecionine retrorsine	Sd Sd
<i>C. verrucosa</i>	1-methylenepyrrolizidine anacrotine crotaverrine acetylcrotaverrine	Sd Sd
<i>C. walker</i>	crotaverrine acetylcrotaverrine	Sd
<i>C. Sp. Aff. Mitchellii</i>	retusamine	WP
<b>RANUNCULACEAE</b>		
<i>Caltha biflora</i>	senecionine	AG
<i>C. leptosepala</i>	senecionine	AG, Rt
<b>SCROPHULARIACEAE</b>		

Plant	Constituent Alkaloids	Plant Part
<i>Castilleja rhexifolia</i>	senecionine	

Adapted from Smith and Culvenor 1981

Abbreviations: WP = whole plant

AG = above ground parts of plant

Rt = root

St = stem

Bk = bark

Lf = leaf

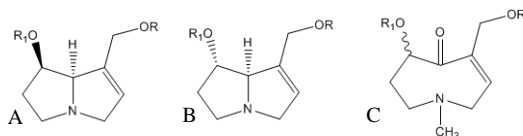
Fl = flower

Sd = seed

## Chemistry/Biochemistry

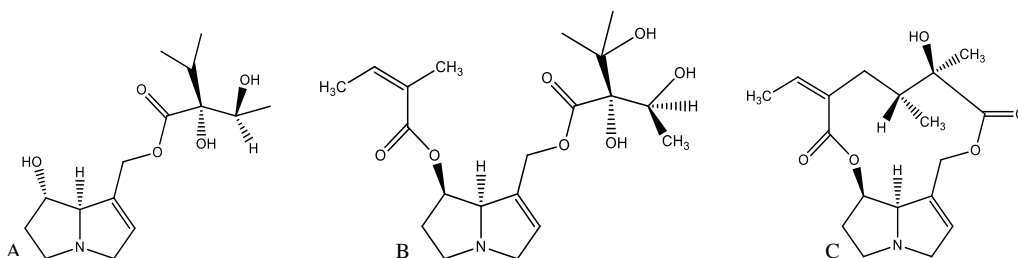
### Structure

Pyrrolizidine alkaloids are composed of two fused five ring structures containing a nitrogen atom in the center called a necine base, and associated acid moieties, which are termed necic acids (Roeder 2000). The necine bases may be either saturated or unsaturated with a 1,2 double bond. Pyrrolizidine alkaloids with the 1,2 double bond have the potential to be toxic/carcinogenic (Mattocks 1986). Nearly all of the necine bases have a hydroxymethyl group at C-1, and most have a hydroxyl group at C-7 (Roeder 2000). Additional hydroxyl groups may also be present particularly at C-2, C-6 or C-7, resulting in the formation of many different necine bases. A large majority of DHPA related toxicity appears to be the result of DHPAs containing one of three necine bases (heliotridine, retronecine, and otonecine) (Fig. 2-1).



**Figure 2-1.** Structures of three necine bases (A) retronecine, (B) heliotridine, (C) otonecine

With the exception of acetic acid, necic acids contain from five to ten carbon atoms (Roeder 1995). They may be branched or simple chains, and also commonly contain various substituents including: hydroxyl, carboxy, methoxy, epoxy, or acetoxy groups. Consequently numerous structural variations can be derived, including various stereoisomers. Necic acids can be esterified to the necine bases at these locations giving rise to monoester, open chain diester, and macrocyclic diester DHPAs (Roeder 2000) (Fig. 2-2).



**Figure 2-2.** Structures of dehydropyrrolizidine alkaloids with A. monoester (echinatine), B. diester (echimidine) and C. macrocyclic diester (senecionine) side chains.

DHPAs are generally referred to by their trivial names as opposed to their chemical names because the chemical names are long and difficult to use (Mattocks 1986). Their trivial names are generally derived from the plants from which they were initially isolated (Mattocks 1986). A different method for naming is used for macrocyclic diesters. For 11 membered ring structures such as monocrotaline, the stem name of crotalanine is used, and for 12 membered rings like senecionine, the stem name senacanine is given (Mattocks 1986).

## Biotransformation

DHPAs are pro-toxins and not inherently toxic. To illustrate this, Mattocks gives multiple points as evidence, including (Mattocks 1986):

1. DHPAs are not locally toxic at the site of injection, or when they are applied to the skin.
2. The alkaloids do not injure some organisms, e.g. cinnabar moth larvae, even though these are able to accumulate relatively large amounts of DHPAs in their tissues (Aplin *et al.*, 1968).
3. The main organ damaged is usually the liver, regardless of the route of administration of the alkaloid; DHPAs are known to be metabolized in the liver.
4. The susceptibility of animals to DHPA intoxication is considerably influenced by treatments which modify the activity of the hepatic drug metabolizing enzymes.
5. Some animals are much more resistant than others to DHPA hepatotoxicity. Guinea pigs can withstand 20 times the dose of retrorsine that is an LD<sub>50</sub> for male rats; stimulation of their liver microsomal enzymes by pretreatment with phenobarbitone increases the susceptibility of guinea pigs fourfold (White *et al.*, 1973). Newborn rats given retrosine within 1 h of birth are more resistant to the chronic hepatotoxicity of the alkaloid than rats aged one day or more when their hepatic microsomal enzyme activity is much greater (Mattocks and White, 1973).
6. DHPAs are chemically rather unreactive. It is unlikely that they would be able to react with cell constituents under physiological conditions. On the other hand, metabolites known to be formed from DHPAs in the liver are highly reactive and more cytotoxic than their parent alkaloids.

As is evident from the points above, the need for bioactivation and the central role of the liver in DHPA-dependent toxicity was understood decades ago. Miranda *et al.* (1991) determined that the major enzyme catalyzing both the bioactivation (formation of 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine or DHP) and detoxification (formation of N-oxide) of senecionine in human liver microsomes was CYP3A4. In a later study Huan *et al.* (1998a) also found that CYP3A isoenzymes played a major role in

bioactivation and detoxification sheep and hamsters, while CYP2B isoenzymes played a minor roll.

Species variation exists in enzymes responsible for DHP formation as well as N-oxidation. Formation of DHP from senecionine in rats is reportedly primarily due to CYP3A2 and N-oxide formation is mainly catalyzed by CYP2C11 (Williams, *et al.* 1989). In guinea pigs, CYP2B isoenzymes are the main catalyst responsible for biotransformation of senecionine and CYP3A isoenzymes play a rather minor role (Chung and Buhler 1994).

Biotransformation also varies by gender at least in some species. Williams *et al.* (1989) found that liver microsomes from male rats were much more efficient at biotransforming senecionine than microsomes from female rats. Both DHP production and N-oxidation was higher with microsomes from male rats. The difference was much greater with N-oxidation than DHP production.

## Toxicology

### **Absorption, Distribution, Metabolism and Excretion (ADME)**

Pharmacokinetic studies have been reported for a few DHPAs or DHPA N-oxides including riddelliine (Williams, *et al.* 2002), monocrotaline (Estep, *et al.* 1991), senecionine (Wang, *et al.* 2011), adonifoline (Wang, *et al.* 2011), and indicine N-oxide (Ames, *et al.* 1982; Kovach, *et al.* 1979). Williams *et al.* (2002) performed kinetics for riddelliine in both rats and mice, but unfortunately examined oral dosing only. Because no IV data are available for comparison, interpretation of the kinetics is

somewhat limited. Senecionine and adonifoline were tested in rats (Wang, *et al.* 2011) and this constitutes the only report of kinetics which includes both IV and oral data. Male Sprague-Dawley rats were used for kinetics testing of monocrotaline IV (Estep, *et al.* 1991). Indicine N-oxide was tested in human adults (Kovach, *et al.* 1979) and children (Ames, *et al.* 1982) as part of a clinical trial to determine its usefulness as a chemotherapeutic agent. Consequently, it was administered IV and no oral kinetics data are available.

A shortfall in the literature evaluating DHPAs is that virtually all of the LD50 data were determined using IP or IV routes in spite of the fact that all natural exposure is oral (Mattocks 1986). In order to more accurately extrapolate the IP and IV LD50 data, more information is needed about the kinetics of the various DHPAs in more species. In spite of these limitations, much is known about the metabolism of DHPAs as it pertains to cytochrome P450s and different metabolites that result from their actions on DHPAs.

#### *Absorption*

As stated above, natural toxicity to DHPAs occurs following oral exposure. After ingestion, DHPAs are absorbed into the portal circulation and carried to the liver. Wang *et al.* (2011) determined that the absorption half-life of senecionine is between 115 and 163 minutes depending on the dose administered. The absorption half-life of adonifoline was somewhat longer, ranging from 95 to 207 minutes (Wang, *et al.* 2011).

#### *Distribution*

The distribution half-lives of senecionine and adonifoline were similar, ranging from 56 to 111 minutes depending on the dose (Wang, *et al.* 2011). The distribution

phase of indicine N-oxide was less than 4 minutes for all but the highest dose given (Kovach, *et al.* 1979). Both senecionine and adonifoline are macrocyclic diesters whereas indicine is a monoester. It is unclear whether the variation present in these two studies is due to the difference in the chemical structures of the DHPAs or if there is some difference in the methodologies that may explain such vast differences.

Estep *et al.* (1991) measured concentrations of <sup>14</sup>C-labeled monocrotaline in erythrocytes, liver, kidney, lung, spleen, cardiac muscle, plasma, and skeletal muscle at 7 hours and again at 24 hours post dosing. At both time points they found the highest concentration of free base in erythrocytes followed by liver. At 24 hours the spleen and lung both had slightly more free base present than the kidneys followed by cardiac muscle, plasma and lastly skeletal muscle (Estep, *et al.* 1991). This group hypothesized that the high concentrations of monocrotaline in red blood cells could play a role in the extrahepatic toxicity seen more commonly with monocrotaline than other DHPAs.

### *Metabolism*

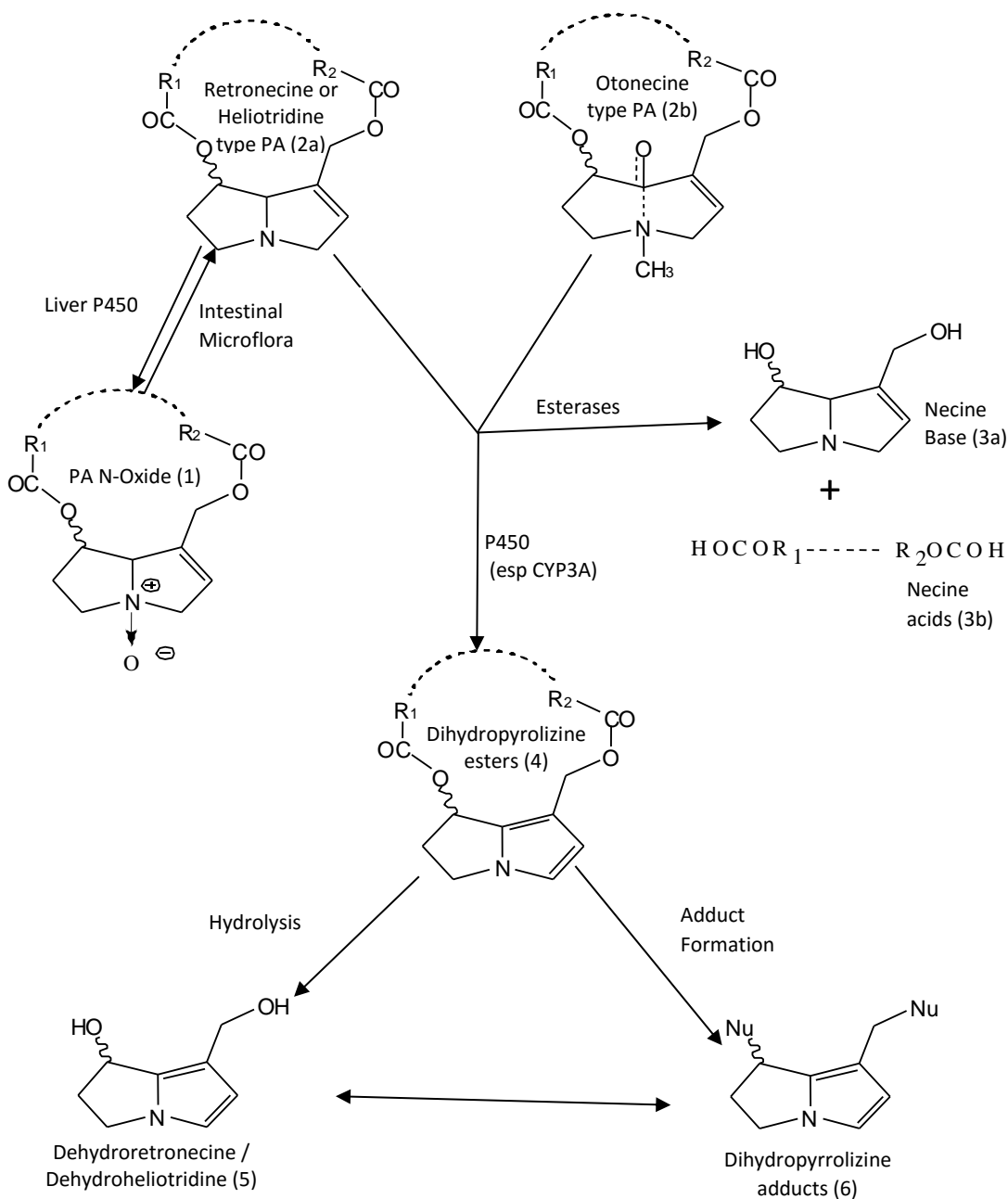
Primary metabolism of DHPAs is achieved by a handful of enzymes belonging to the P450 superfamily. As discussed under bioactivation, although there are multiple P450 enzymes that play a role in DHPA metabolism, the most consistent across species, and the most important from a toxicity standpoint appears to be CYP3A4. Interestingly, both N-oxidation, which increases the water solubility and consequently allows for increased rate of excretion, and formation of the reactive metabolites are mediated through the activity of the same enzyme in some species (Miranda, *et al.* 1991; Williams,

*et al.* 1989). Glutathione conjugation plays a major role in phase II metabolism of the reactive metabolite (Reed, *et al.* 1992; Yan and Huxtable 1995).

DHPAs exist in plants in both free base and N-oxide forms (Fig. 2-3). The N-oxide form can be converted to the free base in the gut via intestinal microflora. In addition, hepatic microsomes are capable of metabolizing the N-oxide to the free base (Wang, *et al.* 2005b). After ingestion, DHPAs are carried first to the liver where they are exposed to P450 enzymes, flavin-containing monooxygenases (FMOs), and esterases. The relative action of each enzyme type is variable depending on the species. In cattle and sheep FMOs had a greater influence on N-oxide formation from senecionine and P450s (CYP3A) had more influence in the formation of DHP (Durringer, *et al.* 2004).

Free bases of retronecine, heliotridine, and otonecine all have the same basic potential metabolic pathways once they reach the liver. They may be acted on by esterases forming non-toxic necine acids (Fig. 2-3 3b) and necine bases (Fig. 2-3 3a). P450 enzymes, particularly isoforms of CYP3A, may convert them to dihydropyrrolizine esters (Fig. 2-3 4), the initial toxic metabolites. Dihydropyrrolizine esters are highly reactive electrophiles at the C-7 and C-9 position, which rapidly bind to tissue nucleophiles (Nu) such as NH, OH, or SH groups found on tissue proteins, nucleic acids or glutathione, forming adducts (Fig. 2-3 6) (Edgar, *et al.* 2011). Dihydropyrrolizine esters may also be hydrolyzed to form dehydroretronecine or dehydroheliotridine (Fig. 2-3 5). Dehydroretronecine and dehydroheliotridine are also electrophilic and





**Figure 2-3.** Dehydropyrrolizidine Alkaloid Metabolism

Dehydropyrrolizidine alkaloids are consumed in either the free base form (2a & 2b) or the N-oxide form (1). If consumed in the N-oxide form (1) some can be converted by intestinal microflora to the free base form (2a or 2b). The free base is metabolized in the liver by one of several mechanisms. It can be N-oxidized increasing its water solubility, acted on by esterases forming non-toxic necine acids (3b) and bases (3a), or bioactivated by P450 enzymes into 6,7-dihydropyrrolizine esters (4). These esters either bind rapidly to nucleophiles forming adducts (6) or are hydrolyzed forming dihydroretronecine or dihydroheliotridine (5), which also have the propensity to bind to nucleophiles and form adducts (5).

readily bind to similar tissue nucleophiles. Additionally over time, adducts may release dehydroretronecine or dehydroheliotridine, which are speculated to bind to other tissue nucleophiles forming new adducts, leading to chronic diseases such as sinusoidal obstructive syndrome, cirrhosis and neoplasia (Edgar, *et al.* 2011).

The initial toxic metabolites (dehydropyrrolizine esters) are so highly reactive that only the alcohols (Fig. 2-3 5) and adducts (Fig. 2-3 6) are measured as products of P450 activation in microsomal preparations (Jago, *et al.* 1970; Mattocks 1986). The rapid nature of metabolism explains why the liver is the primary target organ in most DHPA intoxications. Further metabolism of DHPAs in extrahepatic tissue could account for the presence of pulmonary and renal lesions with DHPAs such as monocrotaline given that CYP 3A activity does occur in both liver and lung (Molyneux, *et al.* 2011). The rate of DHPA metabolism has been increased experimentally by pretreating animals with phenobarbital, which induces P450s, and decreased with chloramphenicol or SKF 525A, which are P450 inhibitors (Allen, *et al.* 1972; White, *et al.* 1983).

### *Excretion*

Studies using radio-labeled monocrotaline, lasiocarpine, senecionine and seneciphylline have shown that approximately 80% of ingested DHPAs are rapidly excreted unchanged in the urine and feces, and that the urine is the most prevalent route (Chan 2001; Eastman, *et al.* 1982). Expiration with carbon dioxide also accounts for approximately 10% of the dose excreted (Chan 2001). Excretion into milk was shown to be a very minor route with 0.04% of both senecionine and seneciphylline excreted in the milk of mice (Eastman, *et al.* 1982).

### **Clinical Signs and Pathology**

Clinical signs and pathologic lesions in DHPA-poisoned animals depending on multiple factors including: the specific DHPA involved, the dose and route of exposure, and the species, age, and sex of the animal. Most DHPAs are primary hepatotoxins, however certain DHPAs also affect other organs. Differences in pathological responses between laboratory and natural exposure exist primarily because of variations in dose and route of exposure. DHPA toxicoses in laboratory animals have been categorized as peracute, acute and chronic forms (Mattocks 1986), whereas naturally-occurring DHPA toxicity in domestic animals has been described as three pathologic expressions representing different types of exposure (Stalker and Hayes 2007). Although similar toxic actions have been demonstrated in many animal species, the quantity of alkaloid required to reach toxic effect can be quite different in different species. Younger animals as well as children tend to be more susceptible than adults, and in some species males tend to be more susceptible than females (Mattocks 1986).

Most clinical signs in DHPA-poisoned animals can usually be attributed to a loss of hepatic function (Galey 1996). The liver plays a major role in metabolism of proteins, lipids, and carbohydrates as well as conversion of excess nitrogen to urea. When these functions are limited there is a lack of energy, clinically expressed as lethargy and weight loss/decreased weight gain, and a buildup of excess nitrogen in the blood resulting in central nervous system dysfunction. The central nervous system dysfunction can result in disorientation leading to common names of DHPA toxicosis such as “walking disease” or “walkabout” (Galey 1996).

DHPAs are hepatotoxic primarily because they are bioactivated in the liver. Pathologic lesions range from zonal hepatic necrosis to fibrosis with or without regeneration depending on chronicity (Stalker and Hayes 2007). Characteristic lesions of DHPA intoxication, such as megalocytosis, typically require more time to develop and are therefore present following more chronic exposures.

Because DHPA metabolites can also affect other organs, death may also be due to renal or pulmonary damage (Stalker and Hayes 2007). Alkaloids from *Crotalaria* affect the widest range of tissues in domestic animals (Stalker and Hayes 2007). Monocrotaline, in particular, is pneumotoxic as well as hepatotoxic (Mattocks 1968; Stalker and Hayes 2007). Pulmonary symptoms have been described in horses, sheep and rats after consumption of various *Crotalaria spp.* plants, and in pigs after eating *Senecio jacobea* (Stalker and Hayes 2007). *Crotalaria spp.* plants are also severely nephrotoxic in pigs (Hooper 1978). In horses and cattle *Trichodesma incanum* has similar effects to *Crotalaria spp.* *Crotalaria retusa*, a plant found in northern Australia, is the one substantial exception. *C. retusa* causes hepatotoxicity, but no pneumotoxicity (Hooper 1978). *Senecio jacobea* has been reported to cause pulmonary disease in pigs, rats and mice, but not in horses, cattle, sheep, or chickens. *S. jacobea* also causes renal tubular epithelial megalocytosis and occasional nephrosis in most species (Hooper 1978). Generally *Heliotropium spp.*, *Amsinkia spp.*, and *Echium spp.* are hepatotoxic, however they may cause renal tubular epithelial megalocytosis, and alkaloids extracted from these genera have been known to cause pulmonary disease in animals acutely poisoned

(Hooper 1978). Sheep, rats, and mice exposed to high doses of Lasiocarpine developed necrotizing enteritis (Hooper 1975).

Megalocytic hepatocytes have morphologic and structural changes when compared to normal hepatocytes. In an ultrastructural study conducted by Allen *et al.* (1970) they found megalocytic hepatocytes to contain large multilobular nuclei with cytoplasmic invaginations. There was a decrease in rough endoplasmic reticulum as well as the number of bound ribosomes. The RNA and nitrogen concentration was the same as controls, but DNA was increased up to 200%. There was also an increase in the size and distribution of Golgi complexes (Allen, *et al.* 1970). These changes are the result of mitotic inhibition (Allen and Hsu 1974) coupled with continued DNA synthesis in some cells. This results in cells with large polyploid nuclei termed megalocytes.

Resistance to chronic toxicity does not necessarily imply resistance to acute toxicity. Sheep died soon after IP injections of senecionine (60 mg kg<sup>-1</sup> BW), lasiocarpine (30 mg kg<sup>-1</sup> BW) and fulvine (45 mg kg<sup>-1</sup> BW) (Hooper 1978). Exposure by the IP route may also have affected the sensitivity of these animals. Several authors have noted that ovine rumen microflora has the ability to detoxify/metabolize DHPAs (Craig, *et al.* 1992; Lanigan 1970; Lanigan and Smith 1970; Wachenheim, *et al.* 1992). However, metabolism by rumen microflora does not appear to be the only factor that determines the resistance of sheep and goats to DHPA intoxication. *S. jacobea* incubated in sheep rumen fluid was more toxic to rats than *S. jacobea* incubated in cattle rumen fluid (Shull, *et al.* 1976).

Mattocks (1986) discussed DHPA toxicity in laboratory animals and categorized observations into either peracute, acute or chronic forms of toxicity. He defines peracute toxicity as rapidly fatal where death ensues within a few minutes to a few hours as a result of a large dose of DHPA (Mattocks 1986). Peracute toxicity is typically due to an IP or IV injection of DHPA such that absorption is very rapid. Consequently the more lipophilic DHPAs are more prone to causing this type of toxicity. This type of toxicity is not considered to be related to the cytotoxic action of the DHPA. It is associated with pharmacological actions of the compound and death may follow convulsions or coma (Mattocks 1986).

#### **Short-term toxicity (Acute)**

Acute toxicity as described by Mattocks (1986) is the result of the hepatotoxic actions of DHPAs. In most cases, acute toxicity is the result of a single exposure and death ensues in a few days as the result of hepatic necrosis. At necropsy, the liver is typically firm and congested with a red granular appearance, and ascites is commonly present. Microscopically there is zonal hemorrhagic hepatic necrosis. Reportedly the zone affected can be different depending primarily on the species, but sometimes on the nutritional status or chemical pretreatment of the animal (Mattocks 1986). Rats, mice, and guinea pigs treated with retrorsine developed centrilobular hepatic necrosis, whereas hamsters treated with retrorsine and monkeys treated with lasiocarpine developed periportal necrosis (Mattocks 1986).

There are numerous studies using scores of DHPAs on numerous different species primarily to determine LD50 information. Unfortunately, nearly all of these studies have

used either an IV or IP route of administration (Mattocks 1986). In virtually all natural exposures, the route of exposure is oral (there are some herbal products marketed for topical use). Some species which are susceptible to IP exposure are resistant to oral exposure, such as sheep (Hooper 1978), hamsters (Huan, *et al.* 1998b) and rabbits (Pierson, *et al.* 1977). Other species such as rats, which are sensitive to oral exposure, are more sensitive to IP exposure (Mattocks and Driver 1987). As a result, it may not be prudent to use only IP-derived data to determine the relative acute toxicity of DHPAs in many species of animals.

### **Chronic Toxicity**

Chronic toxicity may be the result of a single sublethal dose or multiple small doses. Grossly, the liver is atrophic, firm and nodular (Mattocks 1968; Stalker and Hayes 2007). Ascites may also be present. DHPAs inhibit mitosis (Allen and Hsu 1974), but DNA synthesis continues in some cells resulting in cells with large polyploid nuclei termed megalocytes. The characteristic histologic lesions consist of hepatic atrophy, nodular regeneration, megalocytosis, periportal fibrosis and biliary hyperplasia (Stalker and Hayes 2007). Not all alkaloids produce identical lesions. For example, chronic exposure exclusively to *Heliotropium* has been reported to produce firm atrophic livers without nodular regeneration (Stalker and Hayes 2007).

In some cases, toxic or even lethal doses of DHPA may be consumed with mild to no acute clinical signs. *S. jacobea*, *S. longilobus*, and *S. riddellii*, were fed to cattle at lethal doses over a duration of several days. Initial signs of poisoning were absent, mild or transitory. Signs of DHPA toxicosis developed many months after exposure. Death

followed closely after clinical signs (Molyneux, *et al.* 1988). One theory explaining this type of pathogenesis is that tissue-bound DHPA adducts remain a source of ongoing alkylation. Thus, following a dietary exposure, tissue damage continues until the reservoir of labile tissue-bound adducts is eliminated (Edgar, *et al.* 2011)

### **Species Variations**

#### *Swine*

In pigs naturally and experimentally exposed to *Crotalaria retusa*, the major clinical signs were severe nephrosis and uremia, with interstitial pneumonia as the second most common development (Hooper and Scanlan 1977). Both presentations were accompanied by microscopic liver disease. Hepatic and renal megalocytosis were common histologic findings. The clinical disease was most prevalent in growing pigs with a universal depression of appetite and weight. Breeding animals had often did not develop clinical disease, but histologically there was advanced megalocytosis in both the liver and kidneys. This subclinical disease can be highly fatal as 10 of 12 pigs died or were euthanized when moribund when fed diets with 0.02% *Crotalaria retusa* seeds (Hooper and Scanlan 1977).

#### *Poultry*

Outbreaks of DHPA toxicity in chickens and ducks have been characterized by decreased weight gain in growing animals and general ill thrift (Hooper and Scanlan 1977, Pass *et al.* 1979). Gross examination of deceased animals revealed ascites with fibrin frequently present on the surface of most livers (Hooper and Scanlan 1977). In acutely affected animals the major gross finding was yellow to orange friable livers (Pass



*et al.* 1979). Histologically, there was single cell to zonal necrosis of hepatocytes. Hepatocellular megalocytosis was present in chronic cases. In animals exposed to *Crotalaria retusa*, no discernible lesions were present in kidneys of acutely affected animals, but in more chronically affected animals there was renal tubular epithelial megalocytosis (Hooper and Scanlan 1977). In chickens and ducks exposed to *Heliotropium europaeum* renal lesions were mild but consistent and consisted of enlarged nuclei within tubular epithelial cells (Pass, *et al.* 1979). Chickens fed *Crotalaria retusa* seeds at 0.5% of their diets all died, and five of eight of those fed at 0.1% of their diets died (Hooper and Scanlan 1977).

#### *Cattle*

Calves naturally (Baker, *et al.* 1989) or experimentally (Baker, *et al.* 1991) exposed to *Cynoglossum officinale* or experimentally exposed to *Senecio erraticus* (Araya and Fuentealba 1990) all developed hepatocellular megalocytosis if exposed chronically. Renal megalocytosis (Baker, *et al.* 1991) and biliary hyperplasia (Araya and Fuentealba 1990) was also a feature of chronically-exposed animals. Four calves fed *C. officinale* at a rate that provided an approximate daily dose of 60 mg kg<sup>-1</sup> BW of total DHPA died within 48 hours after a single dose (Baker, *et al.* 1991). All four of those animals had massive hepatocellular necrosis and hemorrhage, as well as intestinal hemorrhage most prevalent in the duodenal mucosa. Mesenteric and subcutaneous edema were also common findings in this group (Baker, *et al.* 1991).

### *Horses*

Horses were experimentally exposed to *C. officinale* in a suspension at doses of 5 or 15 mg kg<sup>-1</sup> BW day<sup>-1</sup> for 14 days (Stegelmeier, *et al.* 1996). All of the high dose animals developed severe liver disease within one week which progressed until they were euthanized. Histologically there was extensive hepatocellular necrosis, biliary hyperplasia, and mild periportal fibrosis. The horses treated with 5 mg kg<sup>-1</sup> DHPA day<sup>-1</sup> were depressed for several weeks after dosing and had elevated liver enzyme activities and bile acid concentrations in the serum that resolved within 6-8 weeks. Biweekly liver biopsies of these animals revealed minimal periportal necrosis with fibrosis, which persisted and was accompanied by extensive megalocytosis by week 14. Biliary epithelial cells had an increased mitotic rate commensurate with the other histologic changes (Stegelmeier, *et al.* 1996).

### *Sheep and Goats*

As previously mentioned, sheep and goats are significantly more resistant to DHPA toxicosis than other livestock species. Lactating dairy goats as well as goat kids were fed dried, ground *S. jacobea* as 25% of their diet (Goeger, *et al.* 1982). Four of eleven animals died and had signs of chronic DHPA poisoning such as hepatocellular degeneration, hepatocyte swelling, megalocytosis, and bile duct proliferation. The chronic lethal dose was determined to be between 1.2 and 4 kg of dried plant per kg of body weight. Intakes of 0.05 to 0.20 kg/kg of body weight were found to be lethal in horses and cattle.

After an outbreak of acute DHPA intoxication in sheep presumably caused by *Crotalaria retusa* seeds, sheep were experimentally fed *C. retusa* seeds at doses of 2.5, 5, 10, 20, and 40 g/kg BW (Nobre, *et al.* 2005). The seeds contained 1.4% monocrotaline. Animals which received 5 g/kg BW or more died with signs of acute DHPA intoxication including anorexia, severe depression, mild jaundice, incoordination and recumbence. In another study, three sheep per group were fed a single dose of monocrotaline at 205.2 and 273.6 mg kg<sup>-1</sup> BW (Anjos *et al.* 2010). Two sheep from the 205.2 mg kg<sup>-1</sup> BW and one from the 273.6 mg kg<sup>-1</sup> BW groups died with signs of acute intoxication including apathy, abdominal pain, anorexia, increased respiratory and cardiac rates, and mild yellowish ocular or oral mucosa, followed by marked depression, recumbence and death within one to four days. The two remaining sheep in the 273.6 mg kg<sup>-1</sup> BW group initially showed clinical signs of anorexia during the first three to five days followed by a period of no clinical signs and then a rapid course leading to death at 12 and one at 68 days post-treatment. Acutely intoxicated sheep exhibited periportal hepatic necrosis, and chronically intoxicated sheep had hepatic fibrosis and megalocytosis.

### Carcinogenicity

Numerous studies have examined various aspects of DHPA carcinogenicity. This review will examine those studies aimed at expounding the extent of primary DNA damage caused by DHPAs, including DNA cross-linking, DNA strand breakage, unscheduled DNA synthesis and DNA adduct formation. Next we will review chromosomal damage induced by DHPAs through studies using the micronucleus assay,

chromosomal aberration and sister chromatid exchanges. We will then examine mutations induced by DHPAs which have been extensively studied in bacteria, *Drosophila* and rodents. Finally we will consider neoplastic conditions associated with DHPA exposure.

### **Primary DNA Damage**

#### *DNA cross-linking*

Due to the presence of two functional groups, one at C7 and one at C9, DHPAs have the capacity to bind protein or DNA at two different locations. Petry *et al.* (1984) demonstrated DNA-DNA and DNA-protein cross-linking due to DHPA exposure. Sprague Dawley rats were injected with doses of monocrotaline ranging from 5 to 30 mg kg<sup>-1</sup> BW IP. Using the alkaloid elution technique, it was determined that DNA-DNA intrastrand cross-linking increased in a dose dependent manner and peaked at twelve hours or less, and that after 96 hours additional cross-linking was statistically no different than the control. These cross-links are thought to play a role in carcinogenicity, toxicity, and the antimitotic effect of some DHPAs (Coulombe, *et al.* 1999; Kim, *et al.* 1999; Pereira, *et al.* 1998; Petry, *et al.* 1984; Rieben and Coulombe 2004; Tepe and Williams 1999; Wagner, *et al.* 1993; Weidner, *et al.* 1990).

Studies focusing on DNA-protein cross-linking ability of DHPAs have been conducted using both cells and isolated nuclei. Coulombe *et al.* (1999) used bovine kidney epithelial cells and human breast carcinoma cells to determine that dehydrosenecionine and dehydromonocrotaline cross-linked DNA with actin. This combined with the previous work of Kim *et al.* (1993), suggesting that megalocytic and

antimitotic effects of DHPAs are directly proportional to their ability to cross-link. These led to the conclusion that the ability of DHPAs to cross link DNA with actin may be at least partially responsible for the antimitotic effects of DHPAs. Furthermore the ability to form DNA-protein crosslinks tended to coincide with the toxic potency of the DHPAs in animals, leading to the conclusion that DNA-protein cross-linking is likely involved in PA-related toxicity (Coulombe, *et al.* 1999; Kim, *et al.* 1993; Petry, *et al.* 1984).

Monocrotaline has been used as a model cross-linking DHPA by several researchers. After Petry *et al.* (1984) demonstrated that IP injections of monocrotaline induced DNA-DNA and DNA-protein cross-links in Sprague-Dawley rats, Tepe *et al.* (1999) used the pyrrolic metabolite of monocrotaline (dehydromonocrotaline) to demonstrate its ability to mediate DNA-DNA cross-linking. Pereira *et al.* (1998) also used dehydromonocrotaline (DHM) to study different types of DHPA-induced DNA-DNA cross-links. By using electrophoresis and electron microscopy, they found that DHM produced piperidine and heat-resistant DNA crosslinks. Based on their research they proposed that DHM undergoes a rapid polymerization forming a structure that allows it to cross-link multiple DNA fragments.

There is some disagreement in the literature as to whether or not there is a preferential site for DNA-DNA cross-link formation. Three different groups used DHM as their model to study DNA-DNA cross-linking. Weidner *et al.* (1990) found a preference for cross-linking at 5'-CG sites using DHM and dehydroretrosine with DNA duplex fragments and polyacrilamide gel electrophoresis (PAGE). Pereira *et al.* (1998) found a predilection for 5'-GC and 5'GA sequences using a DNA alkylation method that

causes cleavage of DNA at N7 residue of guanine. Rieben *et al.* (2004) assessed cross-linking by electrophoretic mobility shift assay of <sup>32</sup>P endlabeled oligonucleotides and by HPLC analysis of cross-linked DNAs enzymatically digested to their constituent deoxynucleosides. In this study the degree of cross-linking was dependent on the concentration of DHM, but they found no base sequence preference.

In a study using bovine kidney epithelial cells Hincks *et al.* (1991) tested DHPAs from three different classes, macrocyclic diesters, open chain diesters, and the pyrrolizidine base (retronecine). All of the DHPAs they tested induced DNA-protein cross-links and DNA-DNA cross-links. The relative ability to induce cross-links was determined as follows: seneciophylline > riddelliine > retrorsine > senecionine > heliosupine > monocrotaline > latifoline > retronecine. All but monocrotaline and retronecine tended to form more DNA-protein cross-links than DNA-DNA cross-links.

#### *DNA strand breakage*

DNA strand breaks were not detectable following treatment with senecionine, seneciophylline, riddelliine, retrorsine, monocrotaline, heliosupine, latifoline or jacobine using the alkaline elution assay (Hincks, *et al.* 1991; Petry, *et al.* 1984). Using the more sensitive single cell gel electrophoresis assay (Comet assay) DNA strand breaks were detected in human hepatoma (HepG2) cells treated with isatidine (Uhl, *et al.* 2000) and human glioblastoma cells (GL-15) treated with monocrotaline (Silva-Neto, *et al.* 2010).

#### *Unscheduled DNA synthesis*

Unscheduled DNA synthesis (UDS) is any DNA synthesis that takes place outside of the S-phase of the cell cycle. The UDS assay incorporates tritiated thymidine into the

DNA of cultured mammalian cells during repair which can be detected via autoradiography. Therefore, this test measures the repair of primary DNA damage. This method has been used to detect DNA damage induced by various DHPAs (Griffin and Segall 1986; Mirsalis 1987; Mirsalis, *et al.* 1993; NTP 2003a). Riddelliine induced UDS in rat hepatocytes following in vitro treatment, however it required greater than an LD50 equivalent dose to produce positive response (Mirsalis 1987). In a prechronic (animals survive up to 30 days) toxicity test conducted for the National Toxicology Program, Mirsalis *et al.* (1993) found that riddelliine did not induce an increase in UDS in rat hepatocytes after 5 or 30 days of dosing. However, it did induce an equivocal response in male mice at both time points and a positive response in female mice after 30 days of dosing. UDS was detected in hepatocytes cultured from male and female rats and mice following five days of riddelliine treatment by gavage. UDS was also detected in cultured hepatocytes from all of the same groups except the female rats following a 30 day gavage study with riddelliine (NTP 2003a). Griffin and Segall (1986) detected a positive, dose-related increase in UDS in primary cultures of rat hepatocytes when treated with any of the following compounds: senecionine, retrorsine, seneciphylline, 19-OH-senecionine, trans-4-OH-2-hexenal, trans-4-OH-2-nonenal, and nonenal.

#### *DNA adducts*

DNA adducts are formed when DHPAs are metabolized in vivo or in vitro. After treating rats with tritiated senecionine and seneciphylline Candrian *et al.* (1985) collected protein and DNA from liver, lung and kidney six hours and four to five days post-treatment. Using HPLC and radioactivity analysis this group was able to show that both

alkaloids covalently bound to DNA, and that the binding persisted for at least four days post-treatment (Candrian *et al.* 1985). Using a <sup>32</sup>P-postlabeling/HPLC method, 8 DHR-derived DNA adducts were identified in calf thymus DNA exposed to riddelliine that was metabolized by rat liver microsomes (Yang *et al.* 2001). Similar DNA adduct profiles were detected in livers of female F344 rats fed riddelliine. They were able to determine the structure of two of the eight adducts, which were epimers of DHR-3'-dGMP (Yang, *et al.* 2001).

Chou *et al.* (2004) measured DHR derived adducts at 1,3,7, and 28 days post inoculation in both rats and mice. The maximum concentration was higher in rats than mice and they were more persistent in rats than mice. Concentrations of DHR derived DNA adducts were higher in hepatic endothelial cells of both mice and rats than in hepatic parenchymal cells. Maximum concentration of DHR derived endothelial DNA adducts were higher in male mice than female mice, but they were more persistent in the female mice than male mice. DHR derived DNA adducts tended to be more persistent in endothelial cells than in hepatic parenchymal cells. (Chou, *et al.* 2004)

Employing the same <sup>32</sup>P-postlabeling/HPLC method, the same eight DNA adducts have been isolated from calf thymus DNA incubated with rat liver microsomes and other DHPAs and DHPA containing plants (Chou and Fu 2006; Wang, *et al.* 2005a; Xia, *et al.* 2006; Xia, *et al.* 2004; Xia, *et al.* 2008). The DHPAs and DHPA containing plants tested include: clivorine, heliotrine, lasiocarpine, monocrotaline, retrorsine, comfrey root extract, comfrey compound oil, coltsfoot extract, *Flos farfara* extract, and *Ligularia hodgsonii* extracts.



In an effort to determine the relevance of this information obtained via use of experimental animals, a similar experiment was conducted using human liver microsomes and calf thymus DNA (Xia, *et al.* 2003). When human liver microsomes were exposed to riddelliine the same major metabolites (dehydroriddelliine and riddelliine N-oxide) were isolated in similar concentrations to that obtained from rat liver microsomes under the same experimental conditions. When metabolism was conducted in the presence of calf thymus DNA the same set of DHR-derived DNA adducts were isolated.

Due to the lack of structural information about DNA adducts, the <sup>32</sup>P-postlabeling/HPLC method was limited. As a result a more sensitive and specific liquid chromatography-electrospray ionization-tandem mass spectrometry method was developed (Fu, *et al.* 2010). Using this new technique in combination with a technique for synthesizing larger quantities of DHR-derived DNA adducts, the full structures of all eight adducts were determined (Zhao, *et al.* 2012).

More recently, female rats were gavaged with one of ten different alkaloids (Xia, *et al.* 2013). Nine of these DHPAs contained the crucial C1-C2 double bond including: riddelliine, retrorsine, monocrotaline, lycopsamine, retronecine, lasiocarpine, heliotrine, clivorine and senkirkine. The tenth, platyphylliine is a saturated pyrrolizidine alkaloid, and therefore is not tumorigenic. They were able to demonstrate the presence of the four major DNA adducts (DHP-dG-3, DHP-dG-4, DHP-dA-3, DHP-dA-4) in rats treated with all of the pyrrolizidine alkaloids with the exception of retronecine and the saturated pyrrolizidine alkaloid platyphylliine.

As mentioned under the metabolism section DHPAs are conjugated by glutathione, and glutathione conjugation is considered a major detoxification mechanism (Reed, *et al.* 1992, Yan and Huxtable 1995). However, glutathione conjugated DHPAs may be somewhat unstable and can potentially act as a reservoir for DHP adduct formation. After incubating the DHPA glutathione conjugate 7-glutathionyl-(±)-6,7-dihydro-1-hydroxymethyl-5H-pyrrolizine with purine nucleosides or calf thymus DNA the same major DNA adducts described previously were formed (Xia, *et al.* 2015). Furthermore an *in vivo* study, where lasiocarpine was injected IP into sheep, rats, and mice resulted in severe intestinal lesions (Hooper 1975). Conclusions were made that the most likely cause of the intestinal lesions was glutathione bound DHPA in the bile.

#### *Chromosomal Damage Induced by DHPAs*

Chromosomal damage as a result of exposure to DHPAs has been studied by numerous scientists beginning at least as early as 1972 (Martin, *et al.* 1972). Three basic methods have been used, the micronucleus assay, chromosomal aberration, and sister chromatid exchange. Increases in micronucleus formation, number of chromosomal aberrations and sister chromatid exchanges are indicative of chromosome damage.

#### *Micronucleus assay*

When a portion of a chromosome is not carried to opposite poles during anaphase of either mitosis or meiosis, a micronucleus is formed. This chromosome or chromosome fragment will transfer to one of the daughter cells. The daughter cell with the extra portion of a chromosome will have a nucleus and a second smaller nucleus or micronucleus. An increase in micronucleus formation is indicative of chromosomal

damage. The micronucleus assay typically uses bone marrow or peripheral blood to detect an increase in micronucleus formation.

DHPAs extracted from *Senecio brasiliensis* in 1945 and stored for 23 years in an ambient environment in Brazil were used to determine the stability of DHPAs (Santos-Mello *et al.* 2002). During storage these DHPAs were exposed to variations in temperature and humidity as well as light. The crude alkaloid contained integerrimine, retrorsine and several unidentified impurities. This crude extract as well as a purified extract of integerrimine were injected IP into albino Swiss mice. Both the purified integerrimine and the crude extract induced micronucleus formation in polychromatic erythrocytes from the bone marrow.

Sanderson and Clark, (1993) evaluated the clastogenic activity of multiple chemicals including heliotrine and monocrotaline, in adult mice as well as transplacental exposure to fetal mice. Both heliotrine and monocrotaline increased micronucleus formation in polychromatic erythrocytes found in bone marrow of adult mice and the liver of fetal mice. Heliotrine exposure resulted in the largest increase which peaked 18 hours post injection in adult bone marrow and 24 hours post injection in fetal liver (Sanderson and Clark 1993). This suggests that heliotrine and monocrotaline are mutagenic to both adult and fetal mice.

Monocrotaline or ground seeds from *Crotalaria spectabilis* containing 4.2% monocrotaline were administered in the diet to Swiss-Webster mice for six days. Polychromatic erythrocytes increased in peripheral blood and became constant after three days of treatment (MacGregor, *et al.* 1990). Muller-Tegethoff *et al.* (1997) investigated a

host of compounds using the micronucleus assay, among which were isatidine, monocrotaline and retrorsine. All three were found to induce micronucleus formation.

Exposure to riddelliine did not increase micronucleus formation in mice or rat peripheral blood samples after 4 or 13 weeks at doses of 10 mg kg<sup>-1</sup> for rats and 25 mg kg<sup>-1</sup> for mice given daily as an oral gavage. A much higher dose of 150 mg kg<sup>-1</sup> did result in a weakly positive response in bone marrow and peripheral blood of male mice (Chan 1993; Mirsalis, *et al.* 1993). To give some perspective to the doses used, the oral LD50 in male rats for riddelliine is about 80 mg kg<sup>-1</sup> (Dalefield, *et al.* 2012).

#### *Chromosomal aberration*

The chromosomal aberration test is performed by analyzing cytologic preparations and counting the number of structural chromosomal aberrations present as a proportion of cells in metaphase. Structural chromosomal aberrations include chromatid and chromosome gaps, breaks and fragments.

The majority of studies linking DHPAs to chromosomal abnormalities have used an in vitro model of either V79 Chinese hamster cells (Muller *et al.*, 1992; Takanashi *et al.* 1980) or Chinese hamster ovary cells (Carballo *et al.*, 1992; Chan 1993), or alternatively in vivo models, such as mouse bone marrow (Ribeiro *et al.*, 1993; Gimmler-Luz *et al.*, 1990), rat peripheral blood (Martin *et al.*, 1972), or peripheral blood from children recovering from veno-occlusive disease (Martin *et al.*, 1972).

Some studies used extracts from various parts of DHPA containing plants (Ribeiro *et al.*, 1993; Carballo *et al.* 1992), while others used purified DHPAs (Martin *et al.*, 1972; Muller *et al.*, 1992; Takanashi *et al.* 1980; Chan 1993). In all of these studies, every

DHPA tested resulted in chromosomal aberrations. Ribeiro *et al.* 1993) found that extracts made from fruit obtained from *Crotalaria retusa* resulted in a dose-dependent increase in chromosomal aberrations while extracts made from the leaves of *C. retusa* and fruit from *C. mucronata* had no detectable effect on frequency of chromosomal aberrations.

#### *Sister chromatid exchanges*

Sister chromatid exchange is the exchange of DNA fragments between two identical or sister chromatids. An increase in sister chromatid exchanges has been associated with chromosome damage and tumor induction (Chen *et al.* 2010). Sister chromatid exchanges have been associated with dehydroretronecine, heliotrine, monocrotaline, riddelliine, seneciophylline, senkirkine and several synthetic DHPAs (Bruggeman and Van der Hoeven 1985, Ord *et al.* 1985, Chan 1993). These studies have employed a variety of in vitro models including human lymphocytes (Ord *et al.* 1985), V79 Chinese hamster cells co-cultured with chick embryo hepatocytes (Bruggeman and Van der Hoeven 1985), and CHO cells with and without S9 (Chan 1993).

### **Mutations Induced by DHPAs**

#### *Mutations in bacteria*

Earlier researchers had difficulty in testing DHPA mutagenicity using the *Salmonella typhimurium*/mammalian microsome test (Clark 1976; Wehner, *et al.* 1979), also known as the Ames test, presumably because they lacked a suitable activation enzyme system. Subsequently, although it may lack sensitivity (Rubiolo, *et al.* 1992), the Ames test has been used extensively with suitable activation systems to evaluate the

mutagenic potential of various DHPAs. The mutagenicity of retrorsine was measured using the Ames assay with four strains of Salmonella (TA98, TA100, TA1535, and TA1537) in the presence of S9 (liver cell fragment containing microsomes that can be obtained by taking the Supernatant of a cell homogenate after centrifugation at 9,000 g). It was determined to be mutagenic for TA1535 and TA1537 indicating that it induced both frame shift mutations and base pair substitutions (Wehner, *et al.* 1979). Using TA100 with S9 the following DHPAs/DHPA containing plants have been determined to be mutagenic: clivorine, fukinotoxin, heliotrine, lasiocarpine, ligularidine, LX201, retrorsine, riddelliine, senecivernine, seneciphylline, senkirkine, and extracts from *Senecio inaequidens*, *S. fuchsia*, *S. cacliastershowed*, and *S. jacobea* (Chan 1993; Rubiolo, *et al.* 1992; White, *et al.* 1984a; Yamanaka, *et al.* 1979). Similar tests using isatidine, monocrotaline, and senecionine were negative (Rubiolo, *et al.* 1992). Repair deficient strains of E. coli have been used to test mutagenicity of heliotrine and monocrotaline, resulting in increased mortality of repair deficient bacteria (Green and Muriel 1975).

#### *Mutations in Drosophila*

*Drosophila melanogaster* has been used extensively to study the mutagenic potential of DHPAs via either the wing spot test or the sex-linked recessive lethal test. One group used the wing spot test to assess the genotoxic potential of 15 DHPAs and one DHPA N-oxide (Frei, *et al.* 1992a). All of the DHPAs tested with the exception of supinine (a C9 monoester), were clearly genotoxic. They determined that macrocyclic diesters were the most potently genotoxic, followed by the open chain diesters. The open

chain diesters had a stronger effect than the corresponding C9 monoesters with a hydroxyl group at C7. Supinine, the only DHPA that was not significantly genotoxic, is a C9 monoester that lacks a hydroxyl group at C7. From this they determined that at least one ester linkage and one hydroxyl group appear to be necessary for genotoxicity in the DHPAs tested. The same group also found that an increasing number of hydroxyl groups appeared to reduce genotoxic potency (Frei, *et al.* 1992a)

Brink (1969) demonstrated chromosomal damage in *Drosophila* exposed to heliotrine by documenting increased frequency of dominant lethal, sex chromosome loss, and translocations and sex-ratio shifts. Candrian *et al.* (1984) used milk from rats fed 25mg kg<sup>-1</sup> seneciophylline to feed *drosophila* resulting in a fourfold increase of sex-linked recessive lethals. They also found senkirkine to be mutagenic in *Drosophila*. Integerrimine was also found to be genotoxic using the wing spot test (Campesato *et al.* 1997).

#### *Mutations in Rodents*

The mutagenicity of comfrey (*Symphytum officinale*) as well as riddelliine has been studied using Big Blue transgenic rats (Mei, *et al.* 2004a; Mei, *et al.* 2005; Mei and Chen 2007). Transgenic mutation assays were used to study induction of tissue-specific mutations, and measure mutation frequencies in target tissues. In one study, female Big Blue Rats were exposed to 0.3 mg riddelliine kg<sup>-1</sup> BW, 5 days per week for 12 weeks (Mei, *et al.* 2004a). After the rats were euthanized they isolated endothelial cell fractions and liver parenchymal cell fractions. They then measured the mutation frequency of the cII transgene in both fractions. While the liver parenchymal cell fraction did not have a

significant difference in mutation frequency, the endothelial cell fraction had increased frequency. Molecular analysis of the mutants in the endothelial cell fraction determined that 17% of mutations in riddelliine treated mice were G:C→T:A transversions, whereas G:C→T:A transversions accounted for only 9% of mutations in the controls. The major mutation in control rats were G:C→A:T transversions, which accounted for 54% of their mutations. This hepatic endothelial cell mutation specificity is interesting in light of the fact that riddelliine readily induces hemangiosarcoma in the liver of rats (Chan 1993), and hemangiosarcoma is a tumor originating from endothelial cells. Riddelliine also induced an increase in tandem base substitutions in Big Blue Rats of GG→TT and GG→AT (Mei, *et al.* 2004b).

The mutagenic effect of comfrey on rat lung was also studied using the Big Blue Rat as a model (Mei and Chen 2007). CII gene mutation frequency from the lungs of Big Blue Rats fed 8% comfrey for 12 weeks was significantly greater than that of controls. Much like the riddelliine treated rats in their previous studies, the major mutation in the comfrey treated rats was a G:C→T:A transversion. The major mutation in the control rats was a G:C→A:T transversion, which is also consistent with their previous studies.

B6C3F1 mice were used to examine alterations in the K-ras proto-oncogene, the P53 tumor suppressor gene as well as  $\beta$ -catenin (Hong *et al.* 2003). They examined 12 riddelliine induced hepatic hemangiosarcomas as well as 15 spontaneous subcutaneous hemangiosarcomas and found that 58% of riddelliine induced hemangiosarcomas contained a K-ras codon 12 G→T transversion mutation, and that 75% had a variety of P53 mutations. The spontaneous hemangiosarcomas of the control mice had neither K-



ras nor P53 gene mutations. They determined that K-ras mutations and p53 protein expression in riddelliine-induced hemangiosarcomas likely resulted from the genotoxic effects of riddelliine.

#### *Neoplasms associated with DHPAs*

A large number of experiments beginning as early as the 1950s have been carried out to determine the carcinogenicity of different DHPAs. Table 2-2 is a summary of those experiments organized by type of DHPA base (retronecine, heliotridine, or otonecine). There is substantial variation between studies in route, dose, dosing interval/frequency, duration of dosing and duration of the experiment. In spite of these differences, the majority of experiments concluded that whatever DHPA they were studying caused an increase in tumors within the liver, the majority of which were either hepatocellular or endothelial in origin.

**Table 2-2** Neoplasms associated with different dehydropyrrolizidine alkaloids and dehydropyrrolizidine alkaloid containing plants

PA / Plant	Species	Study Duration	Tumor type(s)	Reference (route of administration)
<b>Retronecine type</b>				
<i>Amsinckia intermedia</i> (Lycopsamine / Intermedine)	Rats	26 – 31.5 Months	Pancreatic Islet Cell Tumors (3/15), Exocrine Pancreatic Tumor (1/15)	(Schoental, Fowler <i>et al.</i> 1970) (gavage)
			Bladder papillary tumor (1/15)	
			Pituitary tumor(1/15)	
Dehydroretronecine	Rats	22 Months	Rhabdomyosarcoma (31/60)	(Allen <i>et al.</i> 1975) (Subcutaneous injection)
Isatididine	Rats	Until death (2 ½ years)	Liver Tumors (4/6)	(Schoental and Head 1957) (water + IP)

PA / Plant	Species	Study Duration	Tumor type(s)	Reference (route of administration)
			Liver Tumors (10/22), (6/7)	(Schoental <i>et al.</i> 1954) (water)
			Liver Tumor (1/5)	(Schoental <i>et al.</i> 1954) (water + IP)
Monocrotaline	Rats	22 Months	Rhabdomyosarcoma (2/60), Hepatocellular carcinoma (2/60), Acute myelogenous leukemia (2/60)	(Allen <i>et al.</i> 1975) (subcutaneous injection)
		500 Days	Insulinoma (16/23)	(Yuzo <i>et al.</i> 1977) (subcutaneous injection)
		2 Years	Pulmonary Adenoma (10/60), Hepatocellular Carcinoma (5/60), Rhabdomyosarcoma (4/60)	(Shumaker <i>et al.</i> 1976) (subcutaneous injection)
		55 Weeks	Hepatocellular Carcinoma (10/42)&(14/35)	(Newberne and Rogers 1973) in (Mattocks 1986) (gavage)
Retrorsine	Rats	Until death	Liver Tumors (2/4)	(Schoental and Head 1957) (injection)
			Hepatomas (4/14)	(Schoental <i>et al.</i> 1954) (water)
Riddelliine	Rats (F344) male	2 Years	Hemangiosarcoma (43/50), Hepatocellular adenoma (4/50), Mononuclear Cell Leukemia (9/50)	(Chan <i>et al.</i> 2003) (gavage)
	female		Hemangiosarcoma (38/50), Hepatocellular adenoma (7/50), Mononuclear Cell Leukemia (14/50)	

PA / Plant	Species	Study Duration	Tumor type(s)	Reference (route of administration)
	Mice (B6C3F <sub>1</sub> ) male	2 Years	Hemangiosarcoma (31/50)	(Chan <i>et al.</i> 2003) (gavage)
	female		Alveolar/bronchiolar adenoma or carcinoma (13/50)	
<i>Senecio cannabifolius</i>	Rats	240 Days	Hemagioendothelial Sarcoma (female 1/11), Liver Cell Adenoma (male 6/10, female 7/11), Other Tumors (male 5/10, female 2/11)	(Hirono <i>et al.</i> 1983)
		240 Days	Hemagioendothelial Sarcoma (male 2/12, female 6/12), Liver Cell Adenoma (male 1/12, female 2/12), Other Tumors (male 8/12, female 3/12)	
<i>Senecio longilobus</i>	Rats	>200 Days	Hepatocarcinoma (16/47), Angiosarcoma (3/47)	Harris and Chen 1970 in (Mattocks 1986) (feed)
<i>Senecio jacobea</i>	Chickens		Liver Tumors (4/43)	(Campbell 1956)(feed)
<i>Senecio nemorensis</i> (senecionine & Fuchsisenecionine)	Rats	114 Weeks	Hepatocellular tumors (4/11 & 13/29), Cholangiogenic tumors (male 1/2 & 2/5) (female 2/11 & 11/29), Hemangiogenic (male 2/10)(female 5/11 & 5/29)	(Habs <i>et al.</i> 1982) (gavage)
Symphytine	Rats	>330 days	Hemangiosarcoma (3/20), Liver adenoma (1/20)	(Hirono <i>et al.</i> 1979) (IP)

PA / Plant	Species	Study Duration	Tumor type(s)	Reference (route of administration)
<i>Symphytum officinale</i>	Rats	600 Days	Hepatocellular adenoma (24/88), Hemangioendothelial Sarcoma (1/88)	(Hirono <i>et al.</i> 1978) (feed)
		280 Days	Hepatocellular adenoma (57/87), Hemangioendothelial Sarcoma (2/87)	
Heliotridine Type				
<i>Heliotropium supinum</i> L. (Supinine, Echinatine, Trachelanthyl-7-angelylheliotridine, Viridofloryl-7-angelylheliotridine, Heliosupine)	Rats	26 – 31.5 Months	Pancreatic Islet Cell Tumors (1/6, 1/2)	(Schoental <i>et al.</i> 1970) (gavage)
Lasiocarpine	Rats	Until moribund or palpable tumor	Hepatocellular Carcinoma (11/18), Squamous Cell Carcinoma (6/18), Pulmonary Adenoma (5/18), Small Intestinal Adenocarcinoma (2/18), Cholangiocarcinoma (1/18), Adenomyoma, ileum (1/18)	(Svoboda and Reddy 1972) (IP)
		59 Weeks	Hemangiosarcoma (9/20) Hepatocellular Carcinoma (7/20), Lymphosarcoma (1/20), Malignant Adnexal Tumor (1/20)	(Rao and Reddy 1978) (feed)
Heliotrine + Nicotinamide	Rats	2 Years	Pancreatic Islet Cell Tumor (3/6), Interstitial Cell Tumor, Testicle (1/6), Hepatoma (1/6)	(Schoental 1975) (gavage)

PA / Plant	Species	Study Duration	Tumor type(s)	Reference (route of administration)
Heliotrine	Rats	2 Years	Pancreatic Islet Cell Tumor (1/1)	
<b>Otonecine Type</b>				
Clivorine	Rats	480 Days	Hemangiosarcoma (2/12), Liver Tumor (6/12), Interstitial Cell Tumor, Testicle (4/12), Pancreatic Acinar Cell Tumor (1/12), Pituitary Adenoma (1/12)	(Kuhara <i>et al.</i> 1980) (water)
<i>Farfugium japonicum</i> (Senkirkine & Petasitenine)	Rats	240 Days	Hemangiosarcoma (6/29), Liver Adenoma (7/29), Adrenal cortical adenoma (7/29), Other Tumors (6/29)	(Hirono <i>et al.</i> 1983) (feed)
<i>Tussilago farfara</i> (Senkirkine)	Rats	>380 Days	Hemagioendothelial Sarcoma (8/12)	(Hirono <i>et al.</i> 1976) (feed)

Some researchers also concluded that there were gender related differences (Chan, *et al.* 2003; Habs, *et al.* 1982; Hirono, *et al.* 1983). Perhaps the most profound is the NTP funded carcinogenicity study using B6C3F<sub>1</sub> mice. When comparing male and female mice at an oral dose of 3 mg kg<sup>-1</sup> BW day<sup>-1</sup> of riddelliine for 105 weeks, 31 of 50 male mice developed hemangiosarcoma in the liver compared to 1 of 50 female mice (Chan, *et al.* 2003). Also of interest in this study is a significant decrease in tumors of hepatocellular origin in both genders when compared to controls. In the male mice, 3 of 50 developed tumors of hepatocellular origin compared to 36 of 50 controls. Female mice followed a similar trend with 0 of 50 compared to 16 of 49 hepatocellular adenomas or carcinomas in controls (Chan *et al.* 2003). This work was supported as the same group also found increased incidence of hepatocellular and endothelial neoplasia in both male

and female F344 rats that were treated with riddelliine at a dose of  $1 \text{ mg kg}^{-1} \text{ BW day}^{-1}$  for 105 weeks.

## Factors Influencing Toxicity

### Species

Vast variations in susceptibility exist between species. Sheep have been used in many parts of the world to graze pastures that are considered too dangerous for other species. One study compared the susceptibility of pigs and chickens to *Crotalaria retusa* and found that chickens required from 5 to 10 times more *Crotalaria retusa* per body weight than pigs to result in lethality (Hooper and Scanlan 1977). In general it appears that horses are similar to cattle and sheep are similar to goats in their susceptibility to DHPAs. Hooper (1978) approximates the ratios of DHPA containing plant required to poison different species as follows: pigs = 1, chickens = 5, cattle and horses = 14, rats = 50, mice = 150, and sheep and goats = 200. Extreme ranges for acute toxicity have also been reported (Mattocks 1986).

Numerous studies have reported variations in susceptibility of different animals to the effects of DHPAs (Chesney and Allen 1973; Huan 1995; Huan, *et al.* 1998a; 1998b; Shull, *et al.* 1976; Swick, *et al.* 1982; White, *et al.* 1973; Winter, *et al.* 1988). Cheeke (1998) divided species into susceptible and resistant groups. Resistant species included sheep, guinea pigs, gerbils, rabbits, hamsters and Japanese quail, whereas rats, cattle, horses, and chickens were reported to be highly susceptible (Huan, *et al.* 1998a). The

difference in susceptibility between species appears to be primarily due to differences in hepatic DHPA metabolism (Huan, *et al.* 1998a).

### **Individual (Animal or Human)**

Some individual animals are able to develop resistance to DHPA intoxication over time. Two of three and three of three sheep fed single doses of *Crotalaria retusa* containing 205.2 and 273.6 mg kg<sup>-1</sup> BW of monocrotaline respectively, died. However, three sheep fed 136.8 mg kg<sup>-1</sup> BW day<sup>-1</sup> for 20 days followed by seven days of 273.6 mg kg<sup>-1</sup> BW day<sup>-1</sup> developed no clinical signs (Anjos, *et al.* 2010). The authors concluded that chronic poisoning at least in sheep is unlikely to occur as a result of repeated non-acutely toxic doses, but is most likely the result of a single toxic dose. It is unclear whether this is due to induction of detoxification enzymes or some other mechanism.

### **Extent of bioactivation**

In one study researchers measured pyrrolic metabolites and N-oxide formation in hepatic microsomal incubations from eight different species (sheep, cattle, gerbils, rabbits, hamsters, Japanese quail, chickens, and rats) (Huan, *et al.* 1998b). In this study microsomes from hamsters produced far more DHP than N-oxide, whereas sheep, another resistant species, produced much more N-oxide than DHP. They determined that at least in their model, there was not a strong correlation between production of pyrrolic metabolites and susceptibility of animals to DHPA toxicity.

Another study using a similar design compared the metabolism of senecionine in guinea pig, rat, cattle, horse, and sheep hepatic microsomes (Winter, *et al.* 1988). They

found that DHP production by guinea pig hepatic microsomes was higher than the other species. Taken together these two studies suggest that at very least bioactivation is not the sole determinant of species susceptibility.

### **Detoxification processes**

Several investigators have examined the effects of glutathione (GSH) conjugation on DHPA metabolism. In vitro methods were used to determine that GSH forms conjugates with metabolites of monocrotaline (Robertson, Seymour *et al.* 1977, Yan and Huxtable 1995), senecionine (Reed *et al.* 1992) and Jacobine (Dueker, Lamé *et al.* 1994). In vivo methods have also been used to isolate and identify a pyrrolic glutathione conjugate metabolite of monocrotaline (Lamé, Morin *et al.* 1990).

Using hepatic microsomes from hamsters and sheep, the effect of GSH conjugation on formation of DHP and N-oxide was determined by adding the reduced form of GSH to the incubation mixture and comparing the formation of DHP and N-oxide with controls (Huan, *et al.* 1998b). When they added 2.0 mM GSH to the incubation mixture, the occurrence of DHP was reduced by 79% and 63% in sheep and hamsters respectively. They found little effect on formation of N-oxide.

Yan and Huxtable (1995) used isolated perfused rat liver to determine the relationship of GSH concentration and metabolism of monocrotaline. When GSH was depleted, although there was no effect on the total release of pyrrolic metabolites, the pattern of pyrrole release was markedly affected. Release of the GSH conjugated DHP in the bile was reduced 72%, protein bound pyrrole concentration in the liver increased, and there was double the release of dehydromonocrotaline into the perfusate. Increase of



GSH resulted in a 54% increase of total pyrrolic metabolites. Release of GSH conjugated DHP in the bile, release of dehydromonocrotaline in the perfusate, and DHP were all increased. They concluded that hepatic GSH concentration regulates the metabolism of monocrotaline and dehydromonocrotaline. Depletion of GSH results in a switch from biliary release of glutathione conjugated DHP to the perfusate release of the highly toxic dehydromonocrotaline. Furthermore, depletion of GSH allows more dehydromonocrotaline in the liver to remain available for macromolecular alkylation (Yan and Huxtable 1995).

### **Environmental copper and other factors**

Interactions between DHPAs and various dietary constituents have been identified. Some have an additive or symbiotic effect with DHPAs increasing the toxicity of the DHPA. Others have a protective effect, and have been used in an attempt to mitigate the effects of DHPAs. In some cases DHPAs have an effect on the absorption or metabolism of dietary constituents, but there is no observable impact on the toxicity of the DHPA.

Interrelationships between minerals and DHPAs are of particular importance. In Australia, enzootic copper poisoning was observed in areas where the copper concentration of the forage was normal (Cheeke, *et al.* 2011). Consumption of DHPA containing plants (*Heliotropium europaeum* and *Echium lycopsis*) predisposed sheep to the hemolytic effects of chronic copper poisoning (Bull, *et al.* 1956). The relationship between DHPA consumption and copper toxicosis in sheep is now well documented (Cheeke, *et al.* 2011). Copper accumulation appears to be related to both the intakes of copper and DHPA and according to Cheeke *et al.* (2011) probably also dependent on the

pattern of DHPA intake. Pair-fed rats were given a diet containing 250 ppm added copper with and without 5% *Senecio jacobea* (Swick *et al.* 1982). At five weeks the rats fed *S. jacobea* had a 7.5 fold increase in hepatic copper concentration when compared to controls. Serum zinc concentration was decreased in the same animals. Another study also using *S. jacobea* in sheep concluded that sheep fed *S. jacobea* do not preferentially accumulate copper in their livers, but that interactions with molybdenum were responsible for increased sensitivity to Tansy ragwort (White, *et al.* 1984b).

Dietary proteins can have the opposite effect on DHPA-induced toxicity. Cheeke and Garmin (1974) found that toxic effects of DHPAs were increased with restricted dietary protein. They observed no gross signs of DHPA toxicity in rats fed 25% dietary protein, but rats fed only 8% dietary protein exhibited severe signs of toxicosis even though their consumption of *S. jacobea* was only 68% of the amount consumed by the high protein group (Cheeke, *et al.* 2011). The protective effects were specifically associated with sulfur containing amino acids. In a later study which compared the effects of cysteine and methionine in rats fed *S. jacobea*, it was determined that the protective effects were due primarily to cysteine (Buckmaster, *et al.* 1976). Rats fed 1% cysteine had double the survival time of controls, and they consumed 300% more total plant resulting in 3X higher DHPA doses. There was no perceptible advantage in those fed methionine. Partial protection against DHPA toxicity by cysteine supplementation has been shown in a number of studies (Buckmaster, *et al.* 1976; Garrett, *et al.* 1984; Miranda, *et al.* 1981). It is presumed that the protective effects of cysteine are due to its effects as a constituent of glutathione (Cheeke, *et al.* 2011). Glutathione conjugates

DHPAs and DHPA metabolites for excretion as mercaptans (Robertson, *et al.* 1977). Simultaneously administering synthetic antioxidants has been found to potentiate the protective effect of dietary cysteine (Cheeke, *et al.* 2011).

The studies demonstrating protective effects of cysteine were carried out in laboratory animals and results may be different in other species. When ponies were fed *S. jacobea* along with a 1% cysteine and .75% butylated hydroxyanisole (a synthetic antioxidant), no beneficial effect was noted (Garrett, *et al.* 1984). A similar study a diet providing methionine hydroxyl analog (a source of rumen nondegradable sulfur amino acid) and ethoxyquin did not alter the toxicity of *S. jacobea* in Hereford steers (Cheeke, *et al.* 1985).

Although in some situations a high protein diet can be protective against DHPA intoxication, such a diet can exacerbate clinical signs in an animal that has already been poisoned (Cheeke, *et al.* 2011). This is particularly true with respect to animals with hepatic encephalopathy. Hepatic encephalopathy is the result of high blood ammonia concentrations affecting the central nervous system. Hyperammonemia is the result of impaired ability of the liver to convert ammonia (an amino acid degradation product) to urea. Consequently, a high protein diet is contraindicated in such situations.

Other nutrients affected by DHPA toxicosis include vitamins A and E and possibly B12. It is not surprising that hepatotoxic agents such as DHPAs effect metabolism of nutrients that are primarily metabolized or stored in the liver such as vit. A. In rats fed *Senecio jacobea*, liver and plasma concentrations of vitamin A were reduced to 50% that of control rats (Moghaddam and Cheeke 1989). Similarly, Vitamin E concentrations

were decreased in broiler chickens fed *Senecio jacobea* (Lulay, *et al.* 2007). This group concluded that the decrease was likely due to reduction in Vitamin E transport proteins. Some reports suggest a possible beneficial effect of vitamin B12 with respect to ruminal detoxification of DHPAs (Cheeke, *et al.* 2011), but no beneficial effect has been shown in other studies when vitamin B12 has been added to diets of ponies (Garrett, *et al.* 1984) and cattle (Cheeke, *et al.* 1985).

## **Compound**

### *Dose, rate*

Edgar *et al.* (2011) point out that DHPA exposures in pigs, poultry, rodents and non-human primates as well as in human cases, resulted in pulmonary hypertension followed by right heart failure with limited liver damage. They present an argument that if the liver has sufficient capacity for repair, it is likely that at some low-doses with slow rates of exposure that liver lesions would be minimal while lung damage could progress until the main lesions seen would be confined to the pulmonary vascular system (Edgar, *et al.* 2011). Intermittent exposure to DHPAs has been shown to allow recovery from antimitotic effects and allow for repair processes to start (Edgar, *et al.* 2011; Hirono, *et al.* 1978; Svoboda and Reddy 1972). Once free from the antimitotic effects, those diseases that require cellular proliferation (such as neoplasia) begin to develop (IPCS 1988; Jago, *et al.* 1969; Mattocks 1986). As a result, Edgar *et al.* (2011) suggests that sporadic small doses, such as those known to exist in foods, may be expected to produce cancer and pulmonary hypertension rather than liver damage (Edgar, *et al.* 2011).

### *Chemical Structure*

There are certain structural features necessary for a DHPA to be potentially hepatotoxic. They must have an unsaturated ring and at least one hydroxyl group attached to the pyrrolizine ring by one carbon (Mattocks 1986). At least one hydroxyl must be esterified, and the acid moiety needs a branched chain. Diesters tend to be more toxic than monoesters (Culvenor, *et al.* 1976; Mattocks 1986).

Once these minimum requirements have been met, there are several other factors that appear to determine the relative toxicity of individual DHPAs. These factors include lipid solubility, base strength, the acid moiety, the necine moiety, and the total alkaloid structure. These factors are often interdependent.

DHPAs which are more lipid soluble are more susceptible to activation in the liver, whereas DHPAs with higher water solubility are more readily excreted and consequently have a lower toxicity (Mattocks 1986). Base strength is closely related to lipophilicity. Generally those DHPAs which are weakly basic tend to be more lipophilic and as a result they also tend to be among the more toxic DHPAs

As noted above, all hepatotoxic DHPAs are esters, or have an acid moiety, and those with two ester groups are substantially more toxic than those with one (Culvenor, *et al.* 1976; Mattocks 1981). Mattocks (1986) hypothesized that the reason for this increase in toxicity is likely a combination of increased lipophilicity as well as greater resistance to hydrolysis. Because hydrolysis increases water solubility and consequently increases the rate of excretion, it is considered a detoxification pathway. Therefore, resistance to hydrolysis enhances the toxicity of DHPAs (Mattocks 1986). Hydrolysis is inhibited by

steric hindrance, which in the case of DHPAs, can be due to bulky carbon groups, acyl substituents,  $\alpha$ ,  $\beta$ -unsaturation in the acid moiety, or two acid moieties that are sufficiently close together (Mattocks 1986). Additionally, the structure of the acid moiety may also effect chemical changes, such as the opening of the second ester linkage in a macrocyclic diester (after hydrolysis of the primary ester) (Mattocks 1986).

The necine moiety must have an unsaturated (3-pyrroline ring) with associated hydroxyl group(s). The location of the hydroxyl groups is of significant importance. As previously mentioned two closely associated ester groups can inhibit hydrolysis, increasing the potential toxicity of the DHPA (Mattocks 1986). Similar to the discussion of the acid and necine moieties, the total alkaloid structure and conformation largely determine the balance between different routes of metabolism, specifically hydrolysis, N-oxidation, and pyrrole formation by controlling access to necessary enzymes.

Culvenor *et al.* (1976) tested 62 DHPAs and DHPA derivatives on 14 day old rats using a single IP injection. In this study they made multiple conclusions with regard to hepatotoxicity of DHPAs. First they determined that diesters of heliotridine and retronecine were approximately four times as toxic as the respective monoesters. Heliotridine esters were two to four times as toxic as retronecine esters (Culvenor, *et al.* 1976). They also found that most compounds that produced chronic liver lesions also produced chronic lung lesions (although in some cases a higher dose was required).

Mattocks and Bird (1983) tested 13 DHPAs using hepatic microsomes from phenobarbitone-pretreated rats. Their results did not support the hypothesis that heliotridine esters are generally more hepatotoxic than retronecine esters. They

concluded that the structure of the acid moiety was likely to have at least as much influence on toxicity as the configuration of the base (Mattocks and Bird 1983).

N-oxides are hepatotoxic but less so than the free base alkaloids when administered intraperitoneally in experimental mammals. There may be no difference, however, following application PO, because microorganisms contribute to the reduction of N-oxides in the gastrointestinal tract of rodents.(Frei, *et al.* 1992a)

### Human Exposure and Disease

Human exposure has recently been reviewed in two articles (Edgar, *et al.* 2011; Molyneux, *et al.* 2011). Exposure in humans differs from animals, particularly grazing animals. In humans exposure tends to be due to contamination of foodstuffs such as cereal grains, milk, eggs, honey and pollen (Edgar, *et al.* 2011) or the result of consumption of DHPA containing herbal remedies (Roeder 1995). Molyneux *et al.* (2011) emphasizes this point, because of the potential difference in resulting clinical signs. In a given herd or flock of animals, exposure is generally a point source such as a particular species of poisonous plant. Humans on the other hand may be exposed to small amounts of DHPAs from multiple sources and therefore one might expect to find quite a variation in exposure in a given population due to dietary preferences. Because of this difference in exposure, one might expect the majority of animals in an affected herd to show clinical signs. Conversely, in a human population few may consume a sufficient dose at a rate that will be overtly toxic. Others may consistently consume a small dose over a long period of time. Still others may be exposed to low-doses in a sporadic nature. This variation can be further compounded by the use of DHPA containing herbal

remedies (Molyneux, *et al.* 2011). As a result it can be exquisitely challenging to understand the true impact of DHPAs on human populations.

In less developed countries in arid environments, diets often rely on a single source of grain which may be the major source of nutrition for the entire population for a period of time. Such situations have resulted in exposure more similar to what is observed in grazing livestock where a large portion of the population is affected. Several large point source outbreaks affecting humans have occurred and are well documented (Molyneux, *et al.* 2011; Roeder 1995).

## **Food Contamination**

### *Grain*

Large scale DHPA poisonings in humans are typically the result of contamination of staple grains by seeds or other parts of DHPA containing plants. These typically occur in developing countries with arid climates resulting in heavy infestation of fields with the more drought resistant weeds containing DHPAs (Molyneux, *et al.* 2011). When the wheat or other cereal grains are harvested they are contaminated with DHPAs, and the DHPAs are incorporated into the flour during milling.

Outbreaks resulting from cereal grain contamination have occurred in Africa, central Asia and India (Molyneux, *et al.* 2011). One particularly devastating outbreak, in which an estimated 35,000 people were exposed, occurred in northwestern Afghanistan in the 1970s following a two year drought (Mohabbat, *et al.* 1976). A clinicopathological study examined 7,200 people from affected villages and found that 22.6% of them had evidence of liver disease. The disease was typically fatal with death occurring 3 – 9



months after the onset of abdominal distension (Mohabbat, *et al.* 1976). The outbreak was caused by contamination of bread with seeds from *Heliotropium spp.* plants.

Another similar outbreak occurred in Tadjakistan in 1993 (Chauvin, *et al.* 1994). A military blockade resulted in a delay of the wheat harvest, allowing time for *Heliotropium lasiocarpium* to mature in the fields. Contaminated wheat was then distributed to the population who milled it and made bread. The result was 3,906 reported cases of hepatotoxicity with a case fatality rate of 1.3% (Chauvin, *et al.* 1994).

#### *Honey and Pollen*

Honey and pollen have been shown to be a source of DHPA exposure (Edgar, *et al.* 2011; Molyneux, *et al.* 2011). DHPA content in honey has been measured and reported by several investigators at concentrations ranging from 1 to over 3900  $\mu\text{g kg}^{-1}$  of honey (Edgar, *et al.* 2011). The primary source for DHPAs in honey has been determined to be pollen from DHPA containing plants such as *Echium vulgare* and species of *Senecio*, *Eupatorium*, *Heliotropium*, *Borago*, *Myosotis*, *Chromolaena*, *Petasites*, *Ageratum*, *Cynoglossum*, *Tussilago* and *Symphytum* (Edgar, *et al.* 2002). Undocumented sources of contamination probably exist as honey attributed to sources of plants that do not contain DHPAs have also been found to contain up to 800  $\mu\text{g}$  of DHPA  $\text{kg}^{-1}$  of honey (Edgar, *et al.* 2011).

Pollen can contain concentrations of DHPAs several thousand times higher than in honey (Edgar, *et al.* 2011). Floral pollen from *Echium vulgare* was found to contain from 8,000 to 14,000  $\mu\text{g kg}^{-1}$  and pollen from commercial sources was found to contain as much as 16,350  $\mu\text{g kg}^{-1}$  (Edgar, *et al.* 2011; Kempf, *et al.* 2010). Kempf *et al.* (2010)

calculated an approximate daily DHPA intake for pollen consumers based on 30% DHPA contamination of pollen and an average DHPA concentration of  $5.17 \mu\text{g g}^{-1}$ . They calculated the average DHPA consumption at  $15 \mu\text{g}$  (retronecine equivalents)  $\text{day}^{-1}$ . This group further compared this DHPA exposure to the maximum daily allowance from German authorities of  $1 \mu\text{g day}^{-1}$ , and concluded that individuals who regularly use pollen products (more than 6 weeks per year) are exposed to at least 300 times this recommended maximum daily dose (Kempf, *et al.* 2010).

### *Milk*

The ability of DHPAs to transfer into milk has been well documented (Candrian, *et al.* 1984; Deinzer, *et al.* 1982; Dickinson 1980; Dickinson, *et al.* 1976; Eastman, *et al.* 1982; Goeger, *et al.* 1982; Hoogenboom, *et al.* 2011; Johnson 1976; Lüthy, *et al.* 1983; Panariti, *et al.* 1997; Schoental 1959; White, *et al.* 1984a). The quantity of DHPA that transfers into milk tends to be quite low. Some researchers measure or estimate it as a percentage of the total DHPA administered to the lactating female in the neighborhood of 0.1% (Hoogenboom, *et al.* 2011) or 0.04% in 16 hours (Eastman, *et al.* 1982). Others have simply measured the DHPA concentration in the milk for example  $9.4$  to  $16.7 \mu\text{g } 100 \text{ mL}^{-1}$  of milk (Dickinson, *et al.* 1976). In these studies unfortunately there is no standard time point used, which makes it difficult to compare. In one study,  $32.4 \text{ mg}$  of  $^{14}\text{C}$ -seneciophylline was administered to sheep daily for five days (Panariti, *et al.* 1997). The maximum concentration in the milk ( $987 \text{ ng mL}^{-1}$ ) was higher than the maximum concentration in the blood ( $518 \text{ ng mL}^{-1}$ ).

In order to determine the toxic potential of DHPAs transferred in milk, models ranging from the Salmonella/mammalian microsome test (White, *et al.* 1984a), through *Drosophila melanogaster* (Candrian, *et al.* 1984) to rats (Goeger, *et al.* 1982; Johnson 1976) rat pups (Aston, *et al.* 1996; Schoental 1959) and calves (Goeger, *et al.* 1982; Johnson 1976) have been used. Milk from goats fed *S. jacobea* resulted in both negative and marginally positive results for different combinations of tester strains and microsome preparations using the Salmonella/mammalian microsome test (White, *et al.* 1984a). *Drosophila* fed with milk from rats fed senkirkine showed an increase in sex-linked recessive lethal mutations (Candrian, *et al.* 1984). Models using rat pups have demonstrated results attributed to DHPA intoxication such as accumulation of hepatic copper (Aston, *et al.* 1996) and death with severe liver lesions (Schoental 1959). Those using rats had mixed results ranging from swollen hepatocytes, biliary hyperplasia megalocytosis, fibrosis (Goeger, *et al.* 1982) to no gross or histologic abnormalities (Johnson 1976). Feeding milk from cows fed with DHPAs has not resulted in grossly or histologically detectable abnormalities (Goeger, *et al.* 1982; Johnson 1976) even when the cows were clinically intoxicated (Goeger, *et al.* 1982). The potential toxicity of DHPA metabolites, and their possible transfer to milk, has yet to be investigated and is therefore unknown (Hoogenboom, *et al.* 2011).

In a study exploring the hypothesis that a second xenobiotic agent is required with excess copper to produce “Indian Childhood Cirrhosis,” retrorsine was fed to female rats during the period when their pups were suckling (Aston, *et al.* 1996).

They measured changes in neonatal copper concentrations, serum ceruloplasmin concentrations and hepatic metallothionein concentrations. They found that those pups whose mother received retrorsine had significantly increased hepatic copper concentrations, decreased serum ceruloplasmin concentrations (interpreted to indicate a decline or failure of copper incorporation into the apo-protein), decreased hepatic metallothionein and serum albumin concentrations (again interpreted to indicate reduced protein synthesis) and reduced hepatic DNA (interpreted to indicate an increase in cell size with a decrease in cell number). This study suggests that even though the concentration of DHPA that transfers into milk tends to be low, it could still pose a threat to humans (particularly pregnant or nursing females).

#### *Eggs, Meat, and Vegetables*

DHPAs have been isolated from eggs at concentrations far exceeding German standards for DHPAs in herbal medicines (Edgar and Smith 2000). In this case, three flocks of laying hens were inadvertently poisoned with seeds from *Heliotropium europium* as well as lesser amounts of other DHPA containing plants. Concentrations of DHPAs ranged from  $1.15 \mu\text{g egg}^{-1}$  to  $9.7 \mu\text{g egg}^{-1}$ . Another group isolated europine, heliotridine, and senecionine from eggs of experimentally infected Japanese quail (Eröksüz, *et al.* 2008). Concentrations of these DHPAs varied depending on the doses given to the quail, but the range was between  $1.46 \mu\text{g g}^{-1}$  and  $19.05 \mu\text{g g}^{-1}$ .

Numerous meat producing livestock are exposed to DHPA containing plants each year, but it is unclear whether residues of DHPAs enter the human food chain in meat (Wiedenfeld, *et al.* 2008). Experiments using radiolabeled DHPAs have demonstrated

that the majority of radioactivity (80%) is eliminated within the first 24 hours (Candrian, *et al.* 1985; Eastman, *et al.* 1982). The remaining radioactivity is presumed to be in the form of DHPA adducts (Wiedenfeld, *et al.* 2008). The potential danger of adducts is not yet understood, but there is concern that these adducts can form in vivo reservoirs resulting in continued damage to adjacent cellular components as well as a source of subsequent exposure to reactive DHPA metabolites via secondary poisoning (Edgar, *et al.* 2011; Wiedenfeld, *et al.* 2008).

Leafy DHPA producing plants such as comfrey are sometimes used intentionally in salads (Edgar, *et al.* 2011). There are also reports of DHPA producing plants being found as unintentional contaminants of foods such as salads and spices (Edgar, *et al.* 2011; Molyneux, *et al.* 2011). In one case a family used DHPA contaminated spices liberally during a time when the mother was pregnant (Rasenack, *et al.* 2003). The baby was delivered pre-term via cesarean section suffering from hepatic veno-occlusive disease, and subsequently died.

### *Herbal Medicines*

A complete review of DHPA toxicity with relation to herbal medicine is far beyond the scope of this review. The following examples are intended to provide some insight into what is a much broader and more complex situation than can be fully explored here. There are an abundance of case reports in multiple countries with different ages and genders of patients. Invariably, those responsible for a patient taking a medicine/herb intended for the medicine/herb to have a beneficial effect on the patient.

As has been shown with other animal species, the very young tend to be the most susceptible in humans as well. A five day old infant was referred to a neonatal intensive care unit with jaundice, hepatomegaly and ascites (Roulet, *et al.* 1988). The cause was determined to be consumption of herbal tea containing senecionine by the mother during pregnancy. In this case the total DHPA consumption was calculated to be  $0.6 \text{ mg kg}^{-1}$ . There is no report of clinical signs in the mother, but the infant died.

Adults tend to be less susceptible, but have also been poisoned by consumption of herbal medicines. Four young Chinese women used “Indian” herbal tea as a treatment for psoriasis (Kumana, *et al.* 1985). Three developed symptoms of veno-occlusive disease (VOD) including ascites and hepatomegaly as well as serum biochemical abnormalities within 19 to 45 days. The remaining patient stopped taking it after 21 days due to the development of a skin rash. The average DHPA consumption of the three women was estimated to be  $18 \text{ mg kg}^{-1}$ . One of the three women died.

Any DHPA containing plant has the potential to result in poisoning, but due to frequency of use some are more commonly reported than others. Comfrey has been the source of poisoning in multiple reports. A thirteen year old boy developed hepatomegaly and ascites after taking comfrey tea as a treatment for Crohn’s disease (Weston, *et al.* 1987), and a 47 year old woman also developed VOD as a result of consuming comfrey tea (Bach, *et al.* 1989). DHPA containing plants have been and continue to be common in various herbal teas, consumption of which has resulted in adverse clinical signs in humans from infants (Sperl, *et al.* 1995) to adults (Ridker, *et al.* 1985).

## **Associated Diseases**

### *Hepatic Veno-occlusive Disease (Sinusoidal Obstructive Syndrome)*

One of the most common clinical signs in the large point source outbreaks is hepatic VOD, more recently termed sinusoidal obstructive syndrome (SOS). In the 1993 Tadjakistan outbreak four different stages of illness were described (Chauvin, *et al.* 1994). The first stage consisted of abdominal pain, nausea or vomiting, and asthenia. Patients suffering from stage one symptoms universally recovered. Stage two consisted of stage one symptoms with the addition of hepatomegaly. Patients who developed ascites in addition to stage two symptoms were classified as stage three. Stage four included all previous symptoms as well as altered consciousness (Chauvin, *et al.* 1994).

VOD is the result of toxic injury to sinusoidal endothelium (Crawford 2004), and is characterized clinically by hepatomegaly, ascites, weight gain and jaundice (Chen and Huo 2010). The injured cells round up and slough off the sinusoidal wall, resulting in downstream embolization and obstruction of sinusoidal blood flow. It is characterized histologically by obliteration of the hepatic vein radicals with various amounts of reticulated collagen. Acutely there is profound centrilobular congestion and hepatocellular necrosis. Over time there is progressive obliteration of the lumen of the hepatic venule. Chronic lesions are characterized by fibrosis radiating out into the parenchyma, with frequent total obliteration of the venule. At this point congestion has been replaced by hemosiderin deposition (Crawford 2004).

### *Other Associated Diseases/Syndromes*

The liver is the major target organ for acute DHPA toxicity and in humans primarily results in sinusoidal obstructive syndrome as described above. Other diseases/syndromes associated with DHPA toxicity include pulmonary hypertension and various forms of cancer. Neither of these has been definitively associated with DHPA exposure in humans up to this point. However, there is at least one case of pulmonary hypertension where DHPA exposure was present and suspected as the possible cause (Gyorik and Stricker 2009). As described previously DHPAs (riddelliine and lasiocarpine) have been listed by the IARC as class 2b carcinogens (IARC 1987; 2002).

Exposure to monocrotaline as well as fulvine have both been associated with pulmonary hypertension particularly in rats (Kay, *et al.* 1971; Wilson, *et al.* 1992). Monocrotaline has also been associated with pulmonary arterial hypertrophy and cor pulmonale in young stump tailed macaques (Allen and Chesney 1972). Monocrotaline pyrrole injected IV into rats produced pulmonary hypertension and right heart hypertrophy in vivo and resulted in DNA cross-linking of pulmonary endothelial cells in vitro (Wagner, *et al.* 1993). Donkeys fed *C. retusa* as well as *C. juncea* developed pulmonary lesions including interstitial fibrosis and Clara cell proliferation (Pessoa, *et al.* 2013).



## References

- Allen JR, Carstens LA, Norback DH, Loh PM. 1970. Ultrastructural and biochemical changes associated with pyrrolizidine-induced hepatic megalocytosis. *Cancer Res.* 30:1857-1866.
- Allen JR, Chesney CF. 1972. Effect of age on development of cor pulmonale in nonhuman primates following pyrrolizidine alkaloid intoxication. *Exp Mol Pathol.* 17:220-232.
- Allen JR, Chesney CF, Frazee WJ. 1972. Modifications of pyrrolizidine alkaloid intoxication resulting from altered hepatic microsomal enzymes. *Toxicol Appl Pharmacol.* 23:470-479.
- Allen JR, Hsu IC. 1974. Antimitotic effects of dehydroretronecine pyrrole. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine* 147:546-550.
- Allen JR, Hsu IC, Carstens LA. 1975. Dehydroretronecine-induced rhabdomyosarcomas in rats. *Cancer Res.* 35:997-1002.
- Ames MM, Miser JS, Smithson WA, Coccia PF, Hughes CS, Davis DM. 1982. Pharmacokinetic study of indicine N-oxide in pediatric cancer patients. *Cancer Chemoth Pharm.* 10:43-46.
- Anjos BL, Nobre VM, Dantas AF, Medeiros RM, Oliveira Neto TS, Molyneux RJ, Riet-Correa F. 2010. Poisoning of sheep by seeds of *Crotalaria retusa*: acquired resistance by continuous administration of low-doses. *Toxicon* 55:28-32. DOI: 10.1016/j.toxicon.2009.06.028.
- Araya O, Fuentealba IC. 1990. Chronic hepato-toxicity of *Senecio erraticus* in calves from two 50-day feeding periods in consecutive years. *Vet Hum Toxicol.* 32:555-557.
- Aston N, Morris P, Tanner S. 1996. Retrorsine in breast milk influences copper handling in suckling rat pups. *J Hepatol.* 25:748-755.
- Bach N, Thung SN, Schaffner F. 1989. Comfrey herb tea-induced hepatic veno-occlusive disease. *Am J Med.* 87:97-99.
- Baker DC, Pfister JA, Molyneux RJ, Kechele P. 1991. *Cynoglossum officinale* toxicity in calves. *J Comp Pathol.* 104:403-410.
- Baker DC, Smart RA, Ralphs M, Molyneux RJ. 1989. Hound's-tongue (*Cynoglossum officinale*) poisoning in a calf. *J Am Vet Med Assoc.* 194:929-930.

- Brink N. 1969. The mutagenic activity of the pyrrolizidine alkaloid heliotrine in *Drosophila melanogaster* II. Chromosome rearrangements. *Mutat Res.*:139-146.
- Bruggeman I, Van der Hoeven J. 1985. Induction of SCEs by some pyrrolizidine alkaloids in V79 Chinese hamster cells co-cultured with chick embryo hepatocytes. *Mutat Res Lett.* 142:209-212.
- Buckmaster GW, Cheeke PR, Shull LR. 1976. Pyrrolizidine alkaloid poisoning in rats: protective effects of dietary cysteine. *J Anim Sci.* 43:464-473.
- Bull L, Dick A, Keast J, Edgar G. 1956. An experimental investigation of the hepatotoxic and other effects on sheep of consumption of *Heliotropium europaeum* L.: Heliotrope poisoning of sheep. *Crop and Pasture Science* 7:281-332.
- Campbell J. 1956. VI.—An Investigation of the Hepatotoxic Effects in the Fowl of Ragwort (*Senecio jacobæa* Linn.), with special reference to the Induction of Liver Tumours with Seneciphylline. *Proceedings of the Royal Society of Edinburgh. Section B. Biology* 66:111-130.
- Campestrato VR, Graf U, Reguly ML, Rodrigues de Andrade HH. 1997. Recombinogenic activity of integerrimine, a pyrrolizidine alkaloid from *Senecio brasiliensis*, in somatic cells of *Drosophila melanogaster*. *Environ Mol Mutagen.* 29:91-97.
- Candrian U, Lüthy J, Graf U, Schlatter C. 1984. Mutagenic activity of the pyrrolizidine alkaloids seneciphylline and senkirkine in *Drosophila* and their transfer into rat milk. *Food Chem Toxicol.* 22:223-225.
- Candrian U, Lüthy J, Schlatter C. 1985. In vivo covalent binding of retronecine-labelled <sup>3</sup>H seneciphylline and <sup>3</sup>H senecionine to dna of rat liver, lung and kidney. *Chem Biol Interact.* 54:57-69.
- Chan P. 1993. NTP technical report on the toxicity studies of Riddelliine (CAS No. 23246-96-0) Administered by Gavage to F344 Rats and B6C3F1 Mice. Toxicity report series 27:1.
- Chan P. 2001. NTP Technical Report on Toxicology and Carcinogenesis Studies of Riddelliine, (CAS No. 23246-96-0) in F344/N Rats and B6C3F1 Mice. NTP TR 508, NIH Publication.
- Chan PC, Haseman JK, Prejean J, Nyska A. 2003. Toxicity and carcinogenicity of riddelliine in rats and mice. *Toxicol Lett.* 144:295-311.
- Chauvin P, Dillon J, Moren A. 1994. An outbreak of Heliotrope food poisoning, Tadjikistan, November 1992-March 1993]. *Santé (Montrouge, France)* 4:263.

- Cheeke P, Riet-Correa F, Pfister J, Schild A, Wierenga T. 2011. Nutritional implications of pyrrolizidine alkaloid toxicosis. In 8th International Symposium on Poisonous Plants (ISOPP8), João Pessoa, Paraíba, Brazil, May 2009. CABI; 163-174.
- Cheeke PR, Schmitz JA, Lassen ED, Pearson EG. 1985. Effects of dietary supplementation with ethoxyquin, magnesium oxide, methionine hydroxy analog, and B vitamins on tansy ragwort (*Senecio jacobea*) toxicosis in beef cattle. *Am J Vet Res.* 46:2179-2183.
- Chen T, Mei N, Fu PP. 2010. Genotoxicity of pyrrolizidine alkaloids. *J Appl Toxicol.:* JAT 30:183-196.
- Chen Z, Huo J-R. 2010. Hepatic veno-occlusive disease associated with toxicity of pyrrolizidine alkaloids in herbal preparations. *Neth J Med.* 68:252-260.
- Chesney CF, Allen JR. 1973. Resistance of the guinea pig to pyrrolizidine alkaloid intoxication. *Toxicol Appl Pharmacol.* 26:385-392.
- Chou MW, Fu PP. 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.
- Chou MW, Yan J, Nichols J, Xia Q, Beland FA, Chan PC, Fu PP. 2004. Correlation of DNA adduct formation and riddelliine-induced liver tumorigenesis in F344 rats and B6C3F1 mice. *Cancer Lett.* 207:119-125.
- Chung W-G, Buhler DR. 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.
- Clark A. 1976. Naturally occurring mutagens. *Mutation Research/Reviews in Genetic Toxicology* 32:361-374.
- Coulombe RA, Jr., Drew GL, Stermitz FR. 1999. Pyrrolizidine alkaloids crosslink DNA with actin. *Toxicol Appl Pharmacol.* 154:198-202.
- Craig AM, Latham C, Blythe L, Schmotzer W, O'Connor O. 1992. Metabolism of toxic pyrrolizidine alkaloids from tansy ragwort (*Senecio jacobea*) in ovine ruminal fluid under anaerobic conditions. *Appl Environ Microb.* 58:2730-2736.
- Crawford JM. 2004. Liver and Biliary Tract. In *Pathologic Basis of Disease*, Kumar V, Abbas A, Fausto N (eds). Elsevier Inc: Philadelphia; 119-120.
- Culvenor CC, Edgar JA, Jago MV, Qutteridge A, Peterson JE, Smith LW. 1976. Hepato- and pneumotoxicity of pyrrolizidine alkaloids and derivatives in relation to molecular structure. *Chem Biol Interact.* 12:299-324.

- Deinzer ML, Arbogast BL, Buhler DR, Cheeke PR. 1982. Gas chromatographic determination of pyrrolizidine alkaloids in goat's milk. *Anal Chem.* 54:1811-1814.
- Dickinson J. 1980. Release of pyrrolizidine alkaloids into milk. In Proceedings of the Western Pharmacology Society. 23:377-379.
- Dickinson J, Cooke M, King R, Mohamed P. 1976. Milk transfer of pyrrolizidine alkaloids in cattle. *J Am Vet Med Assoc.* 169:1192-1196.
- Dueker SR, Lamé MW, Jones AD, Morin D, Segall H. 1994. Glutathione conjugation with the pyrrolizidine alkaloid, jacobine. *Biochem Bioph Res Co.* 198:516-522.
- Duringer JM, Buhler DR, Craig AM. 2004. Comparison of hepatic in vitro metabolism of the pyrrolizidine alkaloid senecionine in sheep and cattle. *Am J Vet Res.* 65:1563-1572.
- Eastman D, Dimenna G, Segall H. 1982. Covalent binding of two pyrrolizidine alkaloids, senecionine and seneciphylline, to hepatic macromolecules and their distribution, excretion, and transfer into milk of lactating mice. *Drug Metab Dispos.* 10:236-240.
- Edgar JA, Colegate SM, Boppre M, Molyneux RJ. 2011. Pyrrolizidine alkaloids in food: a spectrum of potential health consequences. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment* 28:308-324.
- Edgar JA, Roeder E, Molyneux RJ. 2002. Honey from plants containing pyrrolizidine alkaloids: a potential threat to health. *J Agr Food Chem.* 50:2719-2730.
- Edgar JA, Smith LW. 2000. Transfer of pyrrolizidine alkaloids into eggs: food safety implications. In ACS Symposium series. ACS Publications; 118-128.
- Eröksüz Y, Çeribaşı AO, ÇEVİK A, Eroeksuez H, Tosun F, TAMER U. 2008. Toxicity of *Heliotropium dolosum*, *Heliotropium circinatum*, and *Senecio vernalis* in parental quail and their progeny, with residue evaluation of eggs. *Turk. J. Vet. Anim. Sci* 32:475-482.
- Estep J, Lame M, Morin D, Jones A, Wilson D, Segall H. 1991. [14C] Monocrotaline kinetics and metabolism in the rat. *Drug Metab Dispos.* 19:135-139.
- Fletcher MT, McKenzie RA, Blaney BJ, Reichmann KG. 2009. Pyrrolizidine alkaloids in *Crotalaria* taxa from northern Australia: risk to grazing livestock. *J Agr Food Chem.* 57:311-319.
- Frei H, Luthy J, Brauchli J, Zweifel U, Wurgler FE, Schlatter C. 1992. Structure/Activity Relationships of the Genotoxic Potencies of Sixteen Pyrrolizidien Alkaloids Assayed for the Induction of Somatic Mutation and

Recombination in Wing Cells of *Drosophila melanogaster*. Chemical-Biological Interactions 83:1-22.

- Fu PP, Chou MW, Churchwell M, Wang Y, Zhao Y, Xia Q, Gamboa da Costa G, Marques MM, Beland FA, Doerge DR. 2010. High-performance liquid chromatography electrospray ionization tandem mass spectrometry for the detection and quantitation of pyrrolizidine alkaloid-derived DNA adducts in vitro and in vivo. Chem Res Toxicol 23:637-652.
- Galey F. 1996. Disorders caused by toxicants. In Large Animal Internal Medicine, Smith BP (Ed.). Mosby: St. Louis, MO; 1878-1879.
- Garrett BJ, Holtan DW, Cheeke PR, Schmitz JA, Rogers QR. 1984. Effects of dietary supplementation with butylated hydroxyanisole, cysteine, and vitamins B on tansy ragwort (*Senecio jacobea*) toxicosis in ponies. Am J Vet Res. 45:459-464.
- Goeger DE, Cheeke PR, Schmitz JA, Buhler DR. 1982. Effect of feeding milk from goats fed tansy ragwort (*Senecio jacobea*) to rats and calves. Am J Vet Res. 43:1631-1633.
- Goeger DE, Cheeke PR, Schmitz JA, Buhler DR. 1982. Toxicity of tansy ragwort (*Senecio jacobea*) to goats. Am J Vet Res., 43:252-254.
- Green M, Muriel W. 1975. Use of repair-deficient strains of *Escherichia coli* and liver microsomes to detect and characterise DNA damage caused by the pyrrolizidine alkaloids heliotrine and monocrotaline. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 28:331-336.
- Griffin D, Segall H. 1986. Genotoxicity and cytotoxicity of selected pyrrolizidine alkaloids, a possible alkenal metabolite of the alkaloids, and related alkenals. Toxicol Appl Pharmacol. 86:227-234.
- Gyorik S, Stricker H. 2009. Severe pulmonary hypertension possibly due to pyrrolizidine alkaloids in polyphytotherapy. Swiss Med Wkly. 139:210-211.
- Habs H, Habs M, Marquardt H, Röder E, Schmähl D, Wiedenfeld H. 1982. Carcinogenic and mutagenic activity of an alkaloidal extract of *Senecio nemorensis* ssp. *fuchsii*. ARZNEIM.-FORSCH. 32:144-148.
- Hincks JR, Kim HY, Segall HJ, Molyneux RJ, Stermitz FR, Coulombe RA, Jr. 1991. DNA cross-linking in mammalian cells by pyrrolizidine alkaloids: structure-activity relationships. Toxicol Appl Pharmacol. 111:90-98.
- Hirono I, Haga M, Fujii M, Matsuura S, Matsubara N, Nakayama M, Furuya T, Hikichi M, Takanashi H, Uchida E. 1979. Induction of hepatic tumors in rats by senkirkin and symphytine. J Natl Cancer I. 63:469-472.

- Hirono I, Mori H, Culvenor CC. 1976. Carcinogenic activity of coltsfoot, *Tussilago farfara* L. *Gann* 67:125-129.
- Hirono I, Mori H, Haga M. 1978. Carcinogenic activity of *Symphytum officinale*. *J Natl Cancer I.* 61:865-869.
- Hirono I, Ueno I, Aiso S, Yamaji T, Haga M. 1983. Carcinogenic activity of *Farfugium japonicum* and *Senecio cannabifolius*. *Cancer Lett.* 20:191-198.
- Hong H, Ton T, Devereux T, Moomaw C, Clayton N, Chan P, Dunnick J, Sills R. 2003. Chemical-specific alterations in *ras*, *p53*, and  $\beta$ -catenin genes in hemangiosarcomas from B6C3F1 mice exposed *too*-nitrotoluene or riddelliine for 2 years. *Toxicol Appl Pharmacol.* 191:227-234.
- Hoogenboom L, Mulder PP, Zeilmaker MJ, van den Top HJ, Remmelink GJ, Brandon EF, Klijnstra M, Meijer GA, Schothorst R, Van Egmond HP. 2011. Carry-over of pyrrolizidine alkaloids from feed to milk in dairy cows. *Food Additives & Contaminants: Part A* 28:359-372.
- Hooper, P. 1975. "Experimental acute gastrointestinal disease caused by the pyrrolizidine alkaloid, lasiocarpine." *J Comp Pathol.* 85: 341-349.
- Hooper P. 1978. Pyrrolizidine alkaloid poisoning. Pathology with particular reference to differences in animal and plant species. In *Joint United States-Australian Symposium on Poisonous Plants*, Logan, Utah (USA), 19-24 Jun 1977. Academic Press.
- Hooper P, Scanlan W. 1977. *Crotalaria retusa* poisoning of pigs and poultry. *Aust Vet J.* 53:109-114.
- Huan J. 1995. Species difference in bioactivation and detoxification of pyrrolizidine (senecionine) alkaloids. *Toxicol Lett.*, 99: 127-137.
- Huan JY, Miranda CL, Buhler DR, Cheeke PR. 1998a. The roles of CYP3A and CYP2B isoforms in hepatic bioactivation and detoxification of the pyrrolizidine alkaloid senecionine in sheep and hamsters. *Toxicol Appl Pharmacol.* 151:229-235. DOI: 10.1006/taap.1998.8482.
- Huan JY, Miranda CL, Buhler DR, Cheeke PR. 1998b. Species differences in the hepatic microsomal enzyme metabolism of the pyrrolizidine alkaloids. *Toxicol Lett.* 99:127-137.
- IARC. 1987. IARC monographs on the evaluation of carcinogenic risks to humans. Lyon, France. 10 supplement 7.
- IARC. 2002. IARC monographs on the evaluation of carcinogenic risks to humans. Lyon, France. 82.

- International Programme on Chemical Safety (IPCS). 1988. Pyrrolizidine alkaloids. Environmental Health Criteria 80. Geneva (Switzerland): WHO; Available from: <http://www.inchem.org/documents/ehc/ehc/ehc080.htm/> [Accessed 2010 Apr 9].
- Jago MV, Edgar JA, Smith LW, Culvenor CC. 1970. Metabolic conversion of heliotridine-based pyrrolizidine alkaloids to dehydroheliotridine. *Mol Pharmacol.* 6:402-406.
- Jago MV, Lanigan GW, Bingley JB, Piercy DW, Whitem JH, Titchen DA. 1969. Excretion of the pyrrolizidine alkaloid heliotrine in the urine and bile of sheep. *J Pathol.* 98:115-128.
- Johnson A, Molyneux R, Ralphs M. 1989. *Senecio*: a dangerous plant for man and beast. *Rangelands.* 261-264.
- Johnson AE. 1976. Changes in calves and rats consuming milk from cows fed chronic lethal doses of *Senecio jacobea* (tansy ragwort). *Am J Vet Res.* 37:107.
- Johnson AE, Molyneux RJ, Merrill GB. 1985. Chemistry of toxic range plants. Variation in pyrrolizidine alkaloid content of *Senecio*, *Amsinckia*, and *Crotalaria* species. *J Agr Food Chem.* 33:50-55.
- Kay JM, Heath D, Smith P, Bras G, Summerell J. 1971. Fulvine and the pulmonary circulation. *Thorax.* 26:249-261.
- Kempf M, Heil S, Haßlauer I, Schmidt L, von der Ohe K, Theuring C, Reinhard A, Schreier P, Beuerle T. 2010. Pyrrolizidine alkaloids in pollen and pollen products. *Mol Nutr Food Res.* 54:292-300.
- Kim HY, Stermitz FR, Li JK, Coulombe RA, Jr. 1999. Comparative DNA cross-linking by activated pyrrolizidine alkaloids. *Food Chem Toxicol.:* 37:619-625.
- Kim HY, Stermitz FR, Molyneux RJ, Wilson DW, Taylor D, Coulombe RA, Jr. 1993. Structural influences on pyrrolizidine alkaloid-induced cytopathology. *Toxicol Appl Pharmacol.* 122:61-69.
- Kovach JS, Ames MM, Powis G, Moertel CG, Hahn RG, Creagan ET. 1979. Toxicity and pharmacokinetics of a pyrrolizidine alkaloid, indicine N-oxide, in humans. *Cancer Res.* 39:4540-4544.
- Kuhara K, Takanashi H, Hirono I, Furuya T, Asada Y. 1980. Carcinogenic activity of clivorine, a pyrrolizidine alkaloid isolated from *Ligularia dentata*. *Cancer Lett.* 10:117-122.
- Kumana C, Ng M, Lin H, Ko W, Wu P-C, Todd D. 1985. Herbal tea induced hepatic veno-occlusive disease: quantification of toxic alkaloid exposure in adults. *Gut* 26:101-104.

- Lamé MW, Morin D, Jones AD, Segall H, Wilson DW. 1990. Isolation and identification of a pyrrolic glutathione conjugate metabolite of the pyrrolizidine alkaloid monocrotaline. *Toxicol Lett.* 51:321-329.
- Lanigan G. 1970. Metabolism of pyrrolizidine alkaloids in the ovine rumen. II. Some factors affecting rate of alkaloid breakdown by rumen fluid in vitro. *Crop and Pasture Science* 21:633-640.
- Lanigan G, Smith L. 1970. Metabolism of pyrrolizidine alkaloids in the ovine rumen. I. Formation of 7- $\alpha$ -hydroxy-1- $\alpha$ -methyl-8- $\alpha$  pyrrolizidine from heliotrine and lasiocarpine. *Crop and Pasture Science* 21:493-500.
- Lulay A, Leonard S, Traber M, Keller M, Cheeke P, Panter K, Wierenga T, Pfister J. 2007. Effects of dietary pyrrolizidine (*Senecio*) alkaloids on plasma and liver vitamin E distribution in broiler chickens. *Poisonous Plants: Global Research and Solutions*: 77-81.
- Lüthy J, Heim T, Schlatter C. 1983. Transfer of [<sup>3</sup>H] pyrrolizidine alkaloids from *Senecio vulgaris* and metabolites into rat milk and tissues. *Toxicol Lett.* 17:283-288.
- MacGregor JT, Wehr CM, Henika PR, Shelby MD. 1990. The *in vivo* erythrocyte micronucleus test: Measurement at steady state increases assay efficiency and permits integration with toxicity studies. *Fund Appl Toxicol.* 14:513-522.
- Martin PA, Thorburn MJ, Hutchinson S, Bras G, Miller CG. 1972. Preliminary findings of chromosomal studies on rats and humans with veno-occlusive disease. *Brit J Exp Pathol.* 53:374.
- Mattocks A. 1986. Chemistry and toxicology of pyrrolizidine alkaloids. Academic Press London.
- Mattocks AR. 1968. Toxicity of pyrrolizidine alkaloids. *Nature* 217:723-728.
- Mattocks AR. 1981. Relation of structural features to pyrrolic metabolites in livers of rats given pyrrolizidine alkaloids and derivatives. *Chem Biol Interact.* 35:301-310.
- Mattocks AR, Bird I. 1983. Pyrrolic and N-oxide metabolites formed from pyrrolizidine alkaloids by hepatic microsomes in vitro: relevance to in vivo hepatotoxicity. *Chem Biol Interact.* 43:209-222.
- Mattocks AR, Driver HE. 1987. Metabolism and toxicity of anacrotine, a pyrrolizidine alkaloid, in rats. *Chem Biol Interact.* 63:91-104.
- Mei N, Chou MW, Fu PP, Heflich RH, Chen T. 2004a. Differential mutagenicity of riddelliine in liver endothelial and parenchymal cells of transgenic big blue rats. *Cancer Lett.* 215:151-158.



- Mei N, Guo L, Fu PP, Heflich RH, Chen T. 2005. Mutagenicity of comfrey (*Symphytum officinale*) in rat liver. *Br J Cancer*. 92:873-875.
- Mei N, Heflich RH, Chou MW, Chen T. 2004b. Mutations induced by the carcinogenic pyrrolizidine alkaloid riddelliine in the liver cII gene of transgenic big blue rats. *Chem Res Toxicol*. 17:814-818.
- Mei X, Chen T. 2007. The mutant frequencies and types of mutations induced by comfrey in the lungs of transgenic big blue rats. *J Food Drug Anal*. 15:458.
- Miranda CL, Reed RL, Cheeke PR, Buhler DR. 1981. Protective effects of butylated hydroxyanisole against the acute toxicity of monocrotaline in mice. *Toxicol Appl Pharmacol*. 59:424-430.
- Miranda CL, Reed RL, Guengerich FP, Buhler DR. 1991. Role of cytochrome P450III A4 in the metabolism of the pyrrolizidine alkaloid senecionine in human liver. *Carcinogenesis*. 12:515-519.
- Mirsalis JC. 1987. In vivo measurement of unscheduled DNA synthesis and S-phase synthesis as an indicator of hepatocarcinogenesis in rodents. *Cell Biol Toxicol*. 3:165-173.
- Mirsalis JC, Steinmetz KL, Blazak WF, Spalding JW. 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.
- Moghaddam MF, Cheeke PR. 1989. Effects of dietary pyrrolizidine (*Senecio*) alkaloids on vitamin A metabolism in rats. *Toxicol Lett*. 45:149-156.
- Mohabbat O, Shafiq Younos M, Merzad A, Srivastava R, Ghaos Sediq G, Aram G. 1976. An outbreak of hepatic veno-occlusive disease in north-western Afghanistan. *Lancet*. 308:269-271.
- Molyneux RJ, Gardner DL, Colegate SM, Edgar JA. 2011. Pyrrolizidine alkaloid toxicity in livestock: a paradigm for human poisoning? *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment* 28:293-307.
- Molyneux RJ, Johnson AE, Stuart LD. 1988. Delayed manifestation of *Senecio*-induced pyrrolizidine alkaloidosis in cattle: case reports. *Vet Hum Toxicol*. 30:201-205.
- Muller-Tegethoff K, Kersten B, Kasper P, Muller L. 1997. Application of the in vitro rat hepatocyte micronucleus assay in genetic toxicology testing. *Mutat Res-Gen Tox En*. 392:125-138.

- Newberne P, Rogers A. 1973. Nutrition, monocrotaline, and aflatoxin B1 in liver carcinogenesis. *Plant Foods for Man* 1:23-31.
- Nobre V, Dantas A, Riet-Correa F, Barbosa Filho J, Tabosa I, Vasconcelos J. 2005. Acute intoxication by *Crotalaria retusa* in sheep. *Toxicon* 45:347-352.
- NTP. 2003. NTP Technical report on the toxicology and carcinogenesis studies of riddelliine (CAS NO. 23246-96-0) IN F344/N rats and B6C3F1 mice (gavage studies). Program NT (Ed.). US Department of Health and Human Services: North Carolina.
- Ord MJ, Herbert A, Mattocks AR. 1985. The ability of bifunctional and monofunctional pyrrole compounds to induce sister-chromatid exchange (SCE) in human lymphocytes and mutations in *Salmonella typhimurium*. *Mutat Res.* 149:485-493.
- Panariti E, Xinxo A, Leksani D. 1997. Transfer of <sup>14</sup>C-seneciphylline into sheep milk following multiple oral intakes. *DTW. Deutsche tierärztliche Wochenschrift* 104:97-98.
- Pass DA, Hogg GG, Russell RG, Edgar JA, Tence IM, Rikard-Bell L. 1979. Poisoning of chickens and ducks by pyrrolizidine alkaloids of *Heliotropium europaeum*. *Aust Vet J.* 55:284-288.
- Pereira TN, Webb RI, Reilly PE, Seawright AA, Prakash AS. 1998. Dehydromonocrotaline generates sequence-selective N-7 guanine alkylation and heat and alkali stable multiple fragment DNA crosslinks. *Nucleic Acids Res.* 26:5441-5447.
- Pessoa C, Pessoa A, Maia L, Medeiros R, Colegate S, Barros S, Soares M, Borges A, Riet-Correa F. 2013. Pulmonary and hepatic lesions caused by the dehydropyrrolizidine alkaloid-producing plants *Crotalaria juncea* and *Crotalaria retusa* in donkeys. *Toxicon*.
- Petry TW, Bowden GT, Huxtable RJ, Sipes IG. 1984. Characterization of hepatic DNA damage induced in rats by the pyrrolizidine alkaloid monocrotaline. *Cancer Res.* 44:1505-1509.
- Pierson ML, Cheeke PR, Dickinson EO. 1977. Resistance of the rabbit to dietary pyrrolizidine (*Senecio*) alkaloid. *Res Commun Chem Path.* 16:561-564.
- Rao M, Reddy J. 1978. Malignant neoplasms in rats fed lasiocarpine. *Br J Cancer.* 37:289.
- Rasenack R, Müller C, Kleinschmidt M, Rasenack J, Wiedenfeld H. 2003. Venooclusive disease in a fetus caused by pyrrolizidine alkaloids of food origin. *Fetal Diagn Ther.* 18:223-225.

- Reed R, Miranda C, Kedzierski B, Henderson M, Buhler D. 1992. Microsomal formation of a pyrrolic alcohol glutathione conjugate of the pyrrolizidine alkaloid senecionine. *Xenobiotica* 22:1321-1327.
- Ridker, P. M., S. Ohkuma, W. McDermott, C. Trey and R. Huxtable (1985). "Hepatic  
Ridker PM, Ohkuma S, McDermott W, Trey C, Huxtable R. 1985. Hepatic venoocclusive disease associated with the consumption of pyrrolizidine-containing dietary supplements. *Gastroenterology* 88:1050-1054.
- Rieben WK, Jr., Coulombe RA, Jr. 2004. DNA cross-linking by dehydromonocrotaline lacks apparent base sequence preference. *Toxicol Sci.* 82:497-503.
- Robertson K, Seymour J, Hsia M, Allen J. 1977. Covalent interaction of dehydroretronecine, a carcinogenic metabolite of the pyrrolizidine alkaloid monocrotaline, with cysteine and glutathione. *Cancer Res.* 37:3141-3144.
- Roeder E. 1995. Medicinal plants in Europe containing pyrrolizidine alkaloids. *Pharmazie* 50:83-98.
- Roeder E. 2000. Medicinal plants in China containing pyrrolizidine alkaloids. *Pharmazie* 55:711-726.
- Roulet M, Laurini R, Rivier L, Calame A. 1988. Hepatic veno-occlusive disease in newborn infant of a woman drinking herbal tea. *J Pediatr.* 112:433-436.
- Rubiolo P, Pieters L, Calomme M, Bicchi C, Vlietinck A, Vanden Berghe D. 1992. Mutagenicity of pyrrolizidine alkaloids in the *Salmonella typhimurium*/mammalian microsome system. *Mutat Res Lett.* 281:143-147.
- Sanderson B, Clark A. 1993. Micronuclei in adult and foetal mice exposed in vivo to heliotrine, urethane, monocrotaline and benzidine. *Mutat Res-Fund Mol M.* 285:27-33.
- Santos-Mello R, Deimling L, Lauer Júnior C, Almeida A. 2002. Induction of micronuclei by alkaloids extracted from *Senecio brasiliensis* and stored for 23 years. *Mutat Res-Gen Tox En.* 516:23-28.
- Schoental R. 1959. Liver lesions in young rats suckled by mothers treated with the pyrrolizidine (*Senecio*) alkaloids, lasiocarpine and retrorsine. *J Pathol Bacteriol.* 77:485-495.
- Schoental R. 1975. Pancreatic islet-cell and other tumors in rats given heliotrine, a monoester pyrrolizidine alkaloid, and nicotinamide. *Cancer Res.* 35:2020-2024.
- Schoental R, Fowler M, 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.

- Schoental R, Head MA. 1957. Progression of liver lesions produced in rats by temporary treatment with pyrrolizidine (*Senecio*) alkaloids, and the effects of betaine and high casein diet. *Br J Cancer*. 11:535.
- Schoental R, Head MA, Peacock P. 1954. *Senecio* alkaloids: primary liver tumours in rats as a result of treatment with (1) a mixture of alkaloids from *S. jacobea* lin.;(2) retrorsine;(3) isatidine. *Br J Cancer*. 8:458.
- Shull LR, Buckmaster GW, Cheeke PR. 1976. Factors influencing pyrrolizidine (*Senecio*) alkaloid metabolism: species, liver sulfhydryls and rumen fermentation. *J Anim Sci*. 43:1247-1253.
- Shumaker R, Robertson K, Hsu I, Allen J. 1976. Neoplastic transformation in tissues of rats exposed to monocrotaline or dehydroretronecine. *J Natl Cancer I*. 56:787-790.
- Silva-Neto J, Barreto R, Pitanga B, Souza C, Silva V, Silva A, Velozo E, Cunha S, Batatinha M, Tardy M. 2010. Genotoxicity and morphological changes induced by the alkaloid monocrotaline, extracted from *Crotalaria retusa*, in a model of glial cells. *Toxicon* 55:105-117.
- Smith LW, Culvenor CC. 1981. Plant sources of hepatotoxic pyrrolizidine alkaloids. *J Nat Prod*. 44:129-152.
- Sperl W, Stuppner H, Gassner I, Judmaier W, Dietze O, Vogel W. 1995. Reversible hepatic veno-occlusive disease in an infant after consumption of pyrrolizidine-containing herbal tea. *Eur J Pediatr*. 154:112-116.
- Stalker M, Hayes M. 2007. Liver and biliary system. Jubb, Kennedy, and Palmer's *Pathology of Domestic Animals* 2:297-388.
- Stegelmeier BL. 2011. Pyrrolizidine alkaloid-containing toxic plants (*Senecio*, *Crotalaria*, *Cynoglossum*, *Amsinckia*, *Heliotropium*, and *Echium spp.*). *Vet Clin N Am-Food A*. 27:419-428.
- Stegelmeier BL, Edgar JA, Colegate SM, Gardner DR, Schoch TK, Coulombe RA, Molyneux RJ. 1999. Pyrrolizidine alkaloid plants, metabolism and toxicity. *J Nat Toxins*. 8:95-116.
- Stegelmeier BL, Gardner DR, James LF, Molyneux RJ. 1996. Pyrrole detection and the pathologic progression of *Cynoglossum officinale* (houndstongue) poisoning in horses. *J Vet Diagn Invest*. 8:81-90.
- Svoboda DJ, Reddy JK. 1972. Malignant tumors in rats given lasiocarpine. *Cancer Res*. 32:908-913.

- Swick R, Cheeke P, Buhler D. 1982a. Subcellular distribution of hepatic copper, zinc and iron and serum ceruloplasmin in rats intoxicated by oral pyrrolizidine (*Senecio*) alkaloids. *J Anim Sci.* 55:1425-1430.
- Swick R, Cheeke P, Goeger D, Buhler D. 1982b. Effect of dietary *Senecio jacobea* and injected *Senecio* alkaloids and monocrotaline on guinea pigs. *J Anim Sci.* 55:1411.
- Tepe JJ, Williams RM. 1999. Reductive activation of a hydroxylamine hemiacetal derivative of dehydromonocrotaline: the first reductively activated pyrrolizidine alkaloid capable of cross-linking DNA. *Angewandte Chemie International Edition* 38:3501-3503.
- Uhl M, Helma C, Knasmüller S. 2000. Evaluation of the single cell gel electrophoresis assay with human hepatoma (Hep G2) cells. *Mutat Res-Gen Tox En.* 468:213-225.
- Wachenheim D, Blythe L, Craig A. 1992. Characterization of rumen bacterial pyrrolizidine alkaloid biotransformation in ruminants of various species. *Vet Hum Toxicol.* 34:513-517.
- Wagner J, Petry T, Roth R. 1993. Characterization of monocrotaline pyrrole-induced DNA cross-linking in pulmonary artery endothelium. *Am J Physiol-Lung C.* 264:L517-L522.
- Wang C, Li Y, Gao J, He Y, Xiong A, Yang L, Cheng X, Ma Y, Wang Z. 2011. The comparative pharmacokinetics of two pyrrolizidine alkaloids, senecionine and adonifoline, and their main metabolites in rats after intravenous and oral administration by UPLC/ESIMS. *Anal Bioanal Chem.* 401:275-287.
- Wang YP, Fu PP, Chou MW. 2005a. Metabolic activation of the tumorigenic pyrrolizidine alkaloid, retrorsine, leading to DNA adduct formation in vivo. *Int J Environ Res Public Health.* 2:74-79.
- Wang YP, Yan J, Fu PP, Chou MW. 2005b. Human liver microsomal reduction of pyrrolizidine alkaloid N-oxides to form the corresponding carcinogenic parent alkaloid. *Toxicol Lett.* 155:411-420.
- Wehner F, Thiel P, Van Rensburg S. 1979. Mutagenicity of alkaloids in the *Salmonella*/microsome system. *Mutat Res.* 66:187-190.
- Weidner MF, Sigurdsson ST, Hopkins PB. 1990. Sequence preferences of DNA interstrand cross-linking agents: dG-to-dG cross-linking at 5'-CG by structurally simplified analogs of mitomycin C. *Biochemistry* 29:9225-9233.
- Weston C, Cooper B, Davies J, Levine D. 1987. Veno-occlusive disease of the liver secondary to ingestion of comfrey. *Br Med J (Clin Res Ed).* 295:183.

- White IN, Mattocks AR, Butler WH. 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.
- White RD, Krumperman PH, Cheeke PR, Deinzer ML, Buhler DR. 1984a. Mutagenic responses of tansy ragwort (*Senecio jacobea*) plant, pyrrolizidine alkaloids and metabolites in goat milk with the Salmonella/mammalian-microsome mutagenicity test. *J Anim Sci.* 58:1245-1254.
- White RD, Swick RA, Cheeke PR. 1983. Effects of microsomal enzyme induction on the toxicity of pyrrolizidine (*Senecio*) alkaloids. *J Toxicol Environ Health A.* 12:633-640.
- White RD, Swick RA, Cheeke PR. 1984b. Effects of dietary copper and molybdenum on tansy ragwort (*Senecio jacobea*) toxicity in sheep. *Am J Vet Res.* 45:159-161.
- Whitson TD, Burrill LC, Dewey SA, Cudney DW, Nelson B, Lee RD, Parker R. 1996. *Weeds of the West.* Western Society of Weed Science.
- Wiedenfeld H, Erhard R, Bourauel T, Edgar J. 2008. *Pyrrolizidine Alkaloids Structure and Toxicity.* Bonn University Press: Gottingen.
- Williams D, Reed R, Kedzierski B, Dannan G, Guengerich F, Buhler D. 1989. Bioactivation and detoxication of the pyrrolizidine alkaloid senecionine by cytochrome P-450 enzymes in rat liver. *Drug Metab Dispos.* 17:387-392.
- Williams L, Chou MW, Yan J, Young JF, Chan PC, Doerge DR. 2002. Toxicokinetics of riddelliine, a carcinogenic pyrrolizidine alkaloid, and metabolites in rats and mice. *Toxicol Appl Pharmacol.* 182:98-104.
- Wilson D, Segall H, Pan L, Lame M, Estep J, Morin D. 1992. Mechanisms and pathology of monocrotaline pulmonary toxicity. *Crit Rev Toxicol.* 22:307-325.
- Winter C, Segall H, Jones A. 1988. Species differences in the hepatic microsomal metabolism of the pyrrolizidine alkaloid senecionine. *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* 90:429-433.
- Xia Q, Chou MW, Edgar JA, Doerge DR, Fu PP. 2006. Formation of DHP-derived DNA adducts from metabolic activation of the prototype heliotridine-type pyrrolizidine alkaloid, lasiocarpine. *Cancer Lett.* 231:138-145.
- Xia Q, Chou MW, Kadlubar FF, Chan PC, Fu PP. 2003. Human liver microsomal metabolism and DNA adduct formation of the tumorigenic pyrrolizidine alkaloid, riddelliine. *Chem Res Toxicol* 16:66-73.

- Xia Q, Chou MW, Lin G, Fu PP. 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.
- Xia Q, Ma L, He X, Cai L, Fu PP. 2015. "7-Glutathione Pyrrole Adduct-A Potential DNA Reactive Metabolite of Pyrrolizidine Alkaloids." *Chem Res Toxicol*.
- Xia Q, Yan J, Chou MW, Fu PP. 2008. Formation of DHP-derived DNA adducts from metabolic activation of the prototype heliotridine-type pyrrolizidine alkaloid, heliotrine. *Toxicol Lett.* 178:77-82.
- Xia Q, Zhao Y, Von Tungeln LS, Doerge DR, Lin G, Cai L, Fu PP. 2013. Pyrrolizidine Alkaloid-derived DNA Adducts as a Common Biological Biomarker of Pyrrolizidine Alkaloid-induced Tumorigenicity. *Chem Res Toxicol*.
- Yamanaka H, Nagao M, Sugimura T, Furuya T, Shirai A, Matsushima T. 1979. Mutagenicity of pyrrolizidine alkaloids in the Salmonella/mammalian-microsome test. *Mutat Res.* 68:211-216.
- Yan CC, Huxtable RJ. 1995. Relationship between glutathione concentration and metabolism of the pyrrolizidine alkaloid, monocrotaline, in the isolated, perfused liver. *Toxicol Appl Pharmacol.* 130:132-139.
- Yang YC, Yan J, Doerge DR, Chan PC, Fu PP, Chou MW. 2001. Metabolic activation of the tumorigenic pyrrolizidine alkaloid, riddelliine, leading to DNA adduct formation in vivo. *Chem Res Toxicol* 14:101-109.
- Yuzo H, Mariko S, Hiro K. 1977. Experimental insuloma in rats after a single administration of monocrotaline. *Toxicol Lett.* 1:41-44.
- Zhao Y, Xia Q, Gamboa da Costa G, Yu H, Cai L, Fu PP. 2012. Full structure assignments of pyrrolizidine alkaloid DNA adducts and mechanism of tumor initiation. *Chem Res Toxicol* 25:1985-1996.

## CHAPTER III

COMPARATIVE TOXICITY OF SELECT DEHYDROPYRROLIZIDINE  
ALKALOIDS AND N-OXIDES IN CHICKS (*GALLUS GALLUS DOMESTICUS*)

## Abstract

Dehydropyrrolizidine alkaloids (DHPAs) are a large, structurally diverse group of plant-derived pro-toxins that are potentially carcinogenic. With worldwide significance, these alkaloids can contaminate or be naturally present in the human food supply. Due to the difficulty in obtaining large quantities of purified DHPAs, the majority of research has been done using intraperitoneal injections in rodent models, which is often very different than results via normal exposure of ingestion. Furthermore, few studies compare more than a couple of DHPAs, consequently comparisons must be done between studies with differences in age, strain and dosing strategies. To assess the relative toxicity of structurally diverse DHPAs in a biologically relevant manner, chicks were dosed orally with 0.01, 0.04, 0.13 or 0.26 mmol of riddelliine, senecionine, seneciphylline, echimidine, lycopsamine, lasiocarpine, heliotrine, riddelliine N-oxide, senecionine N-oxide or lasiocarpine N-oxide  $\text{kg}^{-1}$  bodyweight for 10 days. After another 7 days, chicks were euthanized. Based on clinical, serum biochemical, and histopathological evaluations as well as “pyrrole” (a DHPA metabolite) tissue adduct accumulation rate, these different DHPAs are grouped in relation to their toxicity. In this model heliotrine, lasiocarpine, and the three macrocyclic diesters (seneciphylline, senecionine and riddelliine) appear to be most toxic followed by the three N-oxides



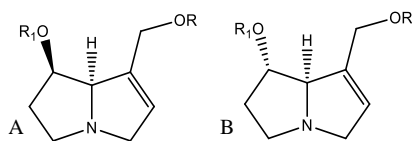
(riddelliine N-oxide, senecionine N-oxide and lasiocarpine N-oxide). Echimidine was next and lycopsamine appear to be the least toxic of the compounds tested in this model. This model is sensitive for comparison of DHPA toxicity, and data obtained from this research can be used to validate previous research in a more biologically relevant exposure.

## Introduction

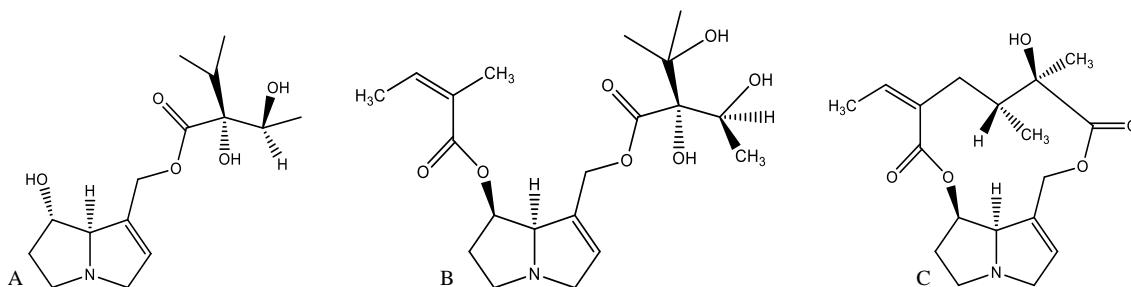
Dehydropyrrolizidine alkaloids (DHPAs) are a large and structurally diverse group of pro-toxins produced by plants assumedly as a protection against herbivory. Present worldwide (Stegelmeier, *et al.* 1999), and estimated to be produced by approximately three percent of flowering plants (Smith and Culvenor 1981); they are likely the most economically important plant toxins. Not only do DHPAs effect livestock, poultry, swine and most likely wildlife, they are also present in numerous herbal preparations, and are potential contaminants of foods, such as grains (Chauvin, *et al.* 1994; Mohabbat, *et al.* 1976), eggs (Edgar and Smith 2000), honey (Edgar, *et al.* 2002), milk (Dickinson, *et al.* 1976), and occasionally fresh salads (Edgar, *et al.* 2011). After they are eaten and absorbed into the body they are metabolized, primarily by CYP3A family enzymes, producing highly reactive dihydropyrrolizine esters.

Even though reported natural exposure is exclusively oral, the vast majority of research devoted to determining the relative toxicity of DHPAs has been done by using a single intraperitoneal injection (IP) in rodents (Mattocks 1986). Although potentially useful as a baseline, data obtained by using this method presupposes that between different DHPAs there are no significant differences in absorption, no significant toxic effects produced by DHPAs prior to reaching the liver, and that absorption rates into the liver either are the same as oral exposure or the rate does not affect the toxicity. Additionally, different studies used relatively resistant rodents with different varieties/strains, strains, and sexes, making comparisons difficult. Using this single injection IP method it has been determined that DHPAs with the heliotridine base are

approximately two times more toxic than those with a retronecine base (Culvenor, *et al.* 1976) (Fig. 3-1). Side chains were also determined to affect the toxicity. Those with two ester linked side chains were found to be more toxic than those with a single ester linked side chain, and those with two ester side chains that connect with each other (macrocyclic diesters) were found to be more toxic than open chain diesters (Culvenor *et al.* 1976, Mattocks 1986) (Fig. 3-2). Based on these guidelines *Senecio douglasii* var *longilobus* (which contains primarily macrocyclic diesters) would be expected to be more toxic than *Cynoglossum officinale* (which contains a mixture of mono and open chain diesters). This does not correlate well with oral exposure studies where they have very similar toxicities. The LD50 for *Cynoglossum officinale* was less than 15 mg kg<sup>-1</sup> for 14 days in horses (Culvenor, *et al.* 1976; Mattocks 1986; Stegelmeier, *et al.* 1996) and cattle (Baker *et al.* 1989), whereas the LD50 for *Senecio douglasii* var *longilobus* was 13 mg kg<sup>-1</sup> for 15 days (Johnson and Molyneux 1984) when orally dosed to cattle. N-oxides are less hepatotoxic than their associated free base when administered IP in experimental animals, but there may be no difference when administered (Frei, *et al.* 1992b). Due to these shortcomings as well as some contradictory experience in the laboratory (Stegelmeier, unpublished data), it was determined that a more biologically relevant model was needed to test relative toxicity of DHPAs.



**Figure 3-1.** Necine bases (A) retronecine & (B) heliotridine



**Figure 3-2.** Necine bases with A. monoester (echinatine), B. diester (echimidine) and C. macrocyclic diester (senecionine) side chains.

Due to the difficulty of obtaining purified DHPAs it was essential that a small sensitive model would be necessary. Hooper (1978) reported the relative species sensitivity to DHPAs as follows: pigs = 1, chickens = 5, cattle and horses = 14, rats = 50, mice = 150, and sheep and goats = 200. It is also well documented that younger individuals are much more sensitive to DHPA intoxication than adults (Mattocks 1986), and that in at least some species males are more sensitive than females. As a result, we used male chicks as a model for DHPA toxicity testing.

## Materials and Methods

### Animals

Three-day-old male, California White chicks (*GALLUS GALLUS DOMESTICUS*) were purchased from Privett Hatchery (Portales, New Mexico) through Intermountain Farmers Association (Hyde Park, Utah). All animals were acclimated for three days prior to initiation of treatment. Chicks were housed in brooder cages that contained heaters, providing a thermal gradient within their microenvironment which ranged from roughly

16 to 25°C. All animals had free access to fresh water and 20% protein poultry starter purchased from Intermountain Farmers Association (Salt Lake City, Utah). The brooder cages were housed in a room with access to ambient sunlight. Because this study was divided into multiple sections and performed at various times throughout the year, room lights were left on if ambient light was less than 12 hours per day, such that the total lighting was at least 12 hours per day. Humidity in the brooder cages was essentially ambient humidity which ranged from approximately 30% to 60% during the course of this study. This research was conducted with the approval of the Utah State University animal care and use committee (IACUC Protocol #2055).

### **Purified Alkaloids**

All of the DHPAs and N-oxides used were from the USDA ARS Poisonous Plant Research Laboratory's collection of dehydropyrrolizidine alkaloids. They were all authenticated using liquid chromatography mass spectrometry and NMR, and estimated to be at least 98% pure.

### **Experimental Design**

Male, California White chicks were randomly divided into groups of five, assigned to a treatment group, and dose with one of the following: Riddelliine, riddelliine N-oxide, senecionine, senecionine N-oxide, seneciphylline, lasiocarpine, lasiocarpine N-oxide, heliotrine, lycopsamine, or vehicle control. Because these compounds differ in molecular weight, the dosages were calculated and dosed in mmols. The doses for all except those animals receiving lycopsamine, or vehicle control were 0.01, 0.04, 0.13, and

0.26 mmol kg<sup>-1</sup> body weight (BW) day<sup>-1</sup>. The vehicle control animals received a volume equivalent to the largest volume received by any group. Because previous IP studies suggested that lycopsamine is less toxic than the other DHPAs used in this study, the animals receiving lycopsamine received 0.04, 0.13, and 0.26 mmol kg<sup>-1</sup> BW day<sup>-1</sup>. Riddelliine is comparatively easier to extract and purify, has been widely studied and was tested for the pilot study for this project, hence riddelliine was used as a positive control and was dosed at 0.13 mmol kg<sup>-1</sup> BW day<sup>-1</sup> for each individual trial. Positive and vehicle control groups consisted of five chicks for each trial. All daily doses were divided into two equal portions, and water was added to all doses such that the total volume was 0.5 mL. One half of each daily dose was given in the morning and one half was given in the afternoon by gavage. Animals were weighed three times per week, and doses were recalculated immediately after weighing and dosages were adjusted ensuring proper treatment. All animals were dosed for 10 days after which they were maintained on the above mentioned commercial chick ration for the following seven days prior to euthanasia with carbon dioxide to allow histologic lesions to fully develop. Animals were monitored at least twice daily, and animals which became moribund were humanely euthanized and necropsied.

### **Tissue Preparation**

After euthanasia, necropsies were performed on all animals. Gross abnormalities were recorded, and tissues including the entire brain, heart, lung, right liver lobe, and spleen, and representative samples of kidney, testicle, crop, proventriculus, ventriculus, small intestine, colon, cecum, and bursa of Fabricius, were collected in 10% neutral

buffered formalin for histopathological analysis. The right liver lobe was sectioned randomly to facilitate morphometric study. This was done by using a random number chart to determine the start location (mm 0-3) and the entire right liver lobe was sectioned every 3 mm transversely along the long axis, laid left face down in the cassette and embedded, sectioned, fixed on glass slides and stained. Blood samples were taken immediately after euthanasia via cardiac puncture, divided into serum and packed cells, and frozen. The left liver lobe of the liver was collected and stored at -80 degrees C for subsequent analyses including DHPA adduct evaluation.

### **Hepatocellular Necrosis Scoring**

Due to the large variation in liver size between animals, all sections of the right liver lobe could occupy from one to four slides. Each slide was graded by examining all sections on that slide and scored for necrosis as follows: 0 - no observed hepatocellular necrosis; 1 - scattered individual cell death; 2 - multifocal areas of necrosis which encompassed up to 25% of the sections on that slide; 3 - larger areas of necrosis were present accounting for between 25 and 50% of the sections; and 4 - greater than 50% of the observed sections were necrotic (Fig. 3-3). The hepatic necrosis score for each animal was computed by taking the average of the necrosis scores for each slide of liver tissue from that animal.

### **Protein “Pyrrole” Adduct Detection**

Procedures for “pyrrole” adduct detection were identical to those described in Brown *et al.* 2015. Briefly, frozen liver lobes were freeze dried and crushed into fine

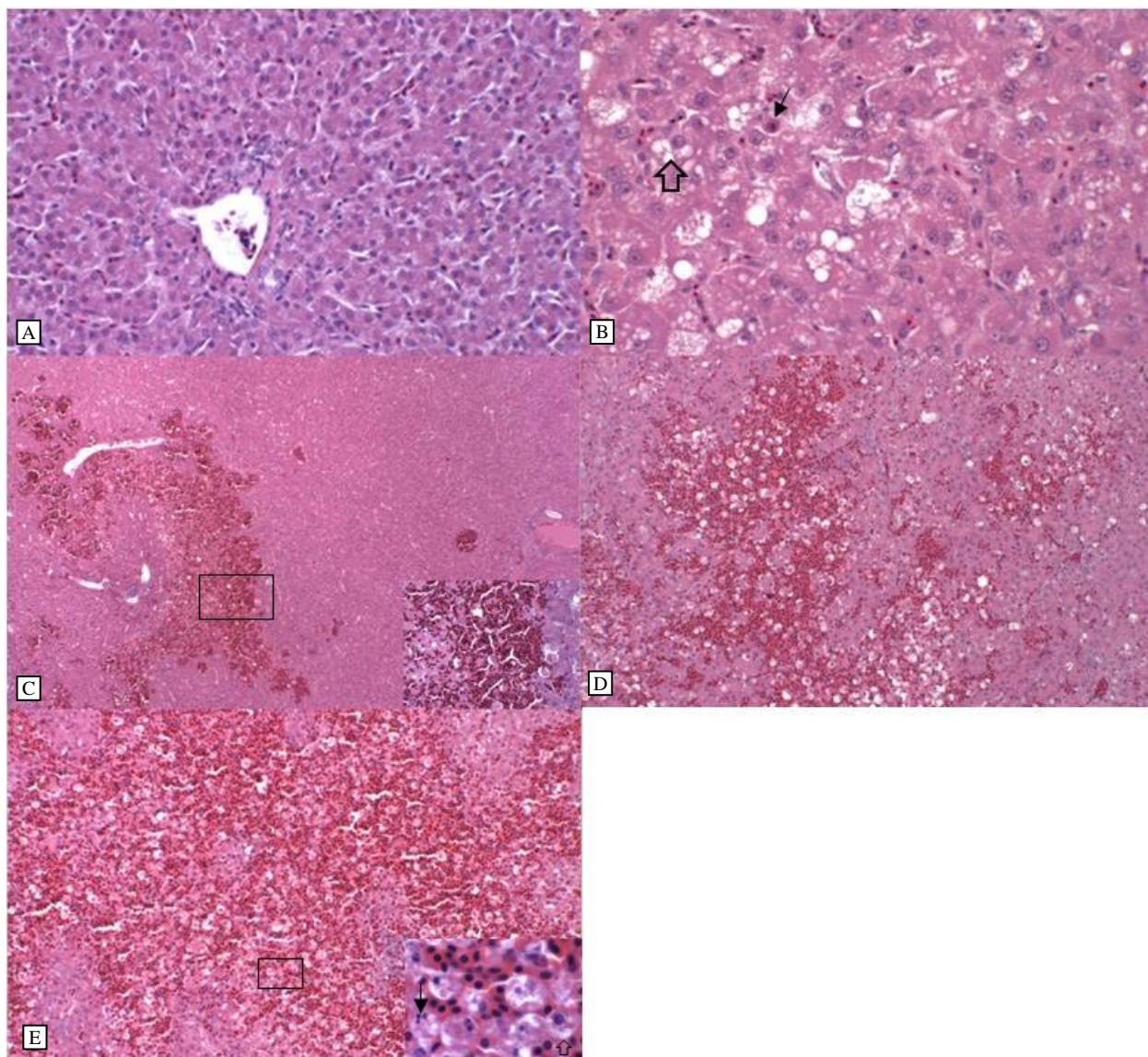
powder. A 25 mg portion of the crushed, freeze dried, chicken liver was mixed with ethanolic silver nitrate and trifluoroacetic acid overnight, centrifuged, mixed with Erlich's solution ( $\rho$ -Dimethylaminobenzaldehyde or DMABA), and analyzed with an HPLC-esi(+) MS/MS system. The pyrrole-DMABA compound eluted with a retention time of 4.4 min and the resulting MS/MS spectrum contained major fragment ions at  $m/z$  252 and 296. The detected "pyrrole" peak area was measured from the reconstructed ion chromatogram ( $m/z = 296$ ) and quantified based on an external calibration curve established from control standards. The resulting "pyrrole" concentration ( $\text{nmol mL}^{-1}$ ) of the injected sample was converted to  $\text{nmol g}^{-1}$  of liver.

#### *Reagents and solvents*

Monocrotaline (crotaline) (used to make the positive control standard), tetrachlorobenzoquinone (o-chloronil, 97%), boron trifluoride diethyl etherate ( $\text{BF}_3 \cdot \text{O}(\text{C}_2\text{H}_5)_2$ ) and silver nitrate were purchased from Sigma-Aldrich.  $\rho$ -Dimethylaminobenzaldehyde (DMABA, Erlich's reagent) was from ICN Biochemical (Cleveland, Ohio). Acetonitrile was HPLC grade (Sigma-Aldrich) and absolute ethanol was reagent grade. 0.1% formic acid for HPLC was prepared with Milli-Q-purified ( $18.2 \text{ M}\Omega \text{ cm}^{-1}$ ) water.

Ethanolic silver nitrate, "pyrrole" calibration standards, and Erlich's solution were prepared as described in Brown *et al.* 2015.





**Figure 3-3.** Sections of livers from chicks exposed to dehydropyrrolizidine alkaloids. A-E H&E. **A.** Hepatocellular necrosis score = 0 (Control) 200X **B.** Hepatocellular necrosis score = 1 There is a single hepatocytes with a pyknotic nucleus (arrow), and multiple hepatocytes are swollen with vacuolated cytoplasm (open arrow) (Riddelliine N-oxide  $0.04 \text{ mmol kg}^{-1}$ ) 200X. **C.** Hepatocellular necrosis score = 2 The inset shows the border of a necrotic area. Hepatocytes are surrounded by hemorrhage. (Lasiocarpine N-oxide  $0.13 \text{ mmol kg}^{-1}$ ) 40X with 200X insert. **D.** Hepatocellular necrosis score = 3 Larger areas of necrosis are present occupying (senecionine  $0.26 \text{ mmol kg}^{-1}$ ) 100X. **E.** Hepatocellular necrosis score = 4 The majority of this section is composed of necrosis and hemorrhage. The inset contains hepatocytes with fragmented, pyknotic nuclei (arrow) as well as individualized hepatocytes surrounded by hemorrhage (open arrow). (Heliotrine  $0.26 \text{ mmol kg}^{-1}$ ) 100X with 400X insert.

### **“Pyrrole” Accumulation Rate Analysis**

Comparison of “pyrrole” concentration between compounds and doses was challenging due to the fact that chicks died at different time points and consequently received different total amounts of DHPA and clearance times. In an effort to account for this difference, a comparison was performed by calculating a “pyrrole” accumulation rate. In any diagnostic test as sensitivity increases, the possibility of false positive results increases. In the present study, a trace amount of “pyrrole” was detected in two of the negative control animals. In order to account for this, the average negative control “pyrrole” concentration was subtracted from each individual “pyrrole” concentration to give a control adjusted “pyrrole” concentration. The control adjusted “pyrrole” concentration was then divided by the number of days the animal survived to account for the differences in total DHPA administered to each animal based on survival time. This control adjusted “pyrrole” accumulation rate was used in a log transformed model to predict the “pyrrole” accumulation rate for each alkaloid at each dose. The data shown in Table 3-3 was back transformed. This data should be used with caution because little is currently known about the potential clearance rate of the pyrrole from the liver. If chicks are able to remove the pyrrole from the liver at a rate relevant to the present study timeline, then this approach for comparison may not be appropriate.

### **Weight Analysis**

With the animal numbers used for each group, there was not sufficient power to determine differences in average daily gain between groups and doses. A repeated measures comparison of weights provides more statistical power. Directly comparing

actual weights would be the preferred method, but because chicks died at different time points throughout the study, this method would preclude comparisons at higher doses of more toxic compounds. In order to account for these challenges, repeated weights were used to create a model estimate for each compound at each dose at the average number of survival days (10 days). There was some difference in initial weights which can affect the weights as measured over time. Consequently, initial weight was used as a covariate. SAS 9.4 was used to create the model estimate. Controls for weight were randomly selected from all of the controls and assigned to each dose for comparison.

### **Serum Biochemistry Analysis**

Frozen serum was sent to a commercial laboratory (Antech Laboratories, Indianapolis, Indiana) for analysis of total protein, glucose, triglycerides, cholesterol, gamma glutamyl transferase (GGT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), sorbitol dehydrogenase (SDH), bile acid, and creatine phosphokinase (CPK). Principle component analysis of serum biochemistries was performed on standardized variables. Three components were used, which explain 77% of the variation in the model. The first component corresponds to hepatic metabolic products. The major contributing variables of the first component are total protein, glucose, cholesterol, and triglycerides. The second component corresponds to hepatocellular damage and is composed of the two hepatocellular leakage enzymes aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) along with a lesser contribution from bile acid. Principle component three is composed primarily of gamma glutamyl transferase (GGT) with a smaller contribution from bile acid. Controls for

principle component analyses were randomly selected from all of the controls and assigned to each dose for comparison.

### **Statistical Analysis**

All statistical analyses were performed using SAS version 9.4 in PROC GLIMIX except for the survival analysis which was performed using PROC LIFETEST. Hepatocellular necrosis scores were compared as multinomial outcomes with alkaloid and dose as fixed factors in the model. A survival analysis was performed using PROC LIFETEST with initial weight as a covariate.

## **Results**

### **Weight**

Within a given alkaloid group, model estimated chick weights decreased as dose increased for all alkaloids except for lycopsamine (Table 3-1). Within the two heliotridine base alkaloids (heliotrine and lasiocarpine), each successive dose resulted in a significant decrease in the projected ten day weight. An estimated weight for the heliotrine 0.26 mmol Kg<sup>-1</sup> group was not calculated because all animals in this group died within one day of initial dose. The three macrocyclic diesters (seneciophylline, senecionine, and riddelliine) all showed a difference between the two lower doses and the two upper doses. There was also a difference between the lower two doses in both senecionine and riddelliine. The three N-oxides (senecionine N-oxide, riddelliine N-oxide, and lasiocarpine N-oxide) animal groups began to become lighter at the 0.13 mmol kg<sup>-1</sup> dose and all three are significantly lighter at 0.26 mmol kg<sup>-1</sup> than at 0.13 mmol kg<sup>-1</sup>.

Echimidine is similar to the N-oxides in that the lower two dosed animal groups are heavier than the higher two doses. There is no difference however, between the two higher doses. There was no difference in weight projections between any doses of lycopsamine.

**Table 3-1.** Projected Weight comparison at the average number of survival days (10) for California White chicks exposed to dehydropyrrolizidine alkaloids. Weights were derived in a mixed model, using initial weight as a covariate. Superscript letters denote statistical differences within a row, and superscript numbers denote statistical differences within a column. A p-value of < 0.05 was considered significant. Explanations for abbreviations are as follows: CNT – control, HEL – heliotrine, LAS – lasiocarpine, SPH – seneciphylline, SEN – senecionine, RID – riddelliine, RNO – riddelliine N-oxide, SNO – senecionine N-oxide, LNO – lasiocarpine N-oxide, ECH – echimidine, LYC – lycopsamine, and NA - not available.

Dose (mmol kg <sup>-1</sup> )	CNT (g)	HEL (g)	LAS (g)	SPH (g)	SEN (g)	RID (g)	RNO (g)	SNO (g)	LNO (g)	ECH (g)	LYC (g)
0.01	105 <sup>abc</sup>	99 <sup>1bc</sup>	109 <sup>1ab</sup>	92 <sup>1c</sup>	92 <sup>1c</sup>	99 <sup>1bc</sup>	96 <sup>1bc</sup>	97 <sup>1bc</sup>	97 <sup>1bc</sup>	120 <sup>1a</sup>	NA
0.04	103 <sup>def</sup>	67 <sup>2a</sup>	84 <sup>2bc</sup>	78 <sup>1ab</sup>	77 <sup>2ab</sup>	78 <sup>2ab</sup>	92 <sup>1bcde</sup>	87 <sup>1bcd</sup>	97 <sup>1cdef</sup>	110 <sup>1f</sup>	108 <sup>ef</sup>
0.13	105 <sup>e</sup>	48 <sup>3ab</sup>	49 <sup>3ab</sup>	46 <sup>2a</sup>	59 <sup>3abc</sup>	53 <sup>3ab</sup>	73 <sup>2bc</sup>	63 <sup>2bc</sup>	75 <sup>2bc</sup>	85 <sup>2d</sup>	102 <sup>e</sup>
0.26	105 <sup>d</sup>	NA	20 <sup>4a</sup>	22 <sup>2ab</sup>	36 <sup>3ab</sup>	35 <sup>3ab</sup>	58 <sup>3b</sup>	38 <sup>3ab</sup>	56 <sup>3b</sup>	78 <sup>2c</sup>	112 <sup>d</sup>

There were some individual differences in weight projections between alkaloids at 0.01 mmol kg<sup>-1</sup>, but at the 0.04 mmol kg<sup>-1</sup> dose alkaloids began to separate somewhat into groups. At the 0.04 mmol kg<sup>-1</sup> dose the two heliotridine based alkaloids as well as the three macrocyclic diesters had significantly lower projections than the controls. This group (the heliotridine based alkaloids and the three macrocyclic diesters) remains the lightest throughout all higher doses, although there is substantial overlap, and in most cases few differences were found between the most toxic group and the three N-oxides.

The three N-oxide groups had lower projections than controls beginning at the 0.13 mmol kg<sup>-1</sup> dose, and with the exception of lasiocarpine N-oxide at 0.04mmol kg<sup>-1</sup> that had significantly lower projections than echimidine. Echimidine, the only open chain diester with a retronecine base, formed its own group. Echimidine exposed chicks were projected heavier than all but the controls and lasiocarpine at the 0.01 mmol dose, and heavier than all but controls, lasiocarpine N-oxide, and lycopsamine at the 0.04 mmol dose. The projected weight of the top two doses of echimidine exposed chicks was less than lycopsamine exposed chicks and controls, but higher than all other groups. Lycopsamine was not different than control at any of the doses used in this study. Based on weight, the rough order of toxicity with the most toxic first is: seneciophylline, heliotrine, lasiocarpine, senecionine riddelliine, senecionine N-oxide, riddelliine N-oxide, lasiocarpine N-oxide, echimidine, and lycopsamine (Table 3-1).

### **Survival Analysis**

Using PROC LIFETEST a survival analysis was performed and the output is shown in Table 3-2. Because this study was designed primarily to maximize the histologic lesions and facilitate their grading, it functions poorly to test survival. The doses, animal numbers and incubation time to set the histologic lesions were not calculated to maximize power in this area. Although perhaps not as sensitive as the weight comparisons the same trends still apply. Heliotrine is the alkaloid that ranks highest in likelihood to result in death. It groups with the macrocyclic diesters and the other heliotridine base, lasiocarpine. Although substantial overlap is present, the next group is made up of the N-oxides followed by echimidine and then lycopsamine. For the

survival analysis the initial weight affected the outcome, and consequently initial weight was used as a covariate.

**Table 3-2.** Survival analysis of California White chicks exposed to dehydropyrrolizidine alkaloids. A higher number denotes an increased likelihood of dying. The key to abbreviations for alkaloids is as follows: HEL – heliotrine, SPH – seneciphylline, RID – riddelliine, LAS – lasiocarpine, SEN – senecionine, SNO – senecionine N-oxide, RNO – riddelliine N-oxide, LNO – lasiocarpine N-oxide, ECH – echimidine, and LYC – lycopsamine. Superscript letters denote statistical significance at  $p < 0.05$

Survival Analysis	
Alkaloid	Log-Rank
HEL	8.782 <sup>a</sup>
SPH	5.406 <sup>ab</sup>
RID	5.077 <sup>abcd</sup>
LAS	4.842 <sup>abc</sup>
SEN	3.623 <sup>abc</sup>
SNO	-0.571 <sup>bcd</sup>
RNO	-1.254 <sup>bcde</sup>
LNO	-4.905 <sup>cde</sup>
ECH	-7.333 <sup>de</sup>
LYC	-13.666 <sup>e</sup>

### Tissue “Pyrrole” Accumulation Rate

DHPA adducts or “Pyrroles” were detected in the livers of chicks treated with every alkaloid at every dose (Table 3-3). Within alkaloids, the “pyrrole” accumulation rate tends to increase with dose such that with the exception of echimidine and lycopsamine, the highest dose of each alkaloid is significantly different than the lowest dose of that same alkaloid. There are no significant differences between any alkaloids in either of the lowest two doses. “Pyrrole” concentrations and accumulation rates for riddelliine were much higher than any other alkaloid. These high rates were consistent and repeatable as riddelliine was used as a positive control for the various experimental phases. Riddelliine

accumulation rate was included in Table 3.3, and was statistically different than all the other DHPAs, but removed from the statistical model comparison to facilitate identifying differences between other compounds. Heliotrine, lasiocarpine, and seneciphylline had a higher “pyrrole” accumulation rate at 0.13 mmol kg<sup>-1</sup> than at the two lower doses, and at the highest dose all alkaloids with the exception of echimidine and lycopsamine had a significantly higher “pyrrole” accumulation rate when compared with all lower doses of the same compound.

With the exception of riddelliine, the highest “pyrrole” accumulation rates for the two high doses were found in the heliotridine base group (heliotrine and lasiocarpine). At the 0.13 mmol kg<sup>-1</sup> dose they were grouped with the remaining two macrocyclic diesters (senecionine and seneciphylline). At the highest dose the “pyrrole” accumulation rate for heliotrine and lasiocarpine was significantly higher than all other groups (except riddelliine). The N-oxides tend to form a group that overlaps with both the higher group just discussed and the lower group, which is composed of echimidine and lycopsamine. Both echimidine and lycopsamine do not show a significant dose dependent increase in “pyrrole” accumulation rate. Based on “pyrrole” accumulation rate, the order of toxicity is as follows: Riddelliine, heliotrine and lasiocarpine, senecionine and seneciphylline, the three N-oxides (riddelliine N-oxide, senecionine N-oxide, and lasiocarpine N-oxide), and echimidine and lycopsamine.



**Table 3-3.** “Pyrrole” accumulation rate comparison in male California White chicks exposed to dehydropyrrolizidine alkaloids. “Pyrrole” accumulation rate was computed by dividing the “pyrrole” concentration ( $\text{nmol g}^{-1}$ ) by the number of days the chick survived. A log transformed model was used to estimate the “pyrrole” accumulation rate for each compound. Data presented in the table is back transformed. Superscript letters denote statistical differences within a row, and superscript numbers denote statistical differences within a column. A p-value of  $< 0.05$  was considered significant. RID column is averages from the respective groups and the numbers in parenthesis are one standard deviation. RID was removed from the model because variation was too great to compare other compounds. Explanations for abbreviations are as follows: CNT – control, HEL – heliotrine, LAS – lasiocarpine, SPH – seneciophylline, SEN – senecionine, RID – riddelliine, RNO – riddelliine N-oxide, SNO – senecionine N-oxide, LNO – lasiocarpine N-oxide, ECH – echimidine, LYC – lycopsamine, and ND - not available

Dose ( $\text{mmol kg}^{-1}$ )	HEL ( $\text{nmol g}^{-1}$ ) $\text{day}^{-1}$	LAS ( $\text{nmol g}^{-1}$ ) $\text{day}^{-1}$	SPH ( $\text{nmol g}^{-1}$ ) $\text{day}^{-1}$	SEN ( $\text{nmol g}^{-1}$ ) $\text{day}^{-1}$	RNO ( $\text{nmol g}^{-1}$ ) $\text{day}^{-1}$	SNO ( $\text{nmol g}^{-1}$ ) $\text{day}^{-1}$	LNO ( $\text{nmol g}^{-1}$ ) $\text{day}^{-1}$	ECH ( $\text{nmol g}^{-1}$ ) $\text{day}^{-1}$	LYC ( $\text{nmol g}^{-1}$ ) $\text{day}^{-1}$	RID ( $\text{nmol g}^{-1}$ ) $\text{day}^{-1}$
0.01	0.002 <sup>1</sup>	0.001 <sup>1</sup>	0.003 <sup>1</sup>	0.001 <sup>1</sup>	0.005 <sup>1</sup>	0.003 <sup>1</sup>	0.003 <sup>1</sup>	0.016	NA	0.022 (0.004)
0.04	0.007 <sup>1</sup>	0.005 <sup>1</sup>	0.006 <sup>1</sup>	0.029 <sup>1</sup>	0.013 <sup>1</sup>	0.006 <sup>1</sup>	0.095 <sup>1</sup>	0.046	0.012	0.058 (0.014)
0.13	0.648 <sup>2ab</sup>	0.687 <sup>2a</sup>	0.559 <sup>2ab</sup>	0.361 <sup>1abc</sup>	0.108 <sup>1bc</sup>	0.027 <sup>1c</sup>	0.019 <sup>1c</sup>	0.142 <sup>abc</sup>	0.001 <sup>c</sup>	2.735 (4.993)
0.26	3.367 <sup>3a</sup>	3.715 <sup>3a</sup>	1.612 <sup>3b</sup>	0.995 <sup>2bc</sup>	0.664 <sup>2cd</sup>	1.241 <sup>2bc</sup>	0.766 <sup>2bcd</sup>	0.259 <sup>de</sup>	0.004 <sup>e</sup>	11.151 (6.414)

## Serum Biochemistry

Serum biochemistries were analyzed using a principle component analysis after standardizing the variables. Two of the variables, creatine phosphokinase (CPK) and sorbitol dehydrogenase (SDH), did not contribute significantly to the variation in the model, and were therefore dropped from the analysis. CPK is a muscle specific enzyme which was originally included in the analysis to provide ability to determine if elevations in AST were of hepatic or muscle origin. In no case was there a rise in AST that corresponded with a rise in CPK. Consequently, it was assumed that elevations in AST were not of muscle origin. Three components were included in the principle component analysis, and they explained 77% of the variation in the model.

As explained previously, the three components corresponded to biologically relevant aspects of hepatic function or damage. Major contributors to component one were total protein, glucose, cholesterol, and triglycerides, all of which correspond to hepatic metabolic functions. In every case the values for component one are lower for the higher two doses than for the lower two doses (Table 3-4A). Echimidine did differ between the 0.01 mmol kg<sup>-1</sup> and 0.26 mmol kg<sup>-1</sup>. Unfortunately, due to animal deaths, blood was not taken from more than one animal in the following groups: heliotrine 0.13 mmol kg<sup>-1</sup>, and lasiocarpine, senecionine, and senecionine N-oxide all at 0.26 mmol kg<sup>-1</sup>. Because it was anticipated that lycopsamine would be less toxic than other compounds the lowest dose tested was 0.04mmol kg<sup>-1</sup>.

Principle component two (Table 3-4B) corresponds to hepatocellular damage with major contributors of AST, LDH, and to a lesser extent bile acid. Only heliotrine and

echimidine show a dose dependent increase in the value for principle component two. At 0.26 mmol kg<sup>-1</sup>, the principle component two value for heliotrine is significantly higher than all other compounds. This corresponds with the histologic characterization of heliotrine. An interesting pattern is present in seneciphylline, riddelliine, riddelliine N-oxide, lasiocarpine N-oxide, and lycopsamine where there is an increase in the value of component two followed by a decrease at the highest dose. This decrease from the 0.13 mmol kg<sup>-1</sup> group to the 0.26 mmol kg<sup>-1</sup> group was only significant for riddelliine and riddelliine N-oxide.

Principle component three, with major contributions from the inducible hepatic enzyme, GGT and bile acid, often demonstrated a trend within a compound where there was an initial increase followed by a decrease (Table 3-4C). This pattern was demonstrated particularly well with heliotrine, seneciphylline, riddelliine, and riddelliine N-oxide. Because the rise and fall in value happened at different doses for different compounds, it can be difficult to interpret differences between values at any given dose. Heliotrine peaks at the 0.04 mmol kg<sup>-1</sup> dose, seneciphylline, riddelliine, and riddelliine N-oxide peak at 0.13 mmol kg<sup>-1</sup>, echimidine appeared to increase at least at the 0.26 mmol kg<sup>-1</sup> dose, and lasiocarpine N-oxide and lycopsamine stayed constant throughout. Lasiocarpine, senecionine, and senecionine N-oxide had increased values at the 0.13 mmol kg<sup>-1</sup> dose, but because only one sample for each of these groups was obtained, estimates were not calculated for the 0.26 mmol dose. Using the dose at which component three peaked, crude toxicity rankings can be made. The most toxic in this case is heliotrine, followed by seneciphylline, riddelliine, senecionine, riddelliine N-

oxide, senecionine N-oxide, and lasiocarpine. The next most toxic compound is echimidine. Lasiocarpine N-oxide and lycopsamine are the least toxic compounds using this criterion.

Toxicity, from a gross pathology perspective, can be ranked based on the lowest dose resulting in ascites. Heliotrine and senecionine are therefore most toxic at 0.04 mmol kg<sup>-1</sup>, followed by seneciophylline, riddelliine, senecionine N-oxide, riddelliine N-oxide, lasiocarpine, and lasiocarpine N-oxide at 0.13 mmol kg<sup>-1</sup>. Echimidine is next with chicks developing ascites at 0.26 mmol kg<sup>-1</sup>, and lycopsamine is least toxic with no animals developing ascites.

### **Histopathology**

The majority of DHPA associated lesions were centered in the liver. There was a range of lesions from massive acute hepatic necrosis, which tended to be more common in high dose animals, to hepatocellular loss with evidence of regeneration. The most severe lesion and the most common cause of mortality prior to the end of the study was centrilobular to massive, diffuse hepatic necrosis. This was characterized by loss of hepatic cords, individualized hepatocytes that often had pyknotic nuclei, collapse of lobular structure with vascular sinusoid expansion and massive vascular congestion and hemorrhage (Fig. 3-3E). Lesions tended to have a patchy distribution, and within the same animal it was common to see large areas of necrosis separated by areas that appeared relatively normal. Although the necrotic areas appeared to be primarily centrilobular, the lesions were not diffuse in most cases, such that centrilobular areas

**Table 3-4. Principle component analysis of serum biochemistry values from California White chicks exposed to dehydroepiandrosterone alkaloids**

This principle component analysis was performed on standardized serum biochemistry variables using three principle components. Principle component one corresponds to metabolic products produced by the liver with major contributing variables of total protein, glucose, cholesterol, and triglycerides. Principle component two corresponds to hepatocellular damage with major contributing variables or Aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and to a lesser extent bile acid. The major contributing variables for component three are gamma glutamyl transferase (GGT) and to a lesser extent bile acid. Superscript letters denote statistical differences within a column, and superscript numbers denote statistical differences within a row. A p-value of < 0.05 was considered significant. Explanations for abbreviations are as follows: CNT – control, HEL – heliotrine, LAS – lasiocarpine, SPH – seneciophylline, SEN – senecionine, RID – riddelline, RNO – riddelline N-oxide, SNO – senecionine N-oxide, LNO – lasiocarpine N-oxide, ECH – echinidine, LYC – lycopsamine, and ND - not done

### Serum Biochemistry Principle Component Analysis

#### A. Principle Component 1 (Metabolic Products)

Dose (mmol kg <sup>-1</sup> )	CNT	HEL	LAS	SPH	SEN	RID	RNO	SNO	LNO	ECH	LYC
0.01	1.18 <sup>a,1,2,3</sup>	1.97 <sup>a,2,3</sup>	0.70 <sup>a,1,2,3</sup>	0.02 <sup>a,1</sup>	0.34 <sup>a,1,2</sup>	0.51 <sup>a,1,2,3</sup>	0.56 <sup>a,1,2,3</sup>	0.41 <sup>a,1,2</sup>	0.84 <sup>a,1,2,3</sup>	2.23 <sup>a,3</sup>	ND
0.04	0.57 <sup>a,1,2</sup>	1.09 <sup>a,2,3</sup>	0.80 <sup>a,2,3</sup>	0.38 <sup>a,1,2</sup>	-1.21 <sup>b,1</sup>	0.34 <sup>a,1,2</sup>	0.59 <sup>a,1,2</sup>	1.20 <sup>a,2,3</sup>	2.55 <sup>b,3</sup>	1.13 <sup>a,b,2,3</sup>	0.15 <sup>a,1,2</sup>
0.13	0.83 <sup>a,3</sup>	ND	-3.29 <sup>b,1</sup>	-3.88 <sup>b,1</sup>	-3.17 <sup>c,1</sup>	-0.93 <sup>a,b,2,3</sup>	-1.72 <sup>b,1,2</sup>	-1.59 <sup>b,1,2</sup>	0.80 <sup>b,3</sup>	0.81 <sup>a,b,3</sup>	-0.31 <sup>a,2,3</sup>
0.26	1.26 <sup>a,4</sup>	0.26 <sup>a,3,4</sup>	ND	-2.66 <sup>b,1,2</sup>	ND	-2.08 <sup>b,1,2,3</sup>	-2.18 <sup>b,1,2,3</sup>	ND	-3.31 <sup>b,3,4</sup>	0.07 <sup>b,3,4</sup>	-0.47 <sup>a,2,3,4</sup>

#### B. Principle Component 2 (Hepatocellular Leakage)

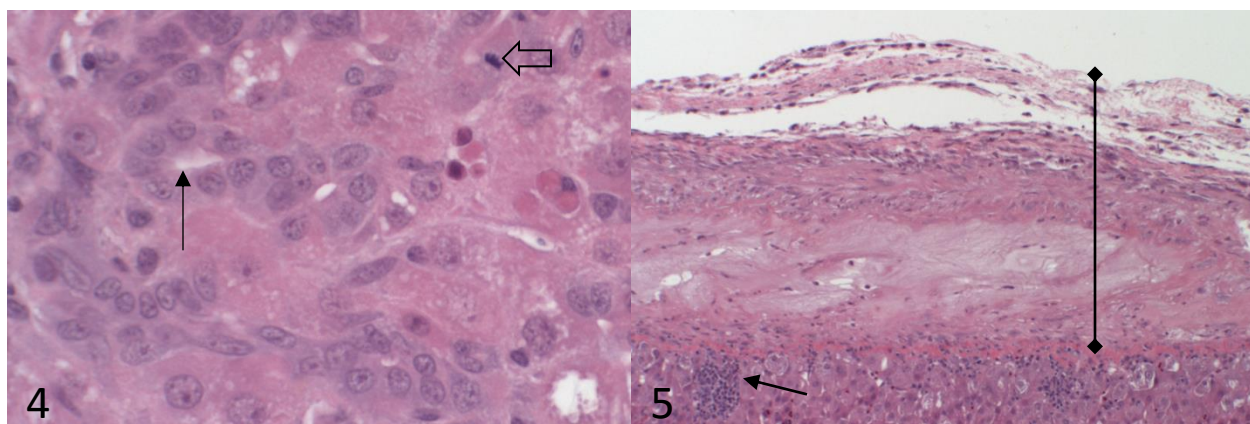
Dose (mmol kg <sup>-1</sup> )	CNT	HEL	LAS	SPH	SEN	RID	RNO	SNO	LNO	ECH	LYC
0.01	-0.43 <sup>a,1,2,3</sup>	-0.40 <sup>a,1,2,3</sup>	-1.35 <sup>a,3</sup>	-0.17 <sup>a,1,2,3</sup>	0.62 <sup>a,1,2,3</sup>	-0.97 <sup>a,2,3</sup>	-0.36 <sup>a,1,2</sup>	-0.49 <sup>a,1,2,3</sup>	-0.77 <sup>a,1,2,3</sup>	-1.00 <sup>a,2,3</sup>	ND
0.04	-0.55 <sup>a,1</sup>	1.32 <sup>b,1</sup>	-0.43 <sup>b,1</sup>	-0.13 <sup>a,1</sup>	-0.30 <sup>a,1</sup>	0.13 <sup>a,b,1</sup>	0.64 <sup>a,b,1</sup>	0.77 <sup>a,1</sup>	-0.17 <sup>a,1</sup>	-0.80 <sup>a,1</sup>	-0.66 <sup>a,1</sup>
0.13	-0.68 <sup>a,2</sup>	ND	-0.06 <sup>a,b,1,2</sup>	-0.08 <sup>a,1,2</sup>	0.35 <sup>a,1,2</sup>	1.88 <sup>b,1,2</sup>	1.39 <sup>b,1</sup>	1.57 <sup>a,1,2</sup>	1.31 <sup>a,1,2</sup>	-0.02 <sup>a,b,1,2</sup>	-0.48 <sup>a,2</sup>
0.26	-0.21 <sup>a,2,3</sup>	6.90 <sup>c,1</sup>	ND	-0.75 <sup>a,2,3</sup>	ND	-1.12 <sup>a,3</sup>	-0.66 <sup>a,3</sup>	ND	-0.59 <sup>a,3</sup>	1.47 <sup>b,2</sup>	-0.93 <sup>a,3</sup>

#### C. Principle Component 3 (Inducible Hepatocellular Enzyme – GGT + Bile Acid)

Dose (mmol kg <sup>-1</sup> )	CNT	HEL	LAS	SPH	SEN	RID	RNO	SNO	LNO	ECH	LYC
0.01	0.11 <sup>a,2,3</sup>	0.40 <sup>a,4</sup>	-0.35 <sup>a,2,3,4</sup>	-1.09 <sup>b,1,2</sup>	-1.46 <sup>b,1</sup>	0.09 <sup>a,b,4</sup>	-0.79 <sup>b,2,3</sup>	-0.81 <sup>b,1,2,3,4</sup>	-0.05 <sup>a,3,4</sup>	0.67 <sup>a,b,4</sup>	ND
0.04	-0.47 <sup>a,3,4</sup>	1.12 <sup>a,1</sup>	-0.57 <sup>a,2,3,4</sup>	0.43 <sup>a,1,2,3</sup>	0.23 <sup>a,1,2,3,4</sup>	-0.01 <sup>b,3</sup>	-0.28 <sup>b,3,4</sup>	0.63 <sup>a,1,2</sup>	0.51 <sup>a,1,2,3</sup>	0.33 <sup>a,b,1,2,3,4</sup>	-0.79 <sup>a,4</sup>
0.13	-0.03 <sup>a,1,2,3</sup>	ND	0.67 <sup>a,1,2</sup>	1.75 <sup>a,1,2</sup>	1.14 <sup>a,1,2,3</sup>	1.00 <sup>a,1</sup>	1.71 <sup>a,1</sup>	1.73 <sup>a,1,2</sup>	0.11 <sup>a,1,2,3</sup>	-0.08 <sup>b,2,3</sup>	-0.80 <sup>a,3</sup>
0.26	-0.05 <sup>a,2,3</sup>	-3.65 <sup>b,1</sup>	ND	-1.25 <sup>b,2</sup>	ND	-0.55 <sup>b,2</sup>	-0.65 <sup>a,b,1,2,3</sup>	ND	0.21 <sup>a,2,3</sup>	0.97 <sup>a,3</sup>	-0.77 <sup>a,2</sup>

were not all equally affected. In severely affected animals the necrosis within lobules was more likely to be massive and there were fewer unaffected/less affected areas. In less severely affected individuals, hepatocytes were often swollen with microvesiculated cytoplasm, and necrotic foci occupied a much smaller proportion of the liver. There were microscopic foci of angiectasis characterized by a focal loss of hepatic cords with the potential space taken up by peripheral blood and typically surrounded by a single layer of endothelium.

More chronic lesions consisted of an overall loss of hepatocytes visualized by a closer proximity between portal areas. These portal areas were often characterized by biliary hyperplasia and bile duct reduplication (Fig. 3-4). The capsule was occasionally



**Figure 3-4.** Liver from a chick exposed to riddelliine N-oxide ( $0.26 \text{ mmol kg}^{-1}$ ) showing regeneration. Regenerative cells often form small ducts (arrow). There is a hepatocyte with a mitotic figure (open arrow).

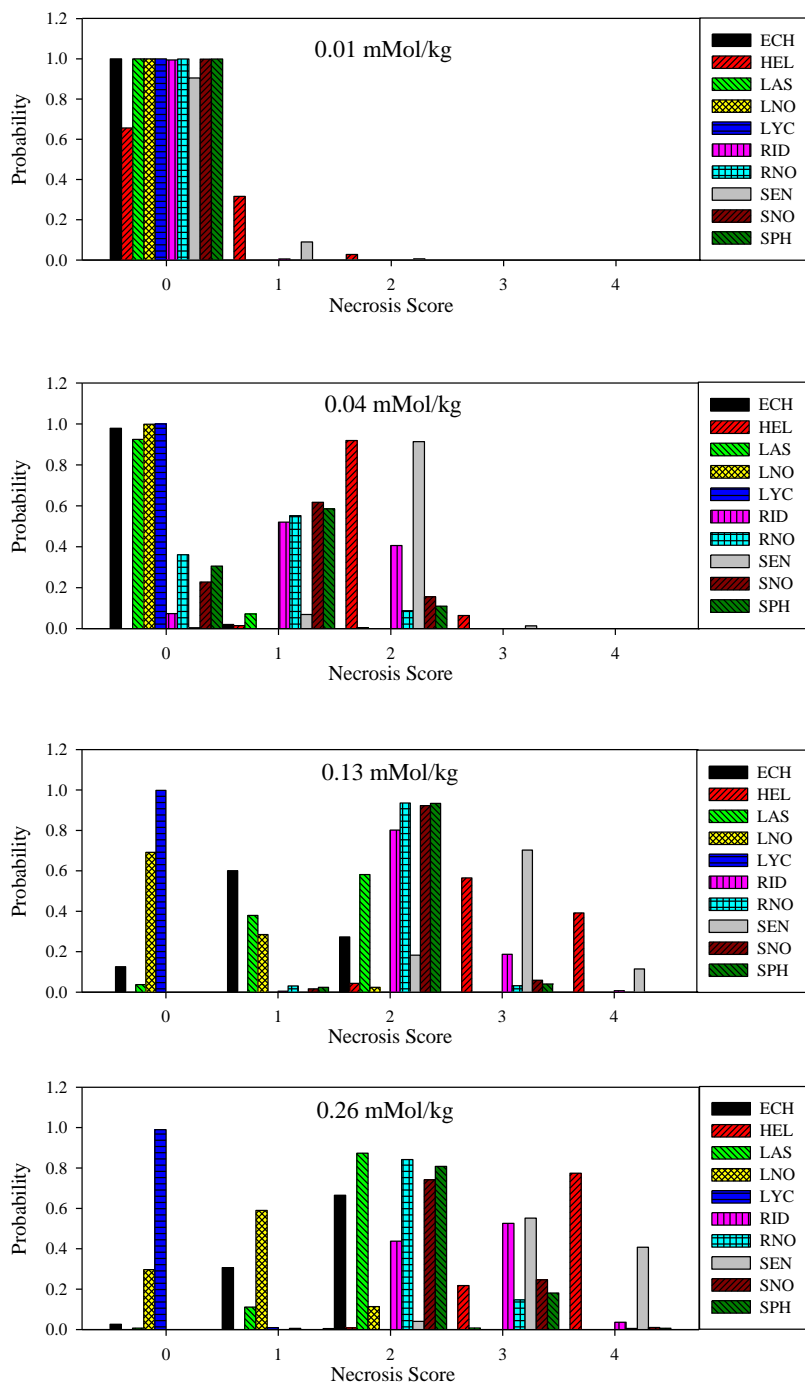
**Figure 3-5.** Liver from a chick exposed to senecionine ( $0.13 \text{ mmol kg}^{-1}$ ) that has capsular serositis. Not the markedly thickened capsule (line). In some cases capsular serositis was accompanied by foci of mononuclear inflammatory cells in the subcapsular hepatocellular parenchyma (arrow).

thickened and composed of a variably dense mixture of inflammatory cells, fibroblasts, an eosinophilic acellular matrix (interpreted as collagen), and fibrin (Fig. 3-5).

Figure 3.6 demonstrates the probability of the hepatocellular necrosis score falling into each category, and is organized by alkaloid and dose. For all alkaloids excepting lycopsamine, the proportion of higher histologic hepatocellular necrosis scores increased as dose increased. Similar to weight and survival data, the heliotrine dosed animals tended to have the highest hepatocellular necrosis scores. At the lowest dose, all groups fall into the normal (0) score except for heliotrine and senecionine. These two alkaloids had a relatively small proportion that fell into a hepatocellular necrosis score of one or much less likely a score of two. At the 0.04 mmol kg<sup>-1</sup> dose all but four alkaloids (lasiocarpine, lasiocarpine N-oxide, echimidine, and lycopsamine) had a greater than 50% proportion that fell into a hepatocellular necrosis score of at least one. At this dose both heliotrine and senecionine had greater than 50% of their scores falling into a score category of 2.

At 0.13 mmol kg<sup>-1</sup> the greatest proportion of lycopsamine and lasiocarpine N-oxide were still normal (score of 0), whereas all other alkaloids fell primarily into a hepatocellular necrosis score of one or higher. At this dose the majority of echimidine scores were in category one. Lasiocarpine, riddelliine, seneciphylline, senecionine N-oxide, and riddelliine N-oxide all had a majority of their scores that fall into the two category. Heliotrine and senecionine both had a majority of hepatocellular necrosis scores that fell into category three, however a substantial proportion (around 40%) of scores from the heliotrine group fell into a category of four.

## Hepatocellular Necrosis



**Figure 3-6. Probability of necrosis score for each alkaloid falling into each of the different categories** Hepatocellular necrosis was scored on a scale of 0 to 4. Necrosis score was compared as a multinomial outcome with alkaloid and dose as fixed factors in the model. The bar graphs demonstrate the probability of the hepatocellular necrosis score for each alkaloid falling into each category. Graphs are arranged by dose.



**Table 3-5.** Comparison of the distribution of hepatocellular necrosis scores from male, California White chicks exposed to dehydropyrrolizidine alkaloids. Comparisons are between all alkaloids at all doses. Superscript letters denote a statistical difference at  $p < 0.05$ .

<b>Hepatocellular Necrosis Score Distribution</b>
Heliotrine <sup>a</sup>
Senecionine <sup>ab</sup>
Riddelliine <sup>abc</sup>
Senicionine N-oxide <sup>abcd</sup>
Seneciphylline <sup>bcd</sup>
Riddelliine N-oxide <sup>bcd</sup>
Lasiocarpine <sup>cde</sup>
Echimidine <sup>de</sup>
Lasiocarpine N-oxide <sup>ef</sup>
Lycopsamine <sup>f</sup>

At the 0,26 mmol kg dose the majority of lycopsamine scores remained in category 0, but other compounds caused more severe effects. The majority of lasiocarpine N-oxide scores moved up to category one for the high dose. The majority of echimidine scores moved up to score category two joining lasiocarpine, seneciphylline, senecionine N-oxide, and riddelliine N-oxide. The major proportion of riddelliine scores moved up to three with senecionine, and the majority of heliotrine scores moved up to category four. For a comparison of patterns for different alkaloid necrosis scores see Table 3-5.

In the highest two dose groups of lasiocarpine, chicks developed a moderate to severe necrotizing colitis (Fig. 3-7), and all five animals in the highest dose group also developed necrotizing enteritis. In the 0.26 mmol kg<sup>-1</sup> group, intestinal lesions were more severe (all were scored as 4 out of 5 where 0 is normal and 5 is severe) than the 0.13 mmol kg<sup>-1</sup> group (all received a score of 2 on the same scale). Lesions were characterized by segmental, mucosal, necrosis with blunting and loss of villi in the small

intestine, and significant loss of mucosal epithelium in the large intestine accompanied by marked attenuation of remaining epithelium. This extrahepatic involvement was sufficient to be the cause of morbidity and mortality in these animals. Similar large intestinal lesions were present in two 0.26 mmol kg<sup>-1</sup> group of riddelliine dosed animals (scored as 3 out of 5), one 0.13 mmol kg<sup>-1</sup> group of riddelliine dosed animals (scored 4 out of 5), and one 0.13 mmol kg<sup>-1</sup> group of lasiocarpine N-oxide animal (scored 2 out of 5). In addition to the large intestinal lesions necrotizing enteritis was observed in five animals in addition to the lasiocarpine highest dose group; one each in the 0.04 and 0.26 mmol kg<sup>-1</sup> lasiocarpine N-oxide groups, the 0.13 and 0.26 mmol kg<sup>-1</sup> heliotrine groups and the 0.13 mmol kg<sup>-1</sup> riddelliine group.



**Figure 3-7.** Large intestine from a chick exposed to lasiocarpine (0.26 mmol kg<sup>-1</sup>). The epithelium is markedly attenuated (closed arrow). Crypt epithelium is also markedly attenuated and the crypts are filled with necrotic debris and occasional inflammatory cells (open arrows).

Histologic ranking based solely on the hepatic necrosis toxicity rankings from most to least toxic are as follows: Heliotrine, senecionine, riddelliine, senecionine N-oxide,

seneciophylline, riddelliine N-oxide, lasiocarpine, echimidine, lasiocarpine N-oxide, and lycopsamine.

Toxicity of the different compounds were ranked based on weight gain, survival time, “pyrrole” accumulation rate, presence of ascites at necropsy, and the histologic hepatocellular necrosis score (Table 3-6). Combining the results from those five factors the DHPAs used in this study can be grouped into four somewhat overlapping groups.

**Table 3-6. Summary of Toxicity Rankings** Male California White chicks were orally exposed to one of 10 different dehydropyrrolizidine alkaloids and toxicity was ranked with output variables of weight gain, survival, pyrrole accumulation rate, presence of ascites at necropsy, and histologic scoring of hepatocellular necrosis. ECH = echimidine, HEL = heliotrine, LAS = lasiocarpine, LNO = Lasiocarpine N-oxide, LYC = lycopsamine, RID = Riddelliine, RNO = Riddelliine N-oxide, SEN = senecionine, SNO = senecionine N-oxide, SPH = seneciophylline

Weight gain	Survival	“Pyrrole” Accumulation Rate	Ascites	Histopathology (Hepatocellular Necrosis)
SPH	HEL	RID	HEL	HEL
HEL	SPH	LAS	SEN	SEN
LAS	RID	HEL	LAS	RID
RID	LAS	SPH	RID	SNO
SEN	SEN	SEN	SPH	SPH
SNO	SNO	RNO	SNO	RNO
RNO	RNO	SNO	RNO	LAS
LNO	LNO	LNO	LNO	ECH
ECH	ECH	ECH	ECH	LNO
LYC	LYC	LYC	LYC	LYC

The most toxic group is composed of heliotrine, lasiocarpine, riddelliine, senecionine, and seneciophylline. The next most toxic group contains the three N-oxides tested (riddelliine N-oxide, senecionine N-oxide, and lasiocarpine N-oxide). These N-oxides tended to be slightly less toxic than their respective free base form in every category.

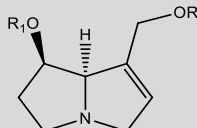
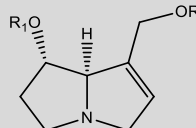
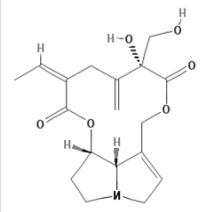
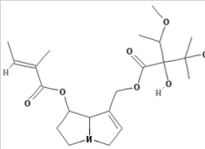
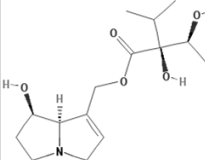
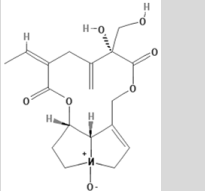
The two remaining DHPAs (echimidine and lycopsamine) were consistently less toxic than all others tested. Of these two, echimidine was more toxic in this model.

## Discussion

The intent of this study was to compare multiple DHPAs with key structural differences in a single more biologically relevant model, and if possible rank them according to toxicity. Comparisons were made with weight gain, survival time, “pyrrole” adduct accumulation rate, gross pathology, histopathology, and serum biochemistries. Serum biochemistry was informative, but did not lend itself to ranking toxicity in this study. The toxicity rankings using the remaining five factors are very similar, although not exactly the same.

Comparing the results of the present study with the previously published results in Table 3-7 it is evident that overall the conclusions are similar. Based on these previous studies it was expected that lasiocarpine and the three macrocyclic diesters would compose the most toxic group. These four compounds were in fact, among the most toxic compounds tested. Heliotrine was far more toxic in this present study than expected, and was in the majority of comparisons the single most toxic DHPA. This suggests that although much of what was found in the present study is similar to previous research, for some compounds (such as heliotrine) the differences could be very substantial. It is important not to underestimate the toxicity of a given DHPA because of assumptions solely based on structure.

**Table 3-7.** Compilation of DHPA relative toxicity data (LD<sub>50</sub>) from published literature. LD<sub>50</sub> data are in mg kg<sup>-1</sup> and all data was obtained using a male rat model and intraperitoneal injection unless otherwise indicated.

	Retronecine 	Heliotridine 
<b>Macrocyclic Diesters</b>  Riddelliine	<b>Riddelliine</b> 105 IV mouse <sup>1</sup> , 80 PO male rat <sup>2</sup> <b>Senecionine</b> 50 <sup>3</sup> , 77 <sup>4</sup> , 85 <sup>5</sup> , 57.3 PO mice <sup>6</sup> <b>Seneciphylline</b> 77 <sup>7</sup>  1 (IARC 1976, Cheeke 1998); 2 (Dalefield, Gosse <i>et al.</i> 2012); 3 (Bull, Culvenor <i>et al.</i> 1968, Mattocks 1972); 4 (WHO 1988); 5 (Cheeke 1998) ; 6 (Wang <i>et al.</i> 2011); 7(Bull, Culvenor <i>et al.</i> 1968, Mattocks 1972)	
<b>Open Chain Diesters</b>  Lasiocarpine	<b>Echimidine</b> 200 (Bull, Culvenor <i>et al.</i> 1968)	<b>Lasiocarpine</b> 77 (Bull, Culvenor <i>et al.</i> 1968, Mattocks 1972) 72 (WHO 1988, Cheeke 1998) 88 IV Rat (Rose, Harris <i>et al.</i> 1959)
<b>Monoesters</b>  Lycopsamine	<b>Lycopsamine</b> 1500 (Cheeke 1998) <b>Intermedine</b> 1500 (Cheeke 1998)	<b>Heliotrine</b> 296 (Bull, Culvenor <i>et al.</i> 1968, Mattocks 1972) 300 (Downing and Peterson 1968, Mattocks and White 1968)
<b>N-Oxides</b>  Riddelliine N-Oxide	<b>Riddelliine N- Oxide</b> ~250 PO male rat estimated from adduct production (Chou, Wang <i>et al.</i> 2003) <b>Senecionine N-Oxide</b> ~2000 (Culvenor, Edgar <i>et al.</i> 1976)	<b>Lasiocarpine N-Oxide</b> 547 (Bull, Dick <i>et al.</i> 1958) ~1100 (Culvenor, Edgar <i>et al.</i> 1976)

Results from in vitro assays using an immortalized chicken hepatocyte cell line were similar to those found in the present study (Field, *et al.* 2015). In both cases heliotrine was found to be similar in toxicity to the heliotridine based open chain diester, lasiocarpine, as well as the retronecine based macrocyclic diesters senecionine and seneciophylline. There is one rather notable difference in which the present study excels. The same three N-oxides were studied in the in vitro work, and they were found to be similar in toxicity to lycopsamine. They were consistently more toxic than lycopsamine in this live chick model. The difference is most likely due to microbial reduction of the N-oxides in the gastrointestinal tract, reducing the N-oxide to the free base form.

Ranking the toxicity of N-oxides based on previously published literature would be difficult. Using data obtained from intraperitoneal exposure studies would be expected to markedly underestimate their toxicity. More work is needed to determine the mechanism of N-oxide DHPA toxicity. Current hypothesis are they reduced to the free base in the gastrointestinal tract via intestinal microbes, or alternatively it has also been shown they can be reduced by the liver xenobiotic enzymes. Based on intraperitoneal studies the three N-oxides would be expected to be similar, although perhaps slightly more toxic than lycopsamine. In the present study, the three N-oxides were consistently more toxic than both lycopsamine and echimidine. They behaved more closely to their associated free base, although somewhat less toxic.

Echimidine and lycopsamine were more similar to each other than might have been expected based on previous research. Echimidine was consistently more toxic than

lycopsamine, but higher doses (particularly of lycopsamine) would need to be used to more accurately determine their relative toxicity.

Another unanticipated result was the very high “pyrrole” accumulation rate of riddelliine. When comparing DNA adduct production between different DHPAs Xia *et al.* (2013) found lasiocarpine exposure resulted in a higher proportion of DNA adducts than riddelliine. It is worth noting however, that riddelliine is the only DHPA classified by the National Toxicology Program as a possible human carcinogen. DHPAs are thought to cause cancer because of their ability to form DNA adducts. If riddelliine has an increased propensity to produce adducts, measured in the present study as “pyrrole” accumulation rate, this markedly higher “pyrrole” accumulation rate may contribute to the carcinogenicity of this compound. On the other hand it may be specific to this model.

Lasiocarpine exposure resulted in profound intestinal lesions. A similar set of lesions was reported by Hooper (1975) in sheep, rats and mice when exposed IP to high doses of lasiocarpine. In that study lesions were most severe in the proximal intestine. In the present study no such pattern was present, but the lesions were very similar. Hooper suggested a possible etiology to be cleavage of glutathione from the pyrrole after biliary excretion into the duodenum. Xia *et al.* (2015) recently reported that glutathione bound pyrrole is unstable and in the presence of DNA bases formed DNA pyrrole adducts in vitro. Alternatively, it is possible that lasiocarpine was bioactivated in enterocytes, and the intestinal lesions developed as a result. However, taking the previous two studies into account in combination with the results of the present study, it appears likely that the

glutathione bound pyrrole is unstable in the intestinal environment, and that lasiocarpine has a particular propensity for creating such intestinal lesions.

In this study weight, survival analysis, and presence of ascites at necropsy, although nonspecific for pathogenesis, appeared to be good indicators ranking of relative DHPA toxicity. These indicators allowed for ranking of different compounds based on toxicity, and the order of toxicity was very similar to the histologic analysis.

The hepatocellular necrosis score was a very good indicator of toxicity for those compounds which appear to be at least primarily hepatotoxic at the doses used, and in this model. If only hepatocellular necrosis was used to rank the toxicity of DHPAs compounds that produce more extrahepatic lesions, such as lasiocarpine, would be ranked as less toxic than they truly are. Consequently, it is important to examine a wide variety of tissues. It is also beneficial to use multiple indicators of toxicity. Histology is probably the best indicator of toxicity for this model.

When performing principle component analysis of the serum biochemistries, the principle components conveniently separated into biologically relevant divisions. Principle component one was composed of total protein, glucose, triglycerides, and cholesterol, all of which are metabolic products produced in the liver. Consequently, a decrease in the value of principle component one corresponds with toxicity. Because a decrease in this component corresponds to decreased hepatic function, it would require a period of time for this value to drop. This time would correspond to a combination of the serum half-lives of the four biochemistries under consideration as well as the amount of time it took for the functional hepatic mass to decrease. In some of the highest dose



groups such as heliotrine, and seneciophylline, the animals did not survive long enough for this change to be manifest and as a result a comparison between the highest doses may not be completely relevant. This also explains why the component one value for the 0.13 mmol kg<sup>-1</sup> group is lower than the value for the 0.26 mmol kg<sup>-1</sup> group for seneciophylline.

For principle component two, because its major contributors are hepatocellular leakage enzymes, a higher value corresponds with increased toxicity. In this case those animals that died or were euthanized prior to the end of the study would be expected to have higher values due both to an expected increase in the number of damaged hepatocytes as well as a shorter time between damage and blood being taken. This is evident in the 0.26 mmol kg<sup>-1</sup> heliotrine group. Because all of the animals in this group died within one day of initiating treatment, it is logical that they would have very high values for component two. Unfortunately, this may make comparisons between other compounds impossible.

Principle component three is slightly more difficult to interpret than the other two. Heliotrine, seneciophylline, riddelliine, riddelliine N-oxide, and lasiocarpine N-oxide all have a distinct pattern to their component three values. There is an initial rise followed by a decrease that is typically below the lowest dose value. Presumably lasiocarpine, senecionine, and senecionine N-oxide would follow this same pattern if the highest doses could be analyzed. The value for echimidine continues to rise at the highest dose tested. It is also interesting that the highest point for seneciophylline, riddelliine, riddelliine N-oxide, and lasiocarpine N-oxide is at the 0.13 mmol kg<sup>-1</sup> dose, whereas for heliotrine the highest value is at the 0.04 mmol kg<sup>-1</sup> dose.

This study was designed to maximize lesions observed histologically. As a result histology, particularly if all lesions are considered, is the most comprehensive indicator of toxicity for this model. Weight was also a sensitive indicator, but tells little about pathogenesis. The reason for decreases in weight gain could be due to decreased food intake, or metabolic deficiency due to liver damage. Survival is of course a valuable indicator, but when used for toxicity modeling, it not as sensitive as would be desired. “Pyrrole” accumulation rate is a valuable indicator, and may help determine the relative carcinogenicity of different DHPAs. Serum biochemistries were the least helpful in determining the relative toxicity of different compounds in this model. There were clearly adverse effects detectable via serum biochemistry, but there is perhaps too much variation in serum biochemistry values in this model to effectively rank toxicity of different compounds.

### Conclusion

The results of this research are vitally important because multiple structurally different DHPAs were compared at biologically relevant doses and routes in the same model. In this model, heliotrine appears to be at least as toxic as retronecine based macrocyclic diesters and heliotridine based diesters, and much more toxic than expected based on previously published IP exposure studies. The N-oxides used in this study were also more toxic than would be expected based on either IP exposure studies or cell based assays. The retronecine based diester, echimidine, was unexpectedly less toxic than

heliotrine, the heliotridine based monoester. Lycopsamine was the least toxic of the compounds assessed in this study.

Tissue “pyrrole” adducts were present in the livers from chicks exposed to all of the compounds, and the accumulation rate can be used as a toxicity indicator. The “pyrrole” accumulation rate was much higher in riddelliine exposed chicks than in any other group, which may be biologically relevant with respect to carcinogenicity. Future research on the relative carcinogenicity of structurally diverse DHPAs would be valuable.

Extrahepatic lesions present in the highest dose of lasiocarpine exposed animals, in combination with results from other studies suggest that excreted conjugated pyrroles may be unstable in the intestine and may cause damage post excretion for some DHPAs. This adds credence to the concern with consumption of tissues such as liver from animals that have been exposed to DHPAs, and this potential instability should be evaluated in future studies.

## References

- Baker DC, Smart RA, Ralphs M, Molyneux RJ. 1989. Hound's-tongue (*Cynoglossum officinale*) poisoning in a calf. J Am Vet Med Assoc. 194:929-930.
- Brown AW, Stegelmeier BL, Colegate SM, Gardner DR, Panter KE, Knoppel EL, Hall JO. 2015. The comparative toxicity of a reduced, crude comfrey (*Symphytum officinale*) alkaloid extract and the pure, comfrey-derived pyrrolizidine alkaloids, lycopsamine and intermedine in chicks (*GALLUS GALLUS DOMESTICUS*). J Appl Toxicol.
- Bull LB, Dick AT, McKenzie JS. 1958. The acute toxic effects of heliotrine and lasiocarpine and their N-oxides on the rat. J Pathol Bacteriol. 75:17-25.
- Bull LF, Culvenor CCJ, Dick AT. 1968. The Pyrrolizidine Alkaloids: Their Chemistry-Pathogenicity and Other Biological Properties. North Holland Publications: Amsterdam.
- Chauvin P, Dillon J, Moren A. 1994. An outbreak of Heliotrope food poisoning, Tadjikistan, November 1992-March 1993]. Santé (Montrouge, France) 4:263.
- Cheeke PR. 1998. Natural Toxicants in Feeds, Forages, and Poisonous Plants. Interstate Publishers Inc.: Danville, IL.
- Chou MW, Wang YP, Yan J, Yang YC, Beger RD, Williams LD, Doerge DR, Fu PP. 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.
- Culvenor CC, Edgar JA, Jago MV, Qutteridge A, Peterson JE, Smith LW. 1976a. Hepato- and pneumotoxicity of pyrrolizidine alkaloids and derivatives in relation to molecular structure. Chem Biol Interact. 12:299-324.
- Culvenor CC, Edgar JA, Jago MV, Qutteridge A, Peterson JE, Smith LW. 1976b. Hepato- and pneumotoxicity of pyrrolizidine alkaloids and derivatives in relation to molecular structure. Chem Biol Interact. 12:299-324.
- Dalefield RR, Gosse MA, Barholomaeus A, Schyvens CG, Hutchinson KJ, Mueller U. 2012. Determination of the single-dose 72-hour oral gavage LD50 values of monocrotaline and riddelliine in male Han Wistar rats using the up-and-down procedure. Journal of Herbal Medicine and Toxicology 6:153-165.
- Dickinson J, Cooke M, King R, Mohamed P. 1976. Milk transfer of pyrrolizidine alkaloids in cattle. J Am Vet Med Assoc. 169:1192-1196.

- Downing DT, Peterson JE. 1968. Quantitative assessment of the persistent antimutagenic effect of certain hepatotoxic pyrrolizidine alkaloid on rat liver. *Aust J Exp Biol Med.* 46:493-502.
- Edgar JA, Colegate SM, Boppre M, Molyneux RJ. 2011. Pyrrolizidine alkaloids in food: a spectrum of potential health consequences. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure, & Risk Assessment* 28:308-324.
- Edgar JA, Roeder E, Molyneux RJ. 2002. Honey from plants containing pyrrolizidine alkaloids: a potential threat to health. *J Agr Food Chem.* 50:2719-2730.
- Edgar JA, Smith LW. 2000. Transfer of pyrrolizidine alkaloids into eggs: food safety implications. In *ACS Symposium series*. ACS Publications; 118-128.
- Field RA, Stegelmeier BL, Colegate SM, Brown AW, Green BT. 2015. An in vitro comparison of the cytotoxic potential of selected dehydropyrrolizidine alkaloids and some N-oxides. *Toxicol* 97:36-45.
- Frei H, Lüthy J, Brauchli J, Zweifel U, Würigler FE, Schlatter C. 1992. Structure/activity relationships of the genotoxic potencies of sixteen pyrrolizidine alkaloids assayed for the induction of somatic mutation and recombination in wing cells of *Drosophila melanogaster*. *Chem Biol Interact.* 83:1-22.
- Hooper P. 1975. Experimental acute gastrointestinal disease caused by the pyrrolizidine alkaloid, lasiocarpine. *J Comp Pathol.* 85:341-349.
- Hooper P. 1978. Pyrrolizidine alkaloid poisoning. Pathology with particular reference to differences in animal and plant species. In *Joint United States-Australian Symposium on Poisonous Plants, Logan, Utah (USA), 19-24 Jun 1977*. Academic Press.
- IARC. 1976. Pyrrolizidine alkaloids. In: *Some naturally occurring substances*. Lyon International Agency for Research on Cancer; 265-342.
- Johnson AE, Molyneux RJ. 1984. Toxicity of threadleaf groundsel (*Senecio douglasii* var *longilobus*) to cattle. *Am J Vet Res.* 45:26-31.
- Mattocks A. 1986. *Chemistry and toxicology of pyrrolizidine alkaloids*. Academic Press London.
- Mattocks AR. 1972. Acute hepatotoxicity and pyrrolic metabolites in rats dosed with pyrrolizidine alkaloids. *Chemical and Biological Interactions* 5:227-242.
- Mattocks AR, White INH. 1968. Toxicity of pyrrolizidine alkaloids. *Nature London* 217:723-728.

- Mohabbat O, Shafiq Younos M, Merzad A, Srivastava R, Ghaos Sediq G, Aram G. 1976. An outbreak of hepatic veno-occlusive disease in north-western Afghanistan. *Lancet*. 308:269-271.
- Rose CL, Harris PN, Chen KK. 1959. *J Pharmacol Exp Ther*. 126:179-184.
- Smith LW, Culvenor CC. 1981. Plant sources of hepatotoxic pyrrolizidine alkaloids. *J Nat Prod*. 44:129-152.
- Stegelmeier BL, Edgar JA, Colegate SM, Gardner DR, Schoch TK, Coulombe RA, Molyneux RJ. 1999. Pyrrolizidine alkaloid plants, metabolism and toxicity. *J Nat Toxins*. 8:95-116.
- Stegelmeier BL, Gardner DR, James LF, Molyneux RJ. 1996. Pyrrole detection and the pathologic progression of *Cynoglossum officinale* (houndstongue) poisoning in horses. *J Vet Diagn Invest*. 8:81-90.
- Wang C, Li Y, Gao J, He Y, Xiong A, Yang L, Cheng X, Ma Y, Wang Z. 2011. The comparative pharmacokinetics of two pyrrolizidine alkaloids, senecionine and adonifoline, and their main metabolites in rats after intravenous and oral administration by UPLC/ESIMS. *Anal Bioanal Chem*. 401:275-287.
- WHO. 1988a. Pyrrolizidine Alkaloids, Health and Safety Guide. World Health Organization Geneva; 20.
- WHO. 1988b. Pyrrolizidine Alkaloids; Environmental Health Criteria. World Health Organization Geneva; 1-345.
- Xia Q, Ma L, He X, Cai L, Fu PP. 2015. 7-Glutathione Pyrrole Adduct-A Potential DNA Reactive Metabolite of Pyrrolizidine Alkaloids. *Chem Res Toxicol*.
- Xia Q, Zhao Y, Von Tungeln LS, Doerge DR, Lin G, Cai L, Fu PP. 2013. Pyrrolizidine Alkaloid-derived DNA Adducts as a Common Biological Biomarker of Pyrrolizidine Alkaloid-induced Tumorigenicity. *Chem Res Toxicol*.

## CHAPTER IV

THE COMPARATIVE TOXICITY OF A REDUCED, CRUDE COMFREY  
(*SYMPHYTUM OFFICINALE*) ALKALOID EXTRACT AND THE PURE,  
COMPHREY-DERIVED PYRROLIZIDINE ALKALOIDS, LYCOPSAMINE AND  
INTERMEDINE IN CHICKS (*GALLUS GALLUS DOMESTICUS*)Short Abstract<sup>1</sup>

Comfrey (*Symphytum officinale*), a commonly used herb, contains pro-toxic dehydropyrrolizidine alkaloids, including lycopsamine and intermedine, and has consequently been internationally regulated with respect to its use. To help further define the toxicity of *S. officinale*, male, California White chicks were used to compare the toxicity of a crude, reduced comfrey alkaloid extract to purified lycopsamine and intermedine. Based on clinical, serum biochemical, tissue adduct concentrations, and histopathological analysis the reduced comfrey extract was more toxic than either pure lycopsamine or intermedine. This suggests a cautionary note when estimates of herbal toxicity are based upon the observed toxicity of isolated toxins.

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Ammon W. Brown, Bryan L. Stegelmeier, Steven M. Colegate, Dale R. Gardner, Kip E. Panter, Edward L. Knoppel, and Jeffery O. Hall (in review). The Comparative Toxicity of a Reduced, Crude Comfrey (*Symphytum officinale*) alkaloid extract and the Pure, Comfrey-Derived Pyrrolizidine Alkaloids, Lycopsamine and Intermedine in Chicks (*Gallus gallus domesticus*). *Journal of Applied Toxicology*.

## Abstract

Comfrey (*Symphytum officinale*), a commonly used herb, contains dehydropyrrolizidine alkaloids that, as a group of bioactive metabolites, are potentially hepatotoxic, pneumotoxic, genotoxic and carcinogenic. Consequently, regulatory agencies and international health organizations have recommended comfrey be used for external use only. However, in many locations comfrey continues to be ingested as a tisane or as a leafy vegetable. The objective of this work was to compare the toxicity of a crude, reduced comfrey alkaloid extract to purified lycopsamine and intermedine that are major constituents of *S. officinale*. Male, California White chicks were orally exposed to daily doses of 0.04, 0.13, 0.26, 0.52, and 1.04 mmol lycopsamine, intermedine or reduced comfrey extract  $\text{kg}^{-1}$  bodyweight for 10 days. After another seven days, chicks were euthanized. Based on clinical signs of poisoning, serum biochemistry, and histopathological analysis, the reduced comfrey extract was more toxic than lycopsamine and intermedine. This work suggests a greater than additive effect of the individual alkaloids and/or a more potent toxicity of the acetylated derivatives in the reduced comfrey extract. It also suggests that safety recommendations based on purified compounds may underestimate the potential toxicity of comfrey.



## Introduction

Comfrey has been used for a wide variety of medicinal purposes for over 2,000 years (Rode 2002), and it continues to be consumed as an herbal tea or a vegetable in many countries (Mei, *et al.* 2010). More recently, controlled studies have found multiple comfrey-based topical treatments to be beneficial for treatment of a variety of muscle and joint pains (Koll, *et al.* 2004; Staiger 2012). The anti-inflammatory and analgesic effects of comfrey are thought to be due to the imidazolidinylurea allantoin and the phenylpropanoid rosmarinic acid (Staiger 2012). Comfrey also contains dehydropyrrolizidine alkaloids (DHPAs) which are pro-toxins that are hepatotoxic, and potentially pneumotoxic, genotoxic, and carcinogenic (Mei, *et al.* 2010). As a result of the health hazards posed by DHPAs, health organizations and food and drug safety agencies in several countries have developed regulations and recommendations regarding the sale and use of comfrey. In 2001 the US Food and Drug Administration sent an advisory letter to manufacturers of dietary supplements requesting that they remove all comfrey products intended for consumption from the market (FDA 2001). In Germany, the Federal Institute for Risk Assessment (BfR) conducted a risk assessment for DHPAs and concluded that exposure should be kept as low as possible limiting tolerable daily intake to 0.007  $\mu\text{g}$  of unsaturated pyrrolizidine alkaloids  $\text{kg}^{-1}$  bodyweight (BW) (BfR 2011). In 2008, the UK Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment released a statement on DHPAs in food, which supported that of the BfR, limiting daily oral exposures to less than 0.007  $\mu\text{g}$  DHPAs  $\text{kg}^{-1}$  BW oral (Committee on Toxicity of Chemicals in Food 2008). The World Health Organization,

Dutch National Institute for Public Health and the Environment, and European Food Safety Authority have all conducted similar reviews with similar concerns and recommendations (EFSA 2011; RIVM 2005; WHO 2011). The Food Standards Australia New Zealand authority recommends a somewhat higher tolerable exposure of 1  $\mu\text{g}$  DHPAs  $\text{kg}^{-1}$  BW per day based exclusively on hepatotoxicity as opposed to potential carcinogenicity (FSANZ 2001).

DHPA-related toxicity has been associated with its *in vivo* oxidation to a didehydropyrrolizidine alkaloid metabolite, referred to as the “pyrrolic” or dihydropyrrolizine (DHP) metabolite, that is a bifunctional alkylating agent of macromolecules forming adducts with proteins and DNA (Edgar, *et al.* 2014). The indirect detection of these “pyrrolic” adducts by their oxidative cleavage from the tissue adducts has been used as an indicator of exposure to the DHPAs (Lin, *et al.* 2011; Mattocks and Jukes 1990; 1992a; 1992b; Stegelmeier, *et al.* 1996; Winter, *et al.* 1990). Therefore, the observation of adverse clinical signs or pathological changes in this study will be correlated with the indirect detection of “pyrrolic” adducts in an attempt to quantify exposure.

A California White male chick model has recently been investigated to assess the comparative toxicity of pure DHPAs in a biologically-relevant manner and using less alkaloid than some other models (Stegelmeier *et al.*, unpublished). Therefore, the objective of this study was to use this model to compare the toxicity of a reduced, crude comfrey alkaloid extract with that of pure lycopsamine and intermedine, the two major alkaloids isolated from the reduced comfrey extract.

## Materials and Methods

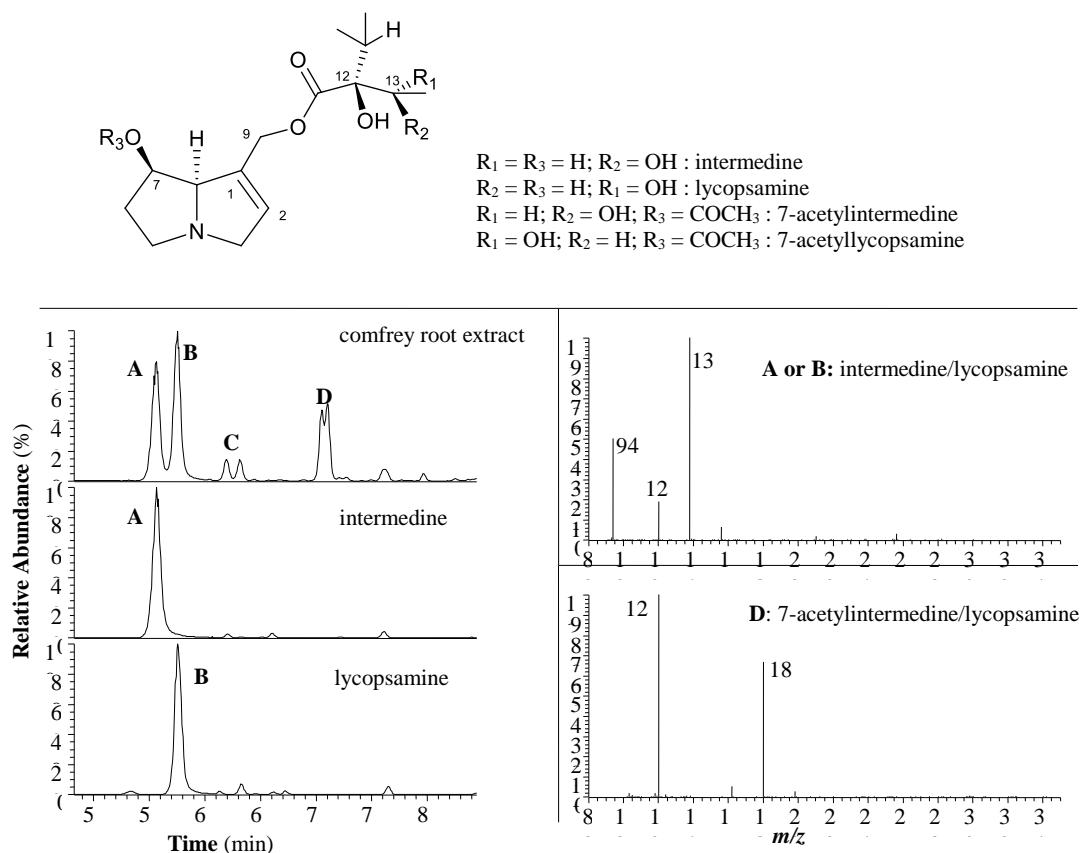
### **Animals**

Three-day-old male, California White chicks (*GALLUS GALLUS DOMESTICUS*) were purchased from Privett Hatchery (Portales, New Mexico) through Intermountain Farmers Association (Hyde Park, Utah). California White chickens are a commercial hybrid resulting from the cross of a White Leghorn hen and a California Grey rooster touted as a hardy breed that is somewhat easier to handle than the White Leghorn. During a 3-day acclimation period before initiation of treatment, the chicks were weighed and outliers culled such that test birds were of a uniform size and body condition. Chicks were housed in heated brooder cages that provided a thermal gradient within their microenvironment ranging from roughly 16 to 25°C. All animals had free access to fresh water and a commercial 20% protein poultry starter purchased from Intermountain Farmers Association (Salt Lake City, Utah). The brooder cages were housed in a windowed room and thus exposed to ambient sunlight. Room lights were used such that there was a minimum of twelve hours of light each day. Humidity in the brooder cages was essentially room-ambient humidity which ranged from approximately 30% to 60% during the course of this study. This research was conducted with the approval of the Utah State University Animal Care and Use Committee (IACUC Protocol #2055).

### **Preparation of Dehydropyrrolizidine Alkaloid Test Samples**

Dry, powdered root of common comfrey (*Symphytum officinale*) was purchased from Starwest Botanicals (Cordova, CA, USA) or from Take Herb (Alhambra, CA,

USA). After mixing the powdered comfrey root from both sources a crude extract of the comfrey root alkaloids (including reduction of N-oxides to their free base forms) and the monoester DHPAs lycopsamine and intermedine (Fig. 4-1) were isolated from a reduced, crude alkaloidal extract of the powdered comfrey as previously described (Colegate, *et al.* 2014). The extract and purified samples were analyzed using high pressure liquid



**Figure 4-1.** The structures of lycopsamine, intermedine and their 7-acetyl derivatives. The HPLC-esi(+)MS ion chromatograms show a high purity for the isolated lycopsamine (**B**) and intermedine (**A**) and a mixture of lycopsamine, intermedine and their 7-acetyl derivatives (**D**) in the comfrey reduced alkaloid sample. Also present in the latter are minor, residual concentrations of intermedine and lycopsamine N-oxides (**C**). The MS/MS profiles confirm the identity of lycopsamine and intermedine, and C7 as the site of acetylation rather than C13.

chromatography coupled to an electrospray ionization mass spectrometer operated in the positive ion full scan and tandem mass spectrometry mode (HPLC-esi(+))MS and MS/MS).

### **Experimental Design**

Eighty chicks were randomly divided into 16 groups of five and assigned to a dose group for comfrey extract, lycopsamine or intermedine. Each test compound(s) was administered at five doses i.e., 0.04, 0.13, 0.26, 0.52, and 1.04 mmols DHPA free base  $\text{kg}^{-1}$  BW per day. Five animals were assigned to the control group that received a volume of absolute ethanol equal to the largest volume received by any of the other groups.

Dosing was based on purified alkaloid toxicity studies using the same model (Stegelmeier, *et al.*, unpublished). In the purified alkaloid studies doses were 0.01, 0.04, 0.13, and 0.26 mmols DHPA free base  $\text{kg}^{-1}$  BW per day. Because lycopsamine was found to be less toxic than the other purified alkaloids tested, the two higher doses were added. Daily doses were divided into two equal portions half of which was given in the morning and the other half was given in the afternoon via gavage. Water was added to all doses such that the final volume was 0.5 mL. All animals were weighed three times per week, and doses were recalculated prior to the next treatment. The chicks were dosed for 10 days and maintained for another seven days before they were euthanized with carbon dioxide for necropsy examination. Chicks were monitored at least twice daily for the duration of the study. Any animals that became moribund were euthanized with carbon dioxide and necropsied.

### **Tissue Preparation**

At post-mortem examination, gross abnormalities were recorded and the entire brain, heart, spleen, and right liver lobe, and representative samples of lung, kidney, testicle, crop, proventriculus, ventriculus, small intestine, colon, cecum and bursa of Fabricius were collected, preserved in neutral buffered formalin, embedded in paraffin and subsequent sections were stained with hematoxylin and eosin via standard procedures for histopathologic analysis. A random number chart was used to determine the start location for sectioning the right liver lobe. The entire right liver lobe was sectioned every 3 mm transversely along the long axis prior to embedding and staining. Blood samples were taken immediately after euthanasia via cardiac puncture, divided into serum and packed cells, and frozen. The left liver lobe was collected and stored at -80°C for “pyrrole” adduct analysis.

### **Histologic Grading**

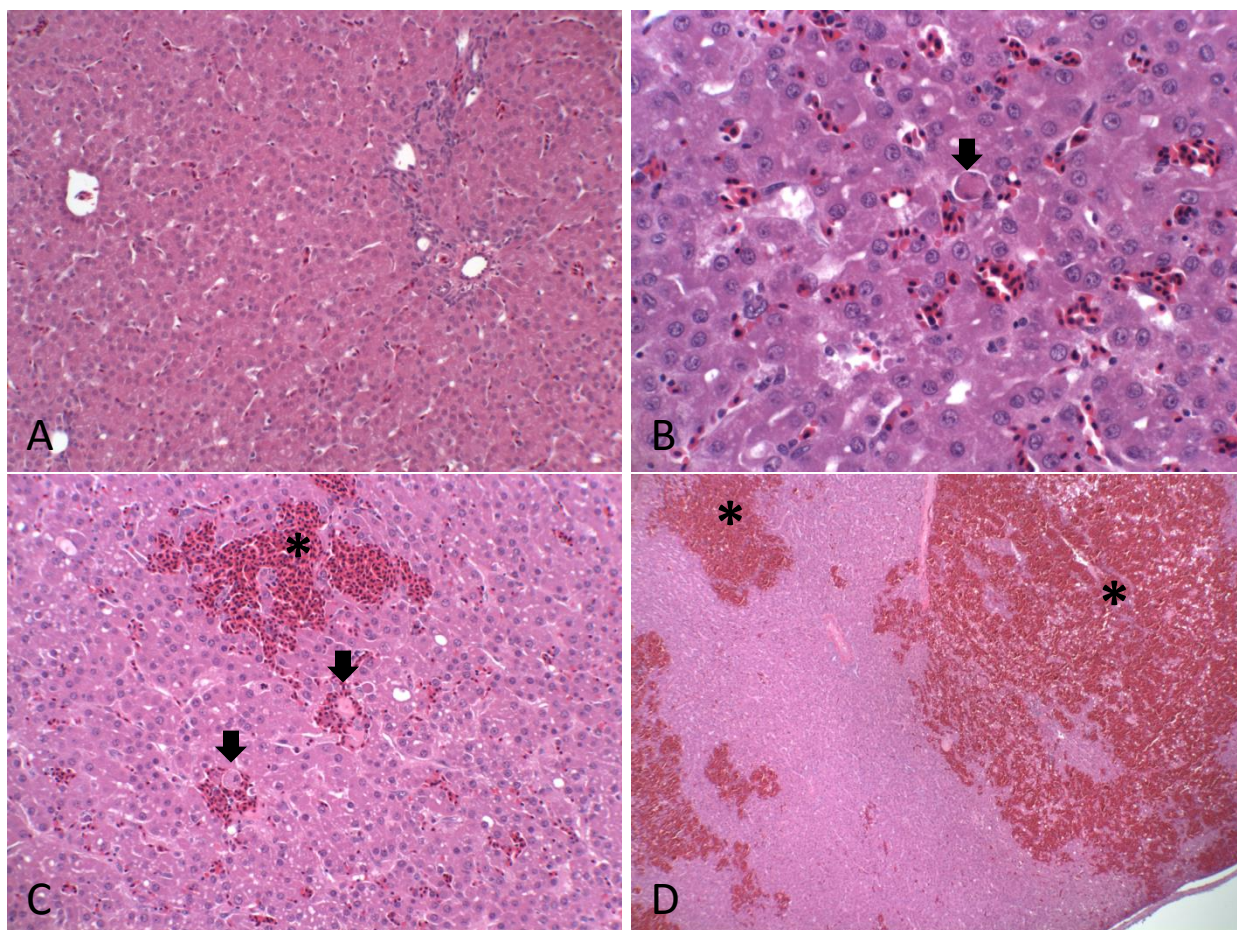
Liver size was quite variable between animals and consequently all sections of the right liver lobe could occupy from one to four slides. Each slide was graded by examining all sections on that slide and scored for necrosis as follows: 0 - no observed hepatocellular necrosis; 1 - scattered individual cell death; 2 - multifocal areas of necrosis which encompassed up to 25% of the sections on that slide; 3 - larger areas of necrosis were present accounting for between 25 and 50% of the sections; and 4 - greater than 50% of the observed sections were necrotic (Fig. 4-2). The hepatic necrosis score for each animal was computed by taking the average of the necrosis scores for each slide of liver tissue from that animal.

### “Pyrrole” Adduct Detection

The indirect detection of hepatic “pyrrolic” adducts was an adaptation of methods originally reported by Mattocks and Jukes (Mattocks and Jukes 1992a; 1992b) and more recently described by Lin *et al.* (Lin, *et al.* 2011) (Fig. 4-3).

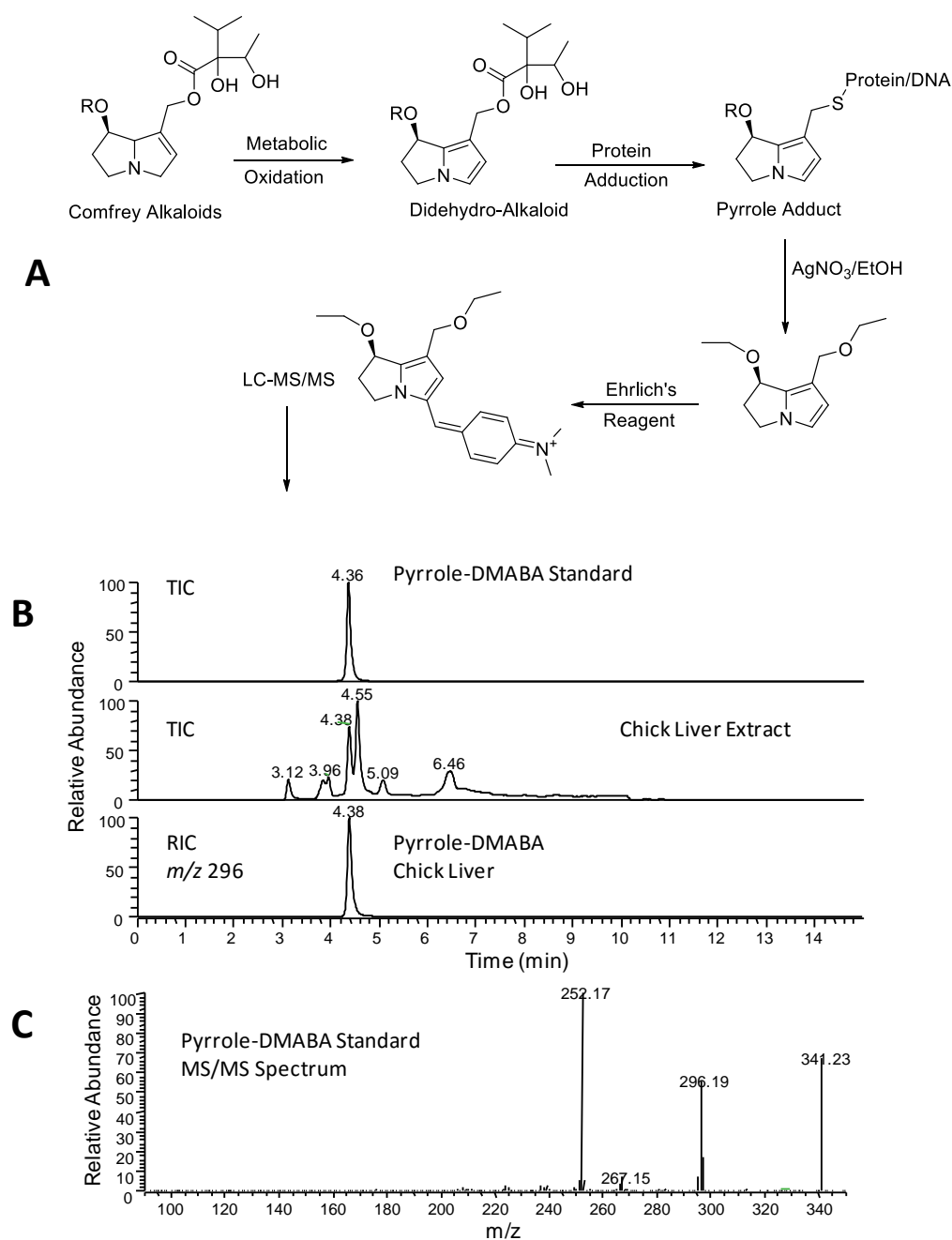
Monocrotaline (crotaline), tetrachloro-*o*-benzoquinone (*o*-chloranil, 97%), boron trifluoride diethyletherate (BF<sub>3</sub>•O(C<sub>2</sub>H<sub>5</sub>)<sub>2</sub>) and silver nitrate were purchased from Sigma-Aldrich (St. Louis, Missouri). *p*-Dimethylaminobenzaldehyde (DMABA) was from ICN Biochemical (Cleveland, Ohio). Acetonitrile was HPLC grade (Sigma-Aldrich), and absolute ethanol was (reagent grade). Formic acid (98%, Sigma Aldrich) used for HPLC was diluted with purified water (18.2 MΩ cm<sup>-1</sup>) (WaterPro PS Station, Labconco, Kansas City, MO, USA) to a concentration of 0.1% (V/V). Ehrlich’s solution was prepared by dissolving DMABA (0.2 g) in ethanol (10 mL) with purified, redistilled BF<sub>3</sub>•O(C<sub>2</sub>H<sub>5</sub>)<sub>2</sub> (2 mL).

Dehydromonocrotaline (DHMC), used for the positive control standard, was prepared by oxidizing monocrotaline using a modification of procedures previously reported (Culvenor, *et al.* 1970). *o*-Chloranil (30 mg) was added to a solution of monocrotaline (30 mg) in chloroform (4 mL) and shaken for 30 to 60 sec. A saturated solution of potassium hydroxide (KOH) containing 2% (w/v) sodium borohydride (NaBH<sub>4</sub>) (4 mL), purchased from Mallinckrodt (Paris, Kentucky) was added to the oxidation solution and shaken until the color faded. The solvent layers were separated by centrifugation; the organic layer was filtered through anhydrous sodium sulfate; and the solvent was removed by roto-evaporation at 40°C and reduced pressure to afford



**Figure 4-2. H&E** Examples of hepatocellular necrosis scores. A. 200X. Necrosis score 0. Normal liver from a control chick. B. 400X. Necrosis score 1. Liver from a chick exposed to  $0.52 \text{ mmol kg}^{-1} \text{ body weight (BW) day}^{-1}$  comfrey extract with a necrosis score of one. Note the individual cell death (arrow). C. 200X. Necrosis score 2. Liver from a chick exposed to  $1.04 \text{ mmol kg}^{-1} \text{ BW day}^{-1}$  comfrey extract with a necrosis score of two. Note the individualized hepatocytes that are surrounded by hemorrhage (arrows) and the lake of hemorrhage (\*) where hepatic cords should be. D. 100X. Necrosis score 3. Liver from a chick exposed to  $1.04 \text{ mmol kg}^{-1} \text{ BW day}^{-1}$  comfrey extract with a necrosis score of three. Note the large lakes of hemorrhage (\*), with occasional rafts of hepatocytes.





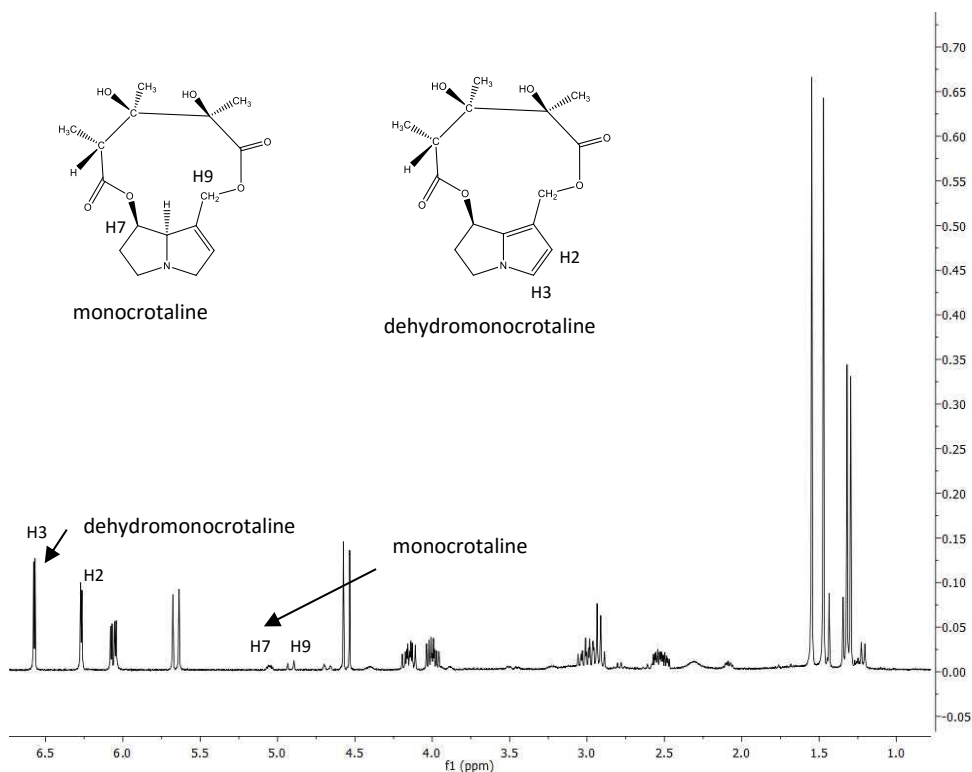
**Figure 4-3.** Schematic outline of the indirect detection of pyrrole liver adducts starting with the metabolic oxidation of comfrey alkaloids, hydrolysis of pyrrolic adducts and formation of pyrrole-DMABA complex (**A**). The HPLC-esi(+)MS/MS total ion chromatograms (TIC) and reconstructed ion chromatograms (RIC) from pyrrole-DMABA standard (top) and then that obtained from chick liver (**B**: middle and bottom). MS/MS spectrum of the pyrrole-DMABA standard (**C**).

the oxidation product (30 mg). Integration and comparison of the H2, H3, H7 and H9 resonance signals for DHMC and monocrotaline in the  $^1\text{H}$ -NMR spectrum (Culvenor *et al.*, 1970) (Fig. 4-4) of the oxidation product indicated an 87:13 mixture of DHMC to residual monocrotaline. A measured quantity of oxidation product was dissolved in absolute ethanol to give a  $2.69 \mu\text{mol mL}^{-1}$  pyrrole equivalent standard stock solution.

Ethanol silver nitrate was prepared by dissolving silver nitrate (625 mg) in deionized water (0.5 mL) with sonication for 1-2 minutes. Absolute ethanol (25 mL) was added, and the mixture was sonicated until all of the silver nitrate had redissolved (approximately 5 min).

“Pyrrole” calibration standards were prepared by diluting an aliquot (50  $\mu\text{L}$ ) of the pyrrole standard stock solution with ethanol (900  $\mu\text{L}$ ) and Erlich’s solution (50  $\mu\text{L}$ ) to afford a “pyrrole” concentration of  $135 \text{ nmol mL}^{-1}$ . An aliquot (50  $\mu\text{L}$ ) was further diluted with ethanol (950  $\mu\text{L}$ ) i.e.,  $6.8 \text{ nmol “pyrrole” mL}^{-1}$  which was further serially diluted (1/5) to give standards at 1.35, 0.27, 0.054, 0.11 and 0.002  $\text{nmol “pyrrole” mL}^{-1}$ .

Entire left liver lobes frozen from individual chicks were freeze-dried using a Labconco® freeze dryer. Portions of the lyophilized liver lobes were placed in conical tubes with small copper coated steel spheres. The conical tubes were then placed in a Retech® MM301 shaker at 20 revolutions  $\text{s}^{-1}$  for 10 min. An accurately weighed portion (ca. 25 mg) of the crushed, lyophilized chicken liver was then placed into a plastic snap-cap conical tube. Ethanol silver nitrate (1.0 mL) was added to the sample and the



**Figure 4-4.**  $^1\text{H-NMR}$  Spectrum of dehydromonocrotaline. The ratio of dehydromonocrotaline to that of the residual monocrotaline was measured from integration of the H2 and H3 protons from dehydromonocrotaline versus the H7 and H9 protons from the residual monocrotaline.

sample mixed for 30 min on an auto rotator. Trifluoroacetic acid (10  $\mu\text{L}$ ) was added to the mixture after 30 min and samples further mixed on the auto rotator overnight (~16 hrs). The samples were then centrifuged at 13000 rpm (16000 G) for 10 min. An aliquot (20  $\mu\text{L}$ ) of the supernatant was added to absolute ethanol (170  $\mu\text{L}$ ) containing of Erlich's reagent (10  $\mu\text{L}$ ) in an HPLC autosampler vial.

The HPLC-esi(+) MS/MS system consisted of a Agilent 1260 Infinity HPLC System (Agilent Technologies, Santa Clara, CA, USA). Samples (5  $\mu\text{L}$ ) were injected onto a Synergi Hydro RP column (75 x 2 mm, 4 $\mu$ ) fitted with a guard column of similar

adsorbent. A gradient flow ( $300 \mu\text{L min}^{-1}$ ) of 0.1% formic acid in water (A) and acetonitrile (B) was used to elute the sample components from the column using the following linear gradient: 20% B (0-1 min); 20% - 45% B (1-2 min); 45% - 75% B (2-11 min); 75% B (11-15 min); 75% - 20% B (15-16 min); and 20% B (16-21 min) for re-equilibration of the column.

Flow from the HPLC column was connected to a Velos Pro LTQ mass spectrometer (Thermo Scientific, Waltham, MA, USA) equipped with a heated electrospray ionization (HESI) source. The capillary temperature was set at  $275^{\circ}\text{C}$ , the ionization spray voltage at 3.45 kV, the HESI source heater temperature at  $305^{\circ}\text{C}$  and the sheath gas flow was 40 units with an auxiliary flow of 5 units. The mass spectrometer was monitored in a positive ion MS/MS mode, scanning product ions from a mass range of  $m/z$  90-350 after fragmentation of the parent ion ( $\text{MH}^+ = 341$ ) using a relative collision energy setting of 45% with high-energy collision induced dissociation (HCD). Under these conditions the “pyrrole”-DMABA compound eluted with a retention time of 4.4 min and the resulting MS/MS spectrum contained major fragment ions at  $m/z$  252 and 296. The detected “pyrrole” peak area was measured from the reconstructed ion chromatogram displaying  $m/z$  296 (Fig. 4-3) and quantitated based on an external calibration curve established from the standards. The resulting “pyrrole” concentration ( $\text{nmol mL}^{-1}$ ) of the injected sample was converted to  $\text{nmol g}^{-1}$  of liver.

### **Serum Biochemistry Analysis**

Frozen serum samples were outsourced (Antech Laboratories, Indianapolis, Indiana) for analysis of total protein, glucose, triglycerides, cholesterol, gamma glutamyl

transferase (GGT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), sorbitol dehydrogenase (SDH), bile acid, and creatine phosphokinase (CPK).

### **Statistical Analysis**

Statistical analyses were performed using Proc GLM and Proc Freq in SAS 9.3 (SAS Institute, Cary, NC). A two way ANOVA with Tukey-Kramer adjustment for multiple comparisons was used to compare average daily gain, liver weight / body weight, necrosis scores, “pyrrole” concentration, and serum biochemistries. A Pearson correlation was used to determine the relationship between pyrrole and dose for each alkaloid. Proc Freq was used to compare the frequency of ascites present at necropsy between different groups. A p-value of <0.05 was considered significant.

## **Results**

### **Test Samples**

HPLC-esi(+)MS and MS/MS analysis showed that the reduced crude extract was comprised mainly of the monoester DHPA lycopsamine, its C13 epimer intermedine, and their C7-acetylated derivatives (Fig. 1). Minor concentrations of intermedine and lycopsamine N-oxides observed were residual due to an incomplete reduction step. Traces (< 1% based on integrated areas of HPLC ion chromatogram peaks) of the open chain diester DHPAs symmlandine and symphytine, were also observed. No other DHPAs reported to be found in comfrey (e.g., echimidine or its N-oxide) were observed in the reduced comfrey extract. Based on the relative integrated areas of the HPLC-MS base

ion chromatogram the ratio of lycopsamine, intermedine and the combined acetylated derivatives was 1.3 : 1.5 : 1.

The test sample solutions were prepared by accurately weighing sub-samples of the crude alkaloidal extract or of the purified lycopsamine and intermedine and dissolving them in ethanol to concentrations of 54.5 mg total alkaloid mL<sup>-1</sup>; 59.8 mg lycopsamine mL<sup>-1</sup>; and 60.4 mg intermedine mL<sup>-1</sup>. The calculated concentrations were confirmed by quantitative HPLC-esi(+)MS analysis against a lycopsamine standard calibration curve. Furthermore, pre- and post-dosing HPLC-esi(+)MS analysis of the samples did not reveal any degradation in ethanol solution over the 10 day dosing period.

## **Clinical**

At the doses used in this study, the chicks in the lycopsamine or intermedine-dosed groups did not show any difference in weight gain over the course of the study compared to controls. The reduced comfrey extract-dosed animals showed a dose-dependent decrease in average daily gain (Table 1). The chicks in the 1.04 and 0.52 mmol kg<sup>-1</sup> groups gained an average of only 1.6 and 4.2 g day<sup>-1</sup> respectively, whereas the controls gained an average of 7.4 g day<sup>-1</sup>. All five of the chicks in the 1.04 and one of the chicks in the 0.52 mmol kg<sup>-1</sup> reduced comfrey extract groups developed ascites over the course of the study (Table 1). Two high dose (1.04 mmol kg<sup>-1</sup>) reduced comfrey extract-exposed chicks had to be euthanized at 7 and 10 days into the study.

**Table 4-1.** Group averages and (SD) of chicks dosed with comfrey, lycopsamine, intermedine and control at 0.4, 0.13, 0.26, 0.52, and 1.04 mmol/kg BW per day. Comparisons were made with average daily gain (ADG), percent of liver weight to total body weight, number of animals with ascites at necropsy, the average necrosis score for the group, the average “pyrrole” concentration in the liver, and serum sorbitol dehydrogenase (SDH), bile acid, and glucose at death. Different superscript letters denote differences at P<0.05. All comparisons were made using a two way ANOVA with the exception of number of animals with ascites where a Fisher’s exact test was used. Numbers in parenthesis are one standard deviation.

	Dose (mmol/kg)	ADG (g/day)	Liver wt. % Body wt.	Ascites	Necrosis Score	“pyrrole” Conc. (nmol/g)	SDH (IU/L)	Bile Acid ( $\mu$ mol/L)	Glucose (mg/dL)
Comfrey	1.04	1.6 <sup>a</sup> (1.0)	2.8 <sup>a</sup> (0.8)	5/5 <sup>a</sup>	2.3 <sup>a</sup> (0.4)	0.59 <sup>a</sup> (0.12)	9.7 <sup>a</sup> (4.5)	117 <sup>a</sup> (86)	187 <sup>a</sup> (101)
	0.52	4.2 <sup>b</sup> (1.8)	3.6 <sup>a,b</sup> (0.6)	1/5 <sup>b</sup>	1.1 <sup>b</sup> (1.2)	0.29 <sup>a,b,c</sup> (0.03)	9.4 <sup>a</sup> (7.6)	56 <sup>b</sup> (55)	349 <sup>b,c,d,e</sup> (67)
	0.26	6.7 <sup>b,c</sup> (1.5)	3.5 <sup>a,b</sup> (0.4)	0/5 <sup>b</sup>	0.6 <sup>b,c</sup> (1.0)	0.24 <sup>b,c</sup> (0.04)	2.5 <sup>b</sup> (1.0)	15 <sup>b</sup> (3)	400 <sup>c,d,e</sup> (57)
	0.13	7.8 <sup>c</sup> (0.9)	3.7 <sup>a,b</sup> (0.5)	0/5 <sup>b</sup>	0.4 <sup>b,c</sup> (0.8)	0.13 <sup>b,c</sup> (0.07)	3.3 <sup>b</sup> (3.2)	24 <sup>b</sup> (17)	383 <sup>b,c,d,e</sup> (45)
	0.04	7.4 <sup>c</sup> (0.8)	3.6 <sup>a,b</sup> (0.2)	0/5 <sup>b</sup>	0.0 <sup>c</sup> (0)	0.00 <sup>c</sup> (0)	2.2 <sup>b</sup> (0.5)	35 <sup>b</sup> (11)	452 <sup>e</sup> (68)
Lycopsamine	1.04	7.2 <sup>c</sup> (1.5)	4.0 <sup>b</sup> (0.5)	0/5 <sup>b</sup>	0.0 <sup>c</sup> (0)	0.38 <sup>a,b</sup> (0.41)	0.7 <sup>b</sup> (0.4)	13 <sup>b</sup> (10)	273 <sup>a,b,c</sup> (33)
	0.52	7.5 <sup>c</sup> (0.8)	4.2 <sup>b</sup> (0.5)	0/5 <sup>b</sup>	0.3 <sup>b,c</sup> (0.7)	0.29 <sup>a,b,c</sup> (0.3)	1.4 <sup>b</sup> (0.9)	12 <sup>b</sup> (8)	355 <sup>b,c,d,e</sup> (88)
	0.26	8.2 <sup>c</sup> (0.6)	4.1 <sup>b</sup> (0.3)	0/5 <sup>b</sup>	0.0 <sup>c</sup> (0)	0.04 <sup>c</sup> (0.06)	0.6 <sup>b</sup> (0.5)	15 <sup>b</sup> (8)	313 <sup>a,b,c,d</sup> (68)
	0.13	7.9 <sup>c</sup> (0.4)	3.9 <sup>a,b</sup> (0.3)	0/5 <sup>b</sup>	0.0 <sup>c</sup> (0)	0.01 <sup>c</sup> (0.02)	0.9 <sup>b</sup> (1.0)	28 <sup>b</sup> (12)	398 <sup>c,d,e</sup> (56)
	0.04	7.9 <sup>c</sup> (0.7)	4.1 <sup>b</sup> (0.4)	0/5 <sup>b</sup>	0.0 <sup>c</sup> (0)	0.00 <sup>c</sup> (0.01)	0.5 <sup>b</sup> (0.6)	23 <sup>b</sup> (10)	403 <sup>c,d,e</sup> (52)
Intermedine	1.04	6.3 <sup>b,c</sup> (2.1)	3.8 <sup>a,b</sup> (0.6)	0/5 <sup>b</sup>	0.1 <sup>b,c</sup> (0.2)	0.07 <sup>b,c</sup> (0.06)	1.7 <sup>b</sup> (1.9)	14 <sup>b</sup> (7)	263 <sup>a,b</sup> (29)
	0.52	8.4 <sup>c</sup> (1.0)	3.9 <sup>b</sup> (0.6)	0/5 <sup>b</sup>	0.1 <sup>b,c</sup> (0.2)	0.01 <sup>c</sup> (0.02)	0.2 <sup>b</sup> (0.2)	15 <sup>b</sup> (7)	344 <sup>b,c,d</sup> (47)
	0.26	7.3 <sup>c</sup> (0.5)	4.0 <sup>b</sup> (0.5)	0/5 <sup>b</sup>	0.0 <sup>c</sup> (0)	0.00 <sup>c</sup> (0)	2.3 <sup>b</sup> (1.2)	13 <sup>b</sup> (4)	357 <sup>b,c,d,e</sup> (56)
	0.13	7.8 <sup>c</sup> (0.9)	4.1 <sup>b</sup> (0.4)	0/5 <sup>b</sup>	0.0 <sup>c</sup> (0)	0.00 <sup>c</sup> (0)	0.6 <sup>b</sup> (0.6)	18 <sup>b</sup> (6)	400 <sup>c,d,e</sup> (50)
	0.04	7.5 <sup>c</sup> (1.2)	4.0 <sup>b</sup> (0.4)	0/5 <sup>b</sup>	0.0 <sup>c</sup> (0)	0.01 <sup>c</sup> (0.03)	1.8 <sup>b</sup> (0.9)	18 <sup>b</sup> (7)	385 <sup>b,c,d,e</sup> (40)
Control	0	7.4 <sup>c</sup> (1.0)	4.0 <sup>b</sup> (0.4)	0/5 <sup>b</sup>	0.0 <sup>c</sup> (0)	0.01 <sup>c</sup> (0.03)	1.8 <sup>b</sup> (0.9)	21 <sup>b</sup> (6)	431 <sup>d,e</sup> (40)

### **Serum Biochemistry**

Serum activity of the cytosolic hepatocellular enzyme SDH was increased in the two highest doses of comfrey (Table 4-1) when compared to controls. Bile acid was also increased in the chicks from the highest dose reduced comfrey extract group when compared to all other groups. Serum glucose concentrations were lower in chicks from all of the 1.04 mmol kg<sup>-1</sup> groups compared to controls but, only the chicks from the 1.04 mmol kg<sup>-1</sup> reduced comfrey extract group were significantly different compared to the lower dose cohorts. No differences were detected between any groups at any concentration for total protein, triglycerides, cholesterol, GGT, AST, LDH, and CPK.

### **Gross Observations and Histopathology**

At necropsy, all livers were weighed after excising and emptying the gall bladders. The percent of liver weight to body weight was low in the chicks from the 1.04 mmol kg<sup>-1</sup> reduced comfrey extract group, which was the only group that differed significantly from controls; however, they were not significantly different from the chicks in other reduced comfrey extract groups or to the chicks in the 1.04 mmol kg<sup>-1</sup> intermedine group.

The most serious histologic change was centrilobular, or zone three, hepatic necrosis that extended through the entire lobule in the most severe cases. In less severely affected individuals there were occasional small foci or individual hepatocytes that were surrounded by hemorrhage and had pyknotic nuclei. Histologic hepatic necrosis scores tended to be higher in the three highest doses of the reduced comfrey extract-treated animals. The average hepatic necrosis scores were significantly higher than controls in both the 1.04 and 0.52 mmol kg<sup>-1</sup> reduced comfrey extract groups, but only the 1.04

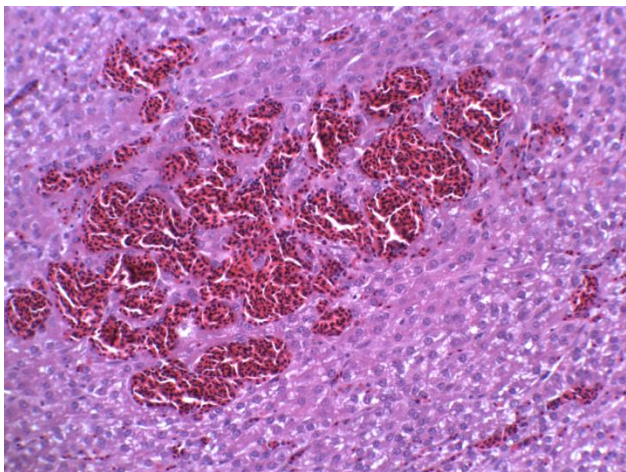


mmol kg<sup>-1</sup> group was significantly higher than the corresponding lycopsamine and intermedine groups.

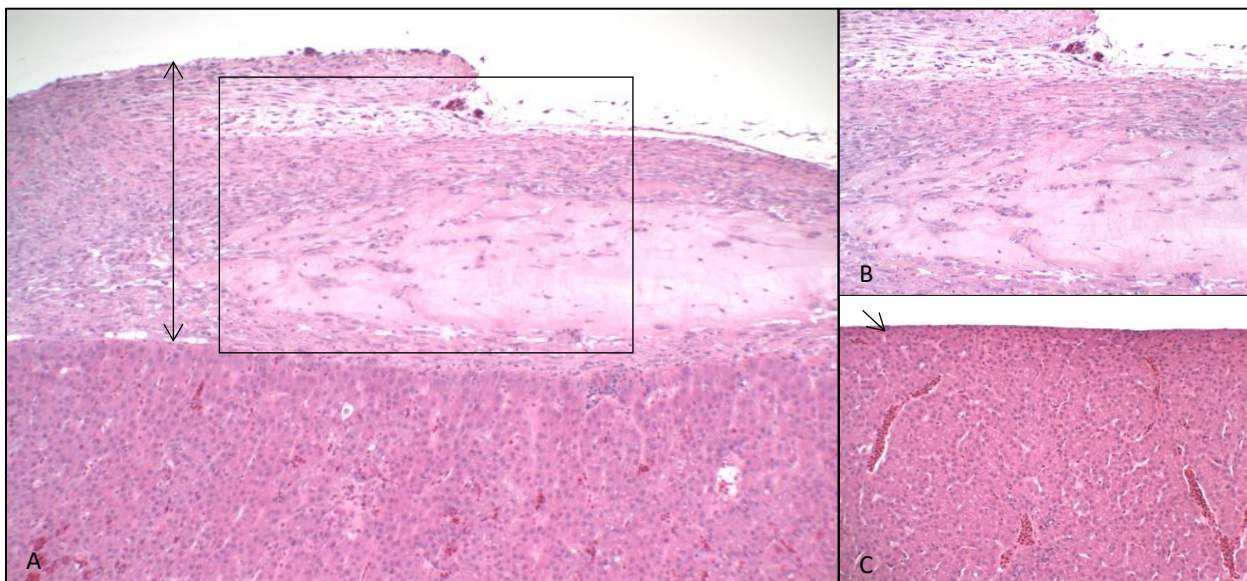
Angiectasis (Fig. 4-5) was present in three, four and three of the 0.26, 0.52, and 1.04 mmol kg<sup>-1</sup> lycopsamine-treated animals respectively. One chick each of the 0.13 and 0.26 mmol kg<sup>-1</sup>, and two and three of the 0.52 and 1.04 mmol kg<sup>-1</sup> reduced comfrey extract groups respectively also had angiectasis. Angiectasis was not observed in any of the animals in the intermedine-dosed groups.

In this study, regeneration was an uncommon finding, only observed in two reduced comfrey extract-treated animals (one each in 0.26 and 1.04 mmol kg<sup>-1</sup> dose groups). It presented primarily in portal areas and was composed of increased numbers of bile ducts with fewer, more basophilic-staining hepatocytes, indicative of immature hepatocytes, and occasional mitotic figures.

Capsular serositis (Fig. 4-6) was only present in chicks from the two higher dose reduced comfrey extract groups, and ranged from mild to severe. The capsules were diffusely and variably thickened and composed of spindle cells (likely fibroblasts), a variably dense eosinophilic matrix, and mononuclear inflammatory cells. Occasionally vascularization of the capsule was present. In the 1.04 mmol kg<sup>-1</sup> dose group four of five animals had a capsular serositis. The one animal that did not was euthanized on day 7 of the study. One of the five animals in the 0.52 mmol kg<sup>-1</sup> group also had capsular serositis.



**Figure 4-5. H&E 200X.** Angiectasis (peliosis hepatis) in a chick liver from a California White chick exposed to  $1.04 \text{ mMol kg}^{-1}$  comfrey.



**Figure 4-6. H&E.** Capsular serositis A. 100X Liver from a comfrey treated chick ( $1.04 \text{ mMol kg}^{-1}$  group) with a proliferative capsule (double headed arrow). B. 200X is an enlargement of the box in A. The capsule is composed of spindle cells and a variably dense fibrillar matrix and occasional small caliber vessels. C. 200X is a normal liver from a control chick. Note the very thin capsule (arrow).

Virtually every section of liver from all animals in all groups, including controls, showed mild to moderate periportal inflammation. There appeared to be no difference in frequency, intensity, type or distribution of inflammation between groups.

### **Tissue “Pyrrole” Adduct Detection**

“Pyrrole” was detected (Fig. 3) in chicks from the 0.13, 0.26, 0.52, and 1.04 mmol kg<sup>-1</sup> BW reduced comfrey extract and lycopsamine-dosed groups. Similarly, “pyrrole” was detected, albeit at lower concentrations than for the chicks in the reduced comfrey extract and lycopsamine groups, in chicks from the 0.04, 0.52, and 1.04 mmol kg<sup>-1</sup> BW intermedine-dosed groups. “Pyrrole” concentration tended to increase with dose for all alkaloids. Correlation coefficients for dose and reduced comfrey extract, lycopsamine and intermedine are 0.93, 0.58, and 0.58 respectively. “Pyrrole” concentration tended to be higher in comfrey extract and lycopsamine exposed chicks however, the variation between animals was too great, with the number of animals used, to determine significance (Table 1). Indeed, among the equivalent-dose groups, the only statistically significant difference that was observed was between the 1.04 mmol kg<sup>-1</sup> groups dosed with the reduced comfrey extract or intermedine.

## **Discussion**

California White chicks are highly sensitive to DHPA toxicity and this model allowed direct comparison of various clinical (general health and weight gains) and pathologic (gross pathology, serum biochemistry, histopathology and tissue adducts) indicators of toxicity. The pure lycopsamine and intermedine, and the reduced, crude

comfrey root alkaloid extract all resulted in some evidence of toxicity. However, the total alkaloid extract of comfrey root was more toxic than an approximately molar equivalent dose of either pure lycopsamine or pure intermedine with respect to clinical, serum biochemical, tissue “pyrrole” accumulation and histopathological comparisons. This is unexpected as lycopsamine and intermedine are the major DHPAs in this comfrey sample and they are enantiomers as they differ only in rotation of the chiral carbon on the ester side chain.

Previously reported toxicities of lycopsamine and intermedine are both  $1500 \text{ mg kg}^{-1}$  (LD50 for single IP injection in rats) (Cheeke and Shull 1985). No data are available for intraperitoneal exposure of comfrey in rats, but a diet containing 30% comfrey or about  $1000 \text{ mg kg}^{-1} \text{ day}^{-1}$  for 21 days resulted in a significantly reduced weight gain (Garrett, *et al.* 1982). It is difficult to compare these estimates because both DHPA content and quantity are highly variable in comfrey (Tice 1997).

It is uncertain why the comfrey extract was more toxic than lycopsamine and intermedine in this study. However, the significant presence of the acetylated derivatives in the comfrey extract may be the key factor. This is especially so since equimolar exposure of the chicks to either lycopsamine or intermedine in the comfrey extract was far less than when these DHPAs were dosed in pure form. The decreased polarity of the acetylated derivatives may affect initial absorption of the pro-toxins resulting in an overall increase in the DHPA load that reaches the liver. However, if absorption rates are approximately the same, then the potential for the acetylated derivatives to be more toxic per se is a consideration. In this regard it has been shown that the acetylated derivatives

were more genotoxic in a wing spot test of *Drosophila melanogaster* following oral administration of the alkaloids (Frei, *et al.* 1992).

Other possible factors that may have affected toxicity are: a greater than additive combined effect of lycopsamine and intermedine, the toxicity of the trace amounts of other minor alkaloids such as symphytine or symlandine, or competition for hepatic metabolizing enzymes from some other compound in the comfrey extract. Data comparing pure intermedine, pure lycopsamine and a combination of the two are not available, thus determining the combined effect is not possible without further research. Symphytine and symlandine are potentially about 10 times more toxic than either lycopsamine or intermedine (LD50 for symphytine is  $130 \text{ mg}^{-1} \text{ kg}^{-1}$  IP, male rat) (Hirono, *et al.* 1979). It would be unlikely for the proportion of symphytine/symlandine present in the comfrey extract to be overtly toxic alone, but it is conceivable that in conjunction with the other DHPAs it may have increased the toxicity.

The last consideration is competition for hepatic metabolizing enzymes from other compounds in the crude comfrey extract. If these other compounds, undetected using the HPLC-esi(+)MS analytical method, have a greater affinity for DHPA-detoxifying enzymes, then conceivably, if upregulation of these enzymes is slow, there will be more DHPA to be activated and lead to “pyrrole”-tissue adducts and consequent damage.

Histologically the lesions tended to be most severe in the comfrey extract groups, followed by the lycopsamine groups, and least severe in the intermedine exposed animals. In the comfrey extract exposed animals the highest dose group had significantly more widespread hepatocellular necrosis. Additionally, only animals exposed to the two

highest doses of comfrey extract developed capsular serositis. Regeneration characterized by oval cell hyperplasia and bile duct reduplication, which is thought to be an attempt to regenerate hepatic parenchyma when parenchymal cells have lost this capacity (Stalker and Hayes 2007), was only observed in two comfrey-treated animals. Angiectasis, which is thought to be a result of loss or weakening of sinusoidal walls and/or supporting tissue (Thoolen, *et al.* 2010) may be an earlier and perhaps more sensitive indicator of hepatic insult in this study. Only animals exposed to pure lycopsamine or the comfrey extract developed angiectasis. Angiectasis is a feature that has been reported in mice exposed to DHPAs (Brown, *et al.* 2015; Ruebner, *et al.* 1970) and may be important because it has been suggested that angiectasis may be a preneoplastic lesion in some DHPA-induced neoplasms. The concentration of “pyrrole” adducts tended to be higher in comfrey extract and lycopsamine exposed chicks compared to those exposed to intermedine which also suggests that lycopsamine may be slightly more toxic than intermedine. If this is the case it suggests that the stereochemistry around the thirteenth carbon is important to toxicity.

The toxicity of the DHPAs is entirely related to metabolic oxidation to form the electrophilic “pyrrolic” mono- and di-alkylating agents that react with nucleophiles on biomacromolecules such as proteins and DNA (Edgar, *et al.* 2014). Therefore, it was recognized that extraction and chemical release of these tissue conjugates could be used diagnostically to confirm a pyrrolizidine alkaloidosis (Mattocks and Jukes 1990; 1992a; 1992b; Winter, *et al.* 1990). Thus, “pyrrole” tissue adducts were originally indirectly detected as the diethylethers of 1-hydroxymethyl-7-hydroxy-6,7-dihydropyrrolizine (the

“pyrrole”) following oxidative cleavage from tissue adducts with silver using GC-MS. The procedure was adapted more recently to use HPLC-MS in which the oxidatively-cleaved “pyrrole”-diethylether was reacted with Ehrlich’s reagent to indirectly detect tissue “pyrrole” adducts in a human patient suffering from DHPA-induced hepatic sinusoidal obstruction syndrome (Lin, *et al.* 2011). In this present study, the generation of a “pyrrole” calibration curve from dehydromonocrotaline enabled a semi-quantitative comparison of in vivo “pyrrole” formation for each of the treatments groups.

The HPLC-MS method has advantages over the previous GC-MS method, specifically it is simpler to perform (it has fewer steps) and it is more reliable and more quantitative. Ideally detection of pyrrole tissue adducts in tissue can be more widely and effectively used in the diagnostic arena. This method needs to be validated in multiple species, and a greater understanding of the kinetics of tissue pyrrole adducts is necessary prior to full implementation as a diagnostic test.

In the present study there appears to be a dose dependent increase in “pyrrole” concentration particularly in the comfrey extract and lycopsamine exposed animals. Similar to the histologic analysis, the comfrey extract exposed animals tend to be most severely affected followed by the lycopsamine, and the intermedine exposed animals appear least affected although there is no statistical difference. Additional research may be helpful to determine the relevance of this trend.

The comfrey extract used in this study is somewhat different than what would be expected from natural exposure. Comfrey (*Symphytum officinale*, *S. uplandicum*) has been reported to produce several monoester and open chain diester DHPAs and their N-

oxides. In this present study, the crude alkaloid extract of the *S. officinale* powdered root was treated in the usual way with zinc and sulphuric acid to reduce most of the N-oxides to the free base DHPA forms. The resultant crude, reduced comfrey extract contained lycopsamine, intermedine and their acetylated derivatives as the predominant alkaloids (Fig. 1). When ingested DHPA-N-oxides are reduced in the gastro-intestinal tract to their parent free base, and although it has been estimated that N-oxides are similar in toxicity to their free base form (Frei, *et al.* 1992), in the present study the toxicity of N-oxides was not tested.

Different comfrey-derived (e.g. leaf, roots, flowers) products, comfrey-based products or products that contain comfrey as a minor additive will contain different concentrations of the alkaloids and their N-oxides. Therefore, any products that include comfrey could be analyzed and the concentrations of the alkaloids determined and assessed against the chick toxicity data presented herein to determine the level of risk. In this study, comfrey root was used as the source of relatively high concentrations of the alkaloids to facilitate the dosing.

In the assessment of risks presented to human health by dietary supplements, medicinal herbs, nutraceuticals or new functional foods, the application of data acquired using various *in vitro* or *in vivo* models is a major challenge with respect to extrapolation to the human situation. Therefore, models are continually changed or modified in an attempt to establish meaningful indications of toxicity. *In vivo* toxicity assessments of the pro-toxic DHPAs are complicated by considerable variations in species-, gender- and age-related susceptibilities. Additionally, assessment of entire products, crude extracts or



purified components presents potential complications associated with any intrinsic additive or protective effects of the whole plant or crude extract relative to the purified components. In this present study, male California White chicks, that have been shown to be sensitive to the DHPAs in terms of dose, duration of exposure and time for clinical and pathological signs to develop, were used to compare the hepatotoxicity of an orally-administered, reduced comfrey extract to that of the purified monoester DHPAs lycopsamine and intermedine (Fig. 4-1).

### Conclusions

The pure lycopsamine and intermedine, and the reduced, crude comfrey root alkaloid extract all resulted in some evidence of toxicity. In this California White male chick model, the total alkaloid extract of comfrey root was more toxic than an approximately equivalent (to the total alkaloid content of the reduced comfrey extract) dose of either pure lycopsamine or pure intermedine with respect to clinical, serum biochemical, tissue “pyrrole” accumulation and histopathological comparisons. This work suggests a greater than additive effect of the individual major components of the reduced comfrey extract and/or a more potent toxicity of the acetylated derivatives in the reduced comfrey extract. This may result in safety recommendations, based on selected purified compounds, underestimating the potential toxicity of comfrey-derived products.

## References

- BfR. 2011. Chemical analysis and toxicity of pyrrolizidine alkaloids and assessment of the health risk posed by their occurrence in honey, BfR opinion no. 038/2011 11.
- Brown AW, Stegelmeier BL, Colegate SM, Panter KE, Knoppel EL, Hall JO. 2015. Heterozygous p53 knockout mouse model for dehydropyrrolizidine alkaloid-induced carcinogenesis. *J. Appl. Toxicol.*
- Cheeke P, Shull L. 1985. Natural toxicants in feeds and livestock. West Port: AVI Publishing Inc.
- Colegate SM, Gardner DR, Betz JM, Panter KE. 2014. Semi-automated Separation of the Epimeric Dehydropyrrolizidine Alkaloids Lycopsamine and Intermedine: Preparation of their N-oxides and NMR Comparison with Diastereoisomeric Rinderine and Echinatine. *Phytochem Analysis.* 25:429-438.
- Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT). 2008. COT statement on pyrrolizidine alkaloids in food. Available: <http://cot.food.gov.uk/pdfs/cotstatementpa200806.pdf> [Accessed 6 June 2015].
- Culvenor C, Edgar J, Smith L, Tweeddale H. 1970. Dihydropyrrolizines. III. Preparation and reactions of derivatives related to pyrrolizidine alkaloids. *Aust J Chem.* 23:1853-1867.
- Edgar JA, Molyneux RJ, Colegate SM. 2014. Pyrrolizidine Alkaloids: Potential Role in the Etiology of Cancers, Pulmonary Hypertension, Congenital Anomalies and Liver Disease. *Chem Res Toxicol* 28: 4-20.
- EFSA. 2011. EFSA Panel on Contaminants in the Food Chain (CONTAM). Scientific Opinion on Pyrrolizidine alkaloids in food and feed. *EFSA Journal* 9:1-134.
- FDA. 2001. FDA advises dietary supplement manufacturers to remove comfrey products from the market. U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition. Available: <http://www.cfsan.fda.gov/~dms/dspltr06.html> [Accessed 6 July 2001].
- Frei H, Lüthy J, Brauchli J, Zweifel U, Würigler FE, Schlatter C. 1992. Structure/activity relationships of the genotoxic potencies of sixteen pyrrolizidine alkaloids assayed for the induction of somatic mutation and recombination in wing cells of *Drosophila melanogaster*. *Chem Biol Interact.* 83:1-22.

- FSANZ. 2001. Pyrrolizidine alkaloids in food. A toxicological review and risk assessment. In Food Standards Australia New Zealand Tech. Report series No. 2. Canberra, Australia.
- Garrett BJ, Cheeke PR, Miranda CL, Goeger DE, Buhler DR. 1982. Consumption of poisonous plants (*Senecio jacobea*, *Symphytum officinale*, *Pteridium aquilinum*, *Hypericum perforatum*) by rats: chronic toxicity, mineral metabolism, and hepatic drug-metabolizing enzymes. *Toxicol Lett.* 10:183-188.
- Hirono I, Haga M, Fujii M, Matsuura S, Matsubara N, Nakayama M, Furuya T, Hikichi M, Takanashi H, Uchida E. 1979. Induction of hepatic tumors in rats by senkirkine and symphytine. *J Natl Cancer I.* 63:469-472.
- Koll R, Buhr M, Dieter R, Pabst H, Predel H-G, Petrowicz O, Giannetti B, Klingenburg S, Staiger C. 2004. Efficacy and tolerance of a comfrey root extract (Extr. Rad. Symphyti) in the treatment of ankle distortions: results of a multicenter, randomized, placebo-controlled, double-blind study. *Phytomedicine.* 11:470-477.
- Lin G, Wang JY, Li N, Li M, Gao H, Ji Y, Zhang F, Wang H, Zhou Y, Ye Y, Xu HX, Zheng J. 2011. Hepatic sinusoidal obstruction syndrome associated with consumption of *Gynura segetum*. *J Hepatol.* 54:666-673.
- Mattocks AR, Jukes R. 1990. Recovery of the pyrrolic nucleus of pyrrolizidine alkaloid metabolites from sulphur conjugates in tissues and body fluids. *Chem Biol Interact.* 75:225-239.
- Mattocks AR, Jukes R. 1992a. Chemistry of sulphur-bound pyrrolic metabolites in the blood of rats given different types of pyrrolizidine alkaloid. *Nat Toxins.* 1:89-95.
- Mattocks AR, Jukes R. 1992b. Detection of sulphur-conjugated pyrrolic metabolites in blood and fresh or fixed liver tissue from rats given a variety of toxic pyrrolizidine alkaloids. *Toxicol Lett.* 63:47-55.
- Mei N, Guo L, Fu PP, Fuscoe JC, Luan Y, Chen T. 2010. Metabolism, genotoxicity, and carcinogenicity of comfrey. *J Toxicol Environ Health A.. Part B, Critical reviews* 13:509-526.
- RIVM. 2005. Advisory Report on Pyrrolizidine Alkaloids in Herb Preparations. Available:[http://www.rivm.nl/en/Documents\\_and\\_publications/Scientific/Reports/2015/april/Pyrrolizidine\\_alkaloids\\_in\\_herbal\\_preparations](http://www.rivm.nl/en/Documents_and_publications/Scientific/Reports/2015/april/Pyrrolizidine_alkaloids_in_herbal_preparations) [Accessed 8 April 2015].
- Rode D. 2002. Comfrey toxicity revisited. *Trends Pharmacol Sci.* 23:497-499.

- Ruebner BH, Watanabe K, Wand J. 1970. Lytic necrosis resembling peliosis hepatis produced by lasiocarpine in the mouse liver. A light and electron microscopic study. *Am J Pathol.* 60:247.
- Staiger C. 2012. Comfrey: a clinical overview. *Phytother Res.* 26:1441-1448.
- Stalker M, Hayes M. 2007. Liver and biliary system. Jubb, Kennedy, and Palmer's *Pathology of Domestic Animals* 2:297-388.
- Stegelmeier BL, Gardner DR, James LF, Molyneux RJ. 1996. Pyrrole detection and the pathologic progression of *Cynoglossum officinale* (houndstongue) poisoning in horses. *J Vet Diagn Invest.* 8:81-90.
- Thoolen B, Maronpot RR, Harada T, Nyska A, Rousseaux C, Nolte T, Malarkey DE, Kaufmann W, Kuttler K, Deschl U, Nakae D, Gregson R, Vinlove MP, Brix AE, Singh B, Belpoggi F, Ward JM. 2010. Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. *Toxicol Pathol.* 38:5s-81s.
- Tice R. 1997. Comfrey and One of Its Constituent Alkaloids, Symphytine: Review of the Toxicological Literature. National Institute of Environmental Health Sciences: Research Triangle Park, North Carolina;
- WHO. 2011. Discussion paper on pyrrolizidine alkaloids, Joint FAO/WHO food standards programme, Codex committee on contaminants in foods. The Hague, The Netherlands.
- Winter H, Seawright AA, Mattocks AR, Jukes R, Tshewang U, Gurung BJ. 1990. Pyrrolizidine alkaloid poisoning in yaks. First report and confirmation by identification of sulphurbound pyrrolic metabolites of the alkaloids in preserved liver tissue. *Aust Vet J.* 67:411-412.

CHAPTER V

HETEROZYGOUS P53 KNOCKOUT MOUSE MODEL FOR  
DEHYDROPYRROLIZIDINE ALKALOID INDUCED CARCINOGENESIS<sup>1</sup>

Abstract

Dehydropyrrolizidine alkaloids (DHPA) are a large, structurally diverse group of plant-derived protoxins that are potentially carcinogenic. With worldwide significance, these alkaloids can contaminate or be naturally present in the human food supply. To develop a small animal model that may be used to compare the carcinogenic potential of the various DHPAs, male heterozygous p53 knockout mice were administered a short-term treatment of riddelliine 5, 15 or 45 mg kg<sup>-1</sup> bodyweight day<sup>-1</sup> by oral gavage for 14 days, or dosed a long-term treatment of riddelliine 1 mg kg<sup>-1</sup> bodyweight day<sup>-1</sup> in pelleted feed for 12 months. Exposure to riddelliine increased the odds of tumor development in a dose-responsive manner (odds ratio 2.05 and Wald 95% confidence limits between 1.2 and 3.4). The most common neoplastic process was hepatic hemangiosarcoma, which is consistent with published lifetime rodent riddelliine carcinogenesis studies. Angiectasis (peliosis hepatis) and other previously unreported lesions were also identified. The results of this research demonstrate the utility of the

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<sup>1</sup>Brown, A. W., Stegelmeier, B. L., Colegate, S. M., Panter, K. E., Knoppel, E. L., & Hall, J. O. (2015). Heterozygous p53 knockout mouse model for dehydropyrrolizidine alkaloid-induced carcinogenesis. *Journal of Applied Toxicology*.

heterozygous p53 knockout mouse model for further investigation of comparative carcinogenesis of structurally and toxicologically different DHPAs and their N-oxides.

## Introduction

The toxic pyrrolizidine alkaloids are a group of approximately 500 structural variants (Wiedenfeld *et al.*, 2008) of monoesters, open chain diesters or macrocyclic diesters of the dehydropyrrolizidine alkaloid (DHPA) necine bases retronecine, heliotridine, and otonecine (Fig. 5-1A). The DHPAs and their co-occurring (usually the major components) N-oxides (Fig. 5-1A) are protoxins requiring activation *in vivo* by cytochrome P450 monooxygenases that result in hepatotoxicity (Campbell, 1956; Gilruth, 1904; Johnson *et al.*, 1985; Vardiman, 1952), pneumotoxicity (Stalker & Hayes, 2007), genotoxicity (Mei *et al.*, 2004, 2005; Mei & Chen, 2007) and carcinogenicity (Allen *et al.*, 1975; Schoental, 1975; Schoental & Cavanagh, 1972; Schoental *et al.*, 1970; Shumaker *et al.*, 1976; Yuzo *et al.*, 1977). In spite of the similarities in metabolism, and toxic responses, the National Toxicology Program (a branch of the US National Institutes of Health) has labeled only one DHPA, riddelliine, as reasonably anticipated to be a human carcinogen (NTP, 2003). This is due, at least in part, to the high cost and long duration of conventional carcinogenicity studies and the availability of sufficient amounts of pure DHPA for such studies. In this latter regard, riddelliine is one of the few DHPAs that can be purified relatively easily in large amounts (Adams *et al.*, 1942). To obtain sufficient evidence to list riddelliine as a potential human carcinogen, the National Toxicology Program assessed years of previous work and then conducted comprehensive animal studies using mice and rats. In these studies, animals were dosed orally 5 days per week with riddelliine for 2 years (Chan *et al.* 2003; NTP, 2003). They determined that male and female F344 rats, and male B6C3F1 mice exposed orally to

riddelliine were at a significantly increased risk of developing hepatic hemangiosarcoma, and that female B6C3F1 mice similarly exposed were at an increased risk of developing hepatocellular adenoma/carcinoma (Chan *et al.*, 2003; NTP, 2003).

For a complete assessment of carcinogenic potential, approximately 950 DHPAs (including their N-oxides) would need to be evaluated. Because of the requirement for large quantities of purified DHPA, and the time and funding impositions, it is not feasible to test all of these compounds using the same animal model as in the National Toxicology Program study. Consequently, the objective of this project was to develop a small animal model that would require less of the pure alkaloid, less time and, hence, less funding to test the carcinogenic potential of DHPAs in a manner relevant to natural exposure in both humans and animals.

The cellular tumor suppressor protein p53 critically regulates the cell cycle to reduce the potential for abnormal proliferation and consequent tumor development. Donehower (1996) showed that p53-deficient mice were more sensitive to carcinogens than normal mice, and that p53-deficient rodent bioassays might require fewer animals and less time to perform. Subsequent studies have shown that heterozygous p53 knockout mice have an advantage over p53 null mice in that they have a longer tumor-free period, allowing sufficient time for carcinogen testing without excessive background spontaneous cancers (Venkatachalam *et al.*, 2001). Therefore, it is hypothesized that p53 knockout mice may provide the basis for a small animal carcinogenesis model that could be completed in less time. In some species, including mice, males tend to be more susceptible than females (Chan *et al.*, 2003; Mattocks, 1986). On this basis, a study was



initiated that represented two potential human exposure scenarios, i.e. a single short duration or intermittent exposure, and a long-term, low-dose continuous exposure to the suspected carcinogenic DHPA, riddelliine.

## Materials and Methods

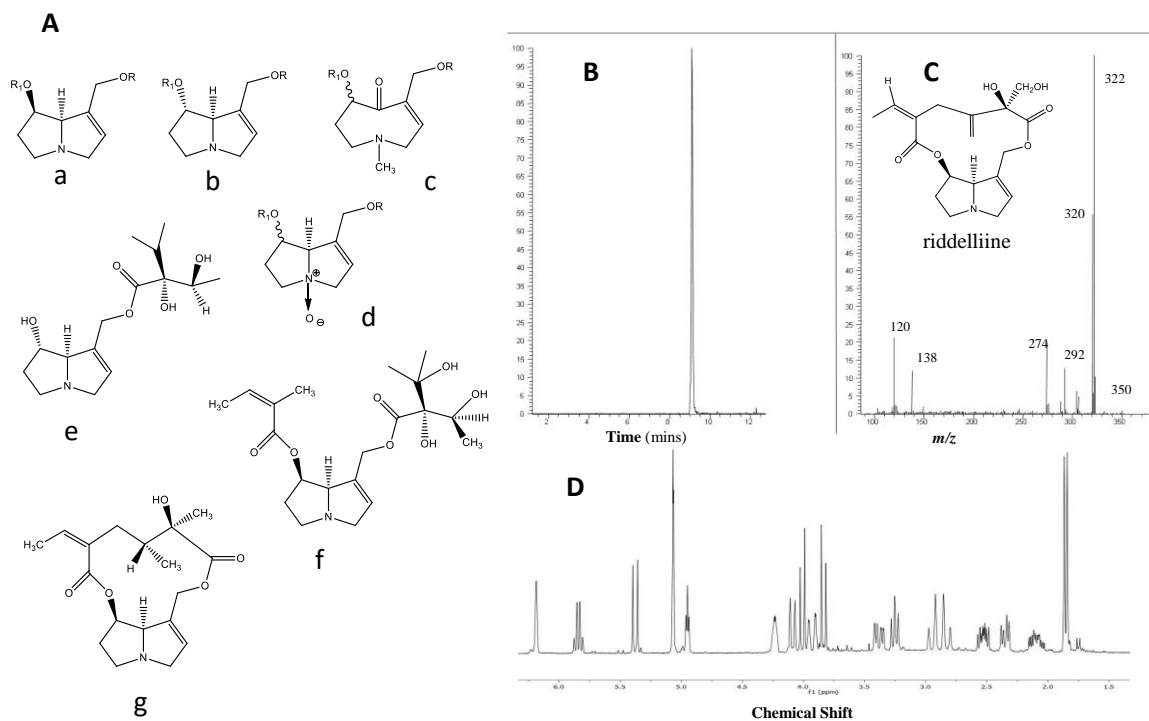
### Animals

Forty-nine, 3 week old, male heterozygous B6, 129-Trp53 mice were purchased from Taconic Farms Inc. (Germantown, NY, USA). Mice were raised to maturity (9 weeks old) and randomly divided into treatment groups. Each group was housed in a group cage and had access to feed (Harlan – Rodent diet 8604) and water ad libitum. Rodent rooms were maintained at between 20 and 22 °C, with a range of 30–60% humidity and a light dark cycle of 12 h. This research was conducted with the approval of the Utah State University animal care and use committee (IACUC Protocol no. 1364).

### Chemicals

Riddelliine was sourced from the Poisonous Plant Research Laboratory collection and originally extracted and purified from *Senecio riddellii* using the method described by Molyneux *et al.* (1991). Cognizant of the potential for riddelliine to form ring opening addition products with hydroxylic solvents (Colegate *et al.*, 2015), the identity and purity ( $\approx 95\%$ ) were confirmed using high-pressure liquid chromatography-positive ion electrospray ionization (+esi) mass spectrometry (Fig. 5-1B), tandem mass spectrometry to reveal structural elements (Fig. 5-1C) and proton ( $^1\text{H}$ ) nuclear magnetic resonance spectroscopy to reveal the characteristic  $^1\text{H}$  spectrum (Fig. 5-1D). The total riddelliine

required for the study was approximately 400 mg, 270 mg of which was dissolved in 100% ethanol at a concentration of 15 mg ml<sup>-1</sup>. The remaining 130 mg was used to make pellets for the 1 mg kg<sup>-1</sup> pellet group.



**Figure 5-1.** Some general structures of dehydropyrrolizidine alkaloids (A) a: retronecine base; b: heliotridine base; c: otonecine base; d: general N-oxide; e: monoester; f: open chain diester; g: macrocyclic diester; and the high-pressure liquid chromatography electrospray ionization mass spectrometry base ion chromatogram (B) and the tandem mass spectrometry of peak 1 (C) and the nuclear magnetic resonance spectrum (D) both consistent with riddelliine.

## Experimental Design

After reaching maturity, mice were randomly divided into four groups of 10 and one group of nine, using a random number chart. Mice in three of the groups of 10 received riddelliine 5, 15 or 45 mg kg<sup>-1</sup> body weight (BW) day<sup>-1</sup> (based upon the group

mean weight at the commencement of dosing and after 1 week of dosing) for 14 days. Thus, a calculated daily dose was administered by oral gavage in two equal portions, each further diluted with water (0.5 ml), one in the morning and one in the afternoon. The other group of 10 mice served as a negative control receiving an equal volume of 100% ethanol in water. After the dosing period, the mice were maintained for another 52 weeks. The remaining group of nine mice was fed a weekly ration of riddelliine-spiked pellets to the equivalent of riddelliine  $1 \text{ mg kg}^{-1} \text{ BW day}^{-1}$  for 12 months.

Mice were monitored daily for deterioration in health and weighed weekly. Animals with grossly visible tumors or significant decline in health were killed with carbon dioxide and necropsied. All remaining animals were killed for post-mortem examination at 1 year after gavage treatment ended. Tissues (including brain, heart, lung, trachea, thyroid gland, esophagus, stomach, small intestine, cecum, colon, pancreas, liver, kidney, urinary bladder, spleen, testicles, epididymides, seminal vesicles, skeletal muscle, bone, bone marrow and any grossly observed masses) were collected from all animals in 10% neutral buffered formalin for histopathology. All tumors were diagnosed based on histomorphology of hematoxylin and eosin-stained sections.

### **Pellet Preparation**

Pellets were made in batches lasting approximately 1 month each, adding riddelliine based on the average monthly weight of the mice in the group and the average daily feed consumption. Harlan – Rodent diet 8604 was ground using a Thomas-Willey Mill “Model #4” with a 1 mm screen. Riddelliine was mixed with the rodent diet using a Waring Laboratory variable speed blender. A small amount of water was sprayed on the

mixture, and pellets were made using a hammer type pellet machine (c. 1950) with an inside diameter of 3.5 cm. The pellets were air dried at ambient temperature ( $\approx 22$  °C), placed into a plastic bag, labeled and stored at  $-20$  °C until used.

### **Statistical Analysis**

Statistical analysis of average daily weight gain was completed using SAS 9.3 using a repeated measures general linear model. Odds ratios for neoplasia were also calculated using SAS 9.3 with a linear regression model with logit transformation.

### **Results**

During the period from commencement of dosing until 4 days after daily gavage ceased, i.e., study days 0–18, the groups receiving riddelliine 5, 15 and 45 mg kg<sup>-1</sup> BW day<sup>-1</sup> by gavage tended to lose weight in a dose-related manner. However, only the 45 mg kg<sup>-1</sup> group was significantly different ( $P < 0.001$ ) compared to the control group, which, along with the spiked pellet, 1 mg kg<sup>-1</sup> group, gained weight over this period. During the following 10 months, all groups gained weight at approximately the same rate. However, at about 10 months after the gavage period all groups receiving riddelliine began to lose weight (Table 5-1) although only the 45 mg kg<sup>-1</sup> group was significantly different compared to the control ( $P < 0.001$ ).

**Table 5-1.** Average Daily Weight Gain (g/day) in male heterozygous p53 knockout mice exposed to Riddelliine.

<sup>a</sup> Mice in these four groups were treated by gavage for 14 days.

<sup>b</sup> Mice in this group were fed riddelliine spiked pellets calculated to provide 1 mg/kg riddelliine per day, for the duration of the experiment.

<sup>1,2,3</sup> Superscript numbers denote statistical significance at  $p = 0.05$ .

	Treatment (Day 0 to 18)	Pre-clinical (Day 18 – 320)	Clinical (Day 320 – 382)
Control <sup>a</sup>	0.033 <sup>1,2</sup>	0.043 <sup>1</sup>	0.028 <sup>1</sup>
5 mg/kg <sup>a</sup>	-0.009 <sup>2</sup>	0.049 <sup>1</sup>	-0.063 <sup>1</sup>
15 mg/kg <sup>a</sup>	-0.036 <sup>2,3</sup>	0.044 <sup>1</sup>	-0.005 <sup>1</sup>
45 mg/kg <sup>a</sup>	-0.131 <sup>3</sup>	0.042 <sup>1</sup>	-0.238 <sup>2</sup>
1 mg/kg <sup>b</sup>	0.121 <sup>1</sup>	0.048 <sup>1</sup>	-0.014 <sup>1</sup>

The odds of having a tumor increase with dose in all individually gavaged animals (riddelliine 5, 15 and 45 mg kg<sup>-1</sup> BW groups) compared to the vehicle-gavaged controls, with an odds ratio of 2.05 and Wald 95% confidence limits between 1.2 and 3.4 (Table 5-2). For the 1 mg kg<sup>-1</sup> group, treatment was associated with a significantly higher prevalence of neoplasia with a  $P = 0.0006$  using the chi-squared test. The likelihood of a neoplasm being large enough to be grossly recognized also appears to increase at the higher doses (Table 5-2). Similarly, it appears that the likelihood of observing an indicative lesion in the location where a neoplasm was detected histologically also increased at higher doses. However, the small group size precluded a statistical confirmation of these observations.

**Table 5-2.** Neoplasms present in male heterozygous p53 knockout mice exposed to riddelliine, arranged by treatment group.

<sup>a</sup> Mice in these four groups were treated by gavage for 14 days.

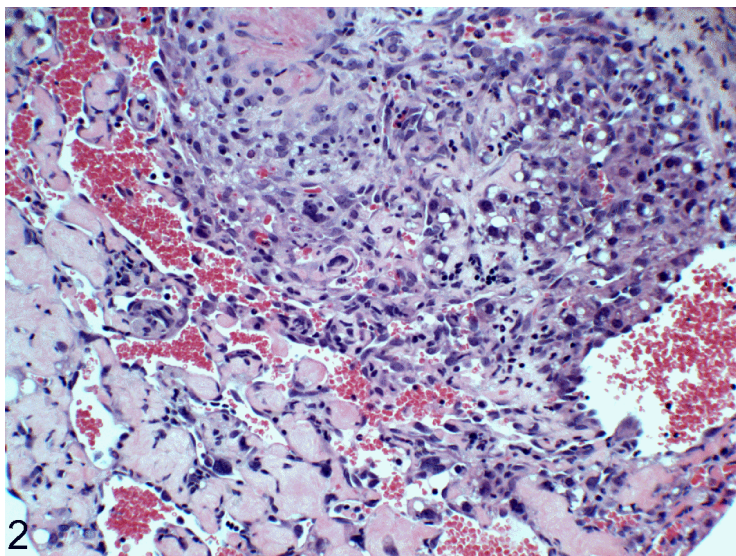
<sup>b</sup> Mice in this group were fed riddelliine spiked pellets calculated to provide 1 mg/kg riddelliine per day, for the duration of the experiment.

<sup>n/d</sup> None detected

	Control	5 mg/kg <sup>a</sup>	15 mg/kg <sup>a</sup>	45 mg/kg <sup>a</sup>	1 mg/kg <sup>b</sup>
Hemangiosarcoma	n/d	1	1	2	5
Carcinoma, Mammary	n/d	n/d	1	2	1
Lymphoma	n/d	1	1	n/d	2
Myeloproliferative Disease	n/d	1	1	n/d	2
Ependymoma, Malignant	n/d	n/d	n/d	3	n/d
Bronchiolo-alveolar Adenoma / Carcinoma	n/d	2	n/d	n/d	n/d
Osteosarcoma	n/d	1	1	n/d	n/d
Adrenocortical Carcinoma	n/d	n/d	1	n/d	n/d
Epithelial Stromal Tumor	n/d	n/d	n/d	1	n/d
Histiocytic Sarcoma	n/d	n/d	n/d	1	n/d
Lacrimal Gland Adenoma	n/d	n/d	n/d	1	n/d
Leydig Cell Tumor	n/d	1	n/d	n/d	n/d
Round Cell Tumor	1	n/d	n/d	n/d	n/d
Total Neoplasms	1	7	6	10	10
Grossly observed lesions in locations with neoplasms	1	3	3	8	8
Grossly observed neoplasms	1	1	3	4	6
Total Animals with Neoplasms	1	5	5	8	8

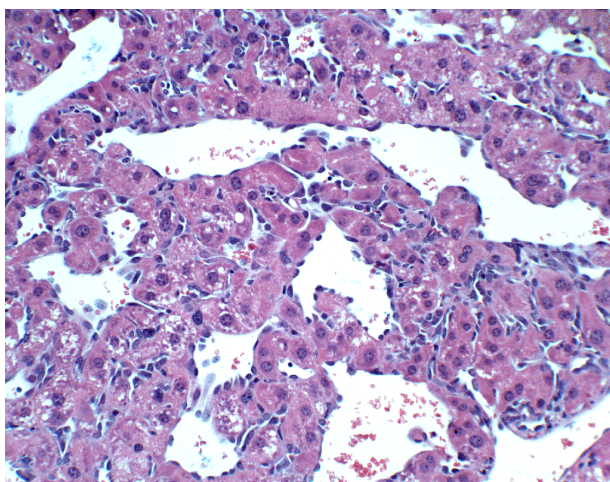
The number and type of neoplasms observed in each group were organized by prevalence (Table 5-2). Because of the indefinable potential for metastasis, when multiple foci of the same neoplasm were present within one animal, only one neoplasm was counted. In the control group, only one animal had a neoplasm, which was a round cell tumor of either lymphocytic or histiocytic origin.

Summed across all the groups, the most common lesions were hepatic vascular lesions, and the most common neoplasm was hemangiosarcoma (Fig. 5-2). The latter was present in all groups except for the control. Seven of the nine hemangiosarcomas observed throughout all the groups were intrahepatic, one was splenic and one was in subcutaneous tissue of the right rear leg. Two hepatic hemangiosarcomas were present in the 5 mg kg<sup>-1</sup> group, one was present in the 45 mg kg<sup>-1</sup> group and four were present in the 1 mg kg<sup>-1</sup> pellet group. The three hepatic hemangiosarcomas in the short exposure groups had one focus observed in each animal. In the 1 mg kg<sup>-1</sup> pellet group, there were 21 foci of hepatic hemangiosarcoma observed in eight different liver lobes (separate lobes within the same animal or different animals). There was no case where an animal had both intrahepatic and extrahepatic hemangiosarcoma.



**Figure 5-2.** Hemangiosarcoma, liver, heterozygous p53 knockout mouse exposed to 1 mg/kg riddelliine for 1 year. Neoplastic endothelial cells form variably sized vascular channels and wrap collagen. Hematoxylin and eosin (HE).

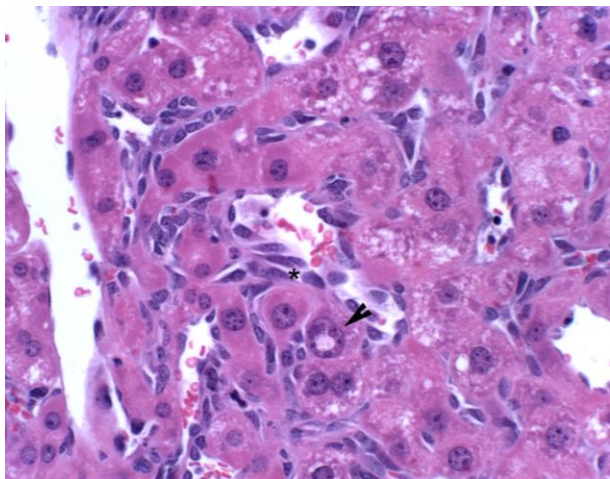
Non-neoplastic, hepatic vascular lesions were present and particularly common in the 1 mg kg<sup>-1</sup> continual exposure group. Variably sized foci of endothelial-lined, blood-filled spaces (peliosis hepatis) were present (one animal with one focus each in the 5 and 15 mg kg<sup>-1</sup> limited exposure groups and eight of nine animals with a total 46 foci in the continual 1 mg kg<sup>-1</sup> exposure group). Endothelial cells lining these lesions were often hyperplastic or dysplastic, and with occasional mitotic figures present (Fig. 5-3). In few instances, endothelial cells tended to pile up on each other (Fig. 5-4).



**Figure 5-3.** Angiectasis (peliosis hepatis), liver, heterozygous p53 knockout mouse exposed to 1 mg kg<sup>-1</sup> riddelliine for 1 year. Hematoxylin and eosin, 100x magnification. There are multifocal dilated, blood-filled, spaces lined with hypertrophic endothelial cells.

There were four cases each of mammary carcinoma, lymphoma, and myeloproliferative disease. The mammary carcinomas were observed in one animal in the 15 mg kg<sup>-1</sup> group, two animals in the 45 mg kg<sup>-1</sup> group and one animal in the 1 mg kg<sup>-1</sup> continuous exposure group. The incidences of lymphoma and myeloproliferative disease were the same with one case each in both the 5 and 15 mg kg<sup>-1</sup> limited exposure groups and two cases in the 1 mg kg<sup>-1</sup> continual exposure group.



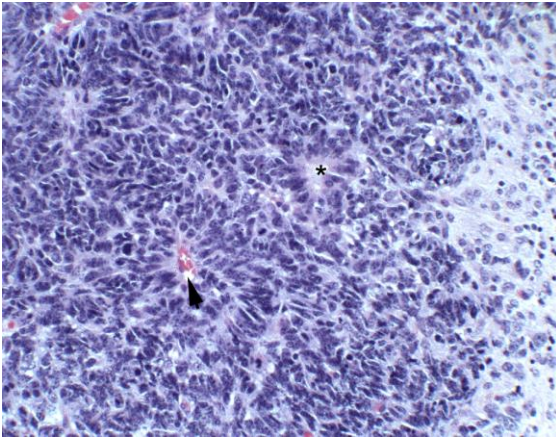


**Figure 5-4.** Angiectasis (peliosis hepatis), liver, heterozygous p53 knockout mouse exposed to 1 mg kg<sup>-1</sup> riddelliine for 1 year. Hematoxylin and eosin, 400x magnification. Multifocally endothelial cells are piled up (\*). There are occasional karyomegalic cells (arrowhead).

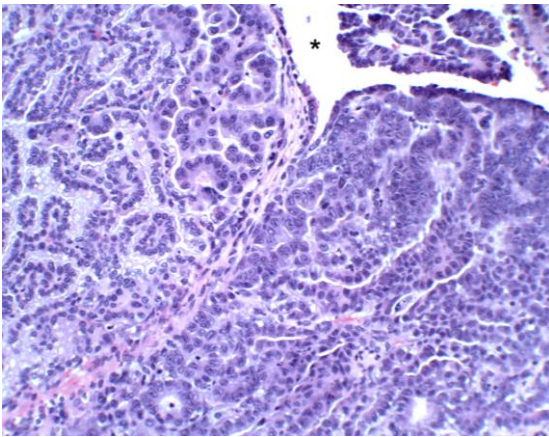
Three malignant ependymomas (Fig. 5-5) were present, all in the 45 mg kg<sup>-1</sup> limited exposure group. All were located near a ventricle in the cerebrum and were composed of proliferative polygonal cells forming abundant rosettes and pseudorosettes. These ependymomas invaded surrounding neuropil, had a high mitotic rate and included multifocal areas of necrosis. In one case, there was invasion through the cribriform plate and into the nasal turbinates.

Multiple neoplasms of lower incidence were also present. Two bronchioloalveolar tumors were present, both within the 5 mg kg<sup>-1</sup> group. One was an adenoma and the other was a carcinoma (Fig. 5-6). Two osteosarcomas were also present, one in the 5 mg kg<sup>-1</sup> group and the other in the 15 mg kg<sup>-1</sup> group. The remaining neoplasms observed, i.e., adrenocortical carcinoma, epithelial stromal tumor (in the seminal vesicle), histiocytic

sarcoma (in the urethra, urinary bladder, and seminal vesicle), lacrimal gland adenoma, Leydig cell tumor and a round cell tumor that was most likely of histiocytic origin (in fibrovascular tissue likely lymph node), all occurred only once.

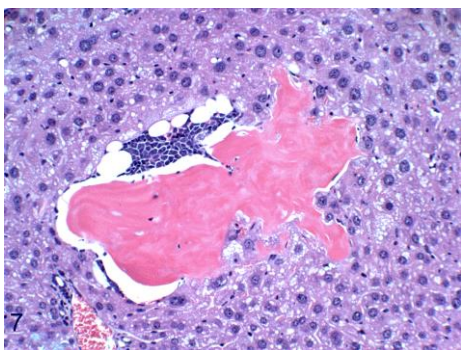


**Figure 5-5.** Malignant ependymoma, cerebrum, heterozygous p53 knockout mouse exposed to 45 mg kg<sup>-1</sup> riddelliine for 14 days. Note rosette (\*) and pseudorosette (arrowhead) formation along with high mitotic rate. Hematoxylin and eosin, 100x magnification.



**Figure 5-6.** Bronchiolo-alveolar carcinoma, lung, heterozygous p53 knockout mouse exposed to 5 mg kg<sup>-1</sup> riddelliine for 14 days. A single bronchiole remains (\*), while the remaining pulmonary parenchyma is replaced by neoplastic polygonal epithelial cells. Hematoxylin and eosin, 100x magnification.

A number of other intrahepatic changes were also present. Osseous metaplasia within the liver (Fig. 5-7) was limited to the 45mg kg<sup>-1</sup> group with 18 foci throughout all animals in this group. Lesions unrelated to treatment were also present, including inflammatory foci and hepatocellular vesicular changes, generally of minimal to mild severity. They were present throughout all groups, including controls, with no significant differences between any groups.



**Figure 5-7.** Osseous metaplasia, liver, heterozygous p53 knockout mouse exposed to 45 mg kg<sup>-1</sup> riddelliine for 14 days. A focus of mature bone, including marrow, replaces pre-existing hepatocytes and mildly compresses adjacent hepatocytes. Hematoxylin and eosin, 200x magnification.

## Discussion

It is well known that at least some DHPAs are carcinogenic in various species of animals (Allen *et al.*, 1975; Hirono *et al.*, 1978; Johnson *et al.*, 1978; Kuhara *et al.*, 1980; Li *et al.*, 2011; Mattocks & Cabral, 1982; NTP, 2003; Shumaker *et al.*, 1976).

Determining the relative carcinogenicity of the several hundred DHPAs is difficult due primarily to the availability of sufficient quantities of DHPAs to conduct such experiments and the cost in time and money to conduct the experiments. This present study, preliminary portions of which were presented at the 9th International Symposium

on Poisonous Plants, was focused on observing any clinical reactions and describing gross and histologic lesions in a small rodent model of DHPA-induced carcinogenesis that requires less time and less DHPA. The combination of lower overall DHPA requirements and a lesser duration of animal exposure and/or post-treatment maintenance time may enable more such studies within time and funding constraints.

Two different dosing strategies, based upon potential human exposures to the DHPAs in the diet, as dietary supplements or as medicinal herbs, were examined. The short duration (2 week) exposure to riddelliine (5, 15 and 45 mg kg<sup>-1</sup> BW day<sup>-1</sup>) was intended to represent “one-of” or intermittent exposure episodes such as very occasional use of a DHPA containing dietary supplement or occasional ingestion of a food, such as contaminated flour. The longer, 12-month duration exposure to riddelliine at 1 mg kg<sup>-1</sup> BW day<sup>-1</sup> was targeted at continual users of DHPA containing products, including herbal products or specialty honeys produced exclusively from DHPA producing plants. In both cases animals received substantially less DHPA than in other models (Table 5-3) (Chan,

**Table 5-3.** Comparison of dose and DHPA requirements using traditional long term models with mice or rats and using male heterozygous p53 knockout mice in the study design used in this study.

	Dose (mg/kg)	Ave. body wt. (kg)	No. doses / animal	Alkaloid needed / animal (mg)
B6C3F <sub>1</sub> Mice	3	0.045	525	70.9
Fischer Rats	1	0.450	525	236.3
Male Heterozygous P53 Knockout Mice	45	0.045	14	28.4
	1	0.045	365	16.4

2001; NTP, 2003).

Between study days 0 and 18, which included the 14 days of gavage treatment, there was a trend of weight loss for all animals gavaged with riddelliine, which was only significant at the highest dose (Table 5-1). For approximately 10 months following the completion of the daily exposure to riddelliine, all animals appeared clinically healthy although the animals that received 15 and 45 mg kg<sup>-1</sup> BW day<sup>-1</sup> always had a lower average BW having failed to regain the weight lost relative to the controls during the gavaging period. Around 10 months all of the groups that received riddelliine began to lose weight (Table 5-1). Over the following 2 months, it was evident that the overall health (specifically, individual animals with rough, unkempt hair coat and losing weight, as well as the presence of grossly visible neoplasms) of all treatment groups was declining resulting in a decision to kill them.

At post-mortem examination, grossly visible tumors included four of the nine hemangiosarcomas (one in the 45 mg kg<sup>-1</sup> group and three in the 1 mg kg<sup>-1</sup> group), all four of the mammary tumors, two of the four lymphomas (both in the 1 mg kg<sup>-1</sup> group), both osteosarcomas, and the adrenocortical carcinoma, histiocytic sarcoma and round cell tumor likely of histiocytic origin.

Compared to the controls, and in the absence of major clinical signs of toxicity until 10 months into the study, the incidence of neoplastic disease increased on exposure to riddelliine regardless of whether that exposure was of higher dose (5, 15 or 45 mg kg<sup>-1</sup> day<sup>-1</sup>) and relatively short duration or low-dose (1 mg kg<sup>-1</sup> day<sup>-1</sup>) with a prolonged

duration. Furthermore, even at the lowest total dose of riddelliine used in this study (5 mg kg<sup>-1</sup> BW day<sup>-1</sup> for 14 days), the risk of tumor development significantly increased.

Low-dose chronic exposure (1 mg kg<sup>-1</sup> group) resulted in a high incidence of hepatic hemangiosarcomas similar to previous studies in which animals were also exposed to low-doses over a long period of time (Chan *et al.*, 2003; NTP, 2003). Furthermore, in light of the fact that there were 21 foci of hepatic hemangiosarcomas in the 1 mg kg<sup>-1</sup> group compared to three foci in all of the short duration groups combined, either the hepatic hemangiosarcomas developed in this group were more prone to metastasis, or there were substantially more de novo hepatic hemangiosarcomas in this group. Given that there were eight separate lobes of liver (separate lobes within the same animal or different animals) involved, it appears that at least a portion of these hemangiosarcomas were derived de novo. Further studies using a similar low-dose and chronic exposure, and including other DHPAs, would be valuable in determining the relevance of this finding to DHPA carcinogenesis.

The short duration exposures (5, 15 and 45 mg riddelliine kg<sup>-1</sup> BW day<sup>-1</sup>) appeared to result in a wider range of neoplasms in extrahepatic tissues compared to the long-term, low-dose (1mg riddelliine kg<sup>-1</sup> BW day<sup>-1</sup>) exposure regimen. While it is evident that, for whatever reason, specific DHPAs can cause either hepatic or pneumotoxic effects (Pessoa *et al.*, 2013; Stalker & Hayes, 2007), it is also reasonable to expect that an increased concentration of systemic DHPA, unchanged by hepatic metabolism, may result if the exposure dose is increased. Metabolized by P450 enzymes found in extrahepatic tissues, these systemic DHPAs can lead to in situ extrahepatic damage. This

may account for a wider variety of tissues developing neoplasia in animals in the limited exposure groups in this study. Studies using different routes of DHPA exposure often resulted in tumors in extrahepatic locations, such as subcutaneous injection resulting in rhabdomyosarcomas (Allen *et al.*, 1975). Alternatively, it is also possible that any one of the reactive compounds from the DHPA metabolic cascade (Edgar *et al.*, 2011), including perhaps a release of adducted metabolites, could escape the liver and subsequently cause damage in distant tissues.

Angiectasis (peliosis hepatis) is thought to be the result of loss or weakening of sinusoidal walls and/or supporting tissue (Thoolen *et al.*, 2010). The presence of peliosis hepatis has been mentioned in one other report in relation to DHPA exposure in mice (Ruebner *et al.*, 1970). In that case, the lesions were not endothelial-lined and were presumed to be due to hepatocellular necrosis. In this study, all the lesions are lined with endothelial cells that commonly round up, and protrude into the vascular lumen and occasionally exhibit dysplastic changes. They are not associated with fibrotic central veins or other discernible vascular lesions that would result in increased pressure within the sinusoids. Foci of angiectasis are more common in the low-dose chronic exposure (1 mg kg<sup>-1</sup> pellet) group. DHPA-DNA adducts have been shown to have a predilection to hepatic endothelial cells (Chou *et al.*, 2004). It is therefore plausible that DHPA-induced endothelial injury can result in angiectasis, and that further exposure to DHPAs could increase the chance of a neoplastic change in that endothelium resulting in hemangiosarcoma.

Osseous metaplasia of the liver also indicates previous damage to hepatic tissue. In this study, foci of osseous metaplasia were found exclusively in the highest short duration dose (45 mg kg<sup>-1</sup>) group, with six of ten animals having at least one focus. It has not been a feature described in other studies.

The purpose of this study was to determine the effectiveness of this p53 knockout mouse model using both long- and short-term exposures to study the carcinogenic potential of DHPAs. The results of the chronic exposure group are very similar to previous reports of low-dose chronic exposure to DHPAs (Chan *et al.*, 2003; NTP, 2003). Hemangiosarcoma as well as leukemia have been reported in association with chronic riddelliine exposure. Although there are many studies relating to the carcinogenicity of DHPAs, due to the wide variation of methodologies, none is truly comparable to the short-term exposure model used in this study. Therefore, it is difficult to determine if previously unreported lesions, such as malignant ependymoma or osseous metaplasia, are relevant to human exposure or if they are exclusive to this more sensitive model.

### Conclusion

This preliminary study clearly demonstrated the positive potential of the male heterozygous p53 knockout mouse model for making comparative assessments of the carcinogenicity of DHPAs. When treated with the macrocyclic DHPA riddelliine, either by daily gavage for 14 days or by continuous, lower concentration dietary exposure, all the treatment groups showed an increased incidence of neoplasia within 12 months. The shorter time required for tumor development, by virtue of the reduced expression by the



p53 gene, leads directly to lower resource requirements to conduct comparative assessments of the carcinogenic potential of the many different DHPAs.

The dosage regimens tested were designed to approximate the pattern, if not the actual exposure types, of two human exposure scenarios, i.e., a low-dose, long-term continuous exposure and a short duration exposure, as might be expected from regular or intermittent use, respectively, of a DHPA containing food, dietary supplement or medicinal herb. Interestingly, though the type and distribution of the neoplasms differed between dose groups, it was found that all riddelliine exposure rates in this study increased the incidence of neoplasia. This suggests DHPA exposure, regardless of the dose, may be a greater carcinogenic risk than previously assessed. This observation supports the very low limits of exposure to DHPAs in general that have been set by European and UK authorities based upon the “single molecular event” potential for genotoxic carcinogenicity.

## References

- Adams R, Hamlin Jr K, Jelinek C, Phillips R. 1942. The structure of riddelliine, the alkaloid in *Senecio riddellii*. I. J. Am. Chem. Soc. 64: 2760–2763.
- Allen JR, Hsu IC, Carstens LA. 1975. Dehydroretronecine-induced rhabdomyosarcomas in rats. Cancer Res. 35: 997–1002.
- Campbell J. 1956. VI. – An investigation of the hepatotoxic effects in the fowl of ragwort (*Senecio jacobæa* Linn.), with special reference to the induction of liver tumours with seneciphylline. Proc. R. Soc. Edinb. [Biol.] 66: 111–130.
- Chan P. 2001. NTP Technical Report on Toxicology and Carcinogenesis Studies of Riddelline (CAS No. 23246-96-0) in F344/N Rats and B6C3F1 Mice. NTP TR 508, NIH Publication.
- Chan PC, Haseman JK, Prejean J, Nyska A. 2003. Toxicity and carcinogenicity of riddelliine in rats and mice. Toxicol. Lett. 144: 295–311.
- Chou MW, Yan J, Nichols J, Xia Q, Beland FA, Chan PC, Fu PP. 2004. Correlation of DNA adduct formation and riddelliine-induced liver tumorigenesis in F344 rats and B6C3F1 mice [Cancer Lett. 193 (2003) 119–125]. Cancer Lett. 207: 119–125.
- Colegate SM, Gardner DR, Resager W, Bollar N, Betz JM, Panter KE. 2015. Hydroxylic Solvent-Induced Ring Opening of the Dehydropyrrolizidine Alkaloids Riddelliine and Seneciphylline: Implications for Toxicity and Analytical Studies. Intl. J. Poisonous Plant Res. (in press).
- Donehower, L. A. 1996. The p53-deficient mouse: a model for basic and applied cancer studies. In Seminars in cancer biology (Vol. 7, No. 5, pp. 269-278). Academic Press.
- Edgar JA, Colegate SM, Boppre M, Molyneux RJ. 2011. Pyrrolizidine alkaloids in food: a spectrum of potential health consequences. Food. Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess. 28: 308–324.
- Gilruth JA. 1904. Hepatic cirrhosis due to ragwort (*Senecio jacobea* ). Div Vet Sci NZ Dept Agric Bull. 9:252–254.
- Hirono I, Mori H, Haga M. 1978. Carcinogenic activity of *Symphytum officinale*. J. Natl Cancer Inst. 61: 865–869.
- Johnson AE, Molyneux RJ, Stuart LD. 1985. Toxicity of Riddell's groundsel (*Senecio riddellii*) to cattle. Am. J. Vet. Res. 46: 577–582.

- Johnson WD, Robertson KA, Pounds JG, Allen JR. 1978. Dehydroretronecine induced skin tumors in mice. *J. Natl Cancer Inst.* 61: 85–89.
- Kuhara K, Takanashi H, Hirono I, Furuya T, Asada Y. 1980. Carcinogenic activity of clivorine, a pyrrolizidine alkaloid isolated from *Ligularia dentata*. *Cancer Lett.* 10: 117–122.
- Li N, Xia Q, Ruan J, Fu PP, Lin G. 2011. Hepatotoxicity and tumorigenicity induced by metabolic activation of pyrrolizidine alkaloids in herbs. *Curr. Drug Metab.* 12: 823–834.
- Mattocks A. 1986. *Chemistry and Toxicology of Pyrrolizidine Alkaloids*. Academic Press: London.
- Mattocks AR, Cabral JR. 1982. Carcinogenicity of some pyrrolic pyrrolizidine alkaloid metabolites and analogues. *Cancer Lett.* 17: 61–66.
- Mei N, Chou MW, Fu PP, Heflich RH, Chen T. 2004. Differential mutagenicity of riddelliine in liver endothelial and parenchymal cells of transgenic big blue rats. *Cancer Lett.* 215: 151–158.
- Mei N, Guo L, Fu PP, Heflich RH, Chen T. 2005. Mutagenicity of comfrey (*Symphytum officinale*) in rat liver. *Br. J. Cancer* 92: 873–875.
- Mei X, Chen T. 2007. The mutant frequencies and types of mutations induced by comfrey in the lungs of transgenic big blue rats. *J. Food Drug Anal.* 15: 458.
- Molyneux RJ, Johnson AE, Olsen JD, Baker DC. 1991. Toxicity of pyrrolizidine alkaloids from Riddell groundsel (*Senecio riddellii*) to cattle. *Am. J. Vet. Res.* 52: 146–151.
- NTP. 2003. NTP Technical report on the toxicology and carcinogenesis studies of riddelliine (CAS NO. 23246-96-0) IN F344/N rats and B6C3F1 mice (gavage studies). Program NT (Ed.). US Department of Health and Human Services: North Carolina.
- Pessoa CR, Pessoa AF, Maia LA, Medeiros RM, Colegate SM, Barros SS, Soares MP, Borges AS, Riet-Correa F. 2013. Pulmonary and hepatic lesions caused by the dehydropyrrolizidine alkaloid-producing plants *Crotalaria juncea* and *Crotalaria retusa* in donkeys. *Toxicol.* 71: 113–120.
- Ruebner BH, Watanabe K, Wand J. 1970. Lytic necrosis resembling peliosis hepatis produced by lasiocarpine in the mouse liver. A light and electron microscopic study. *Am. J. Pathol.* 60: 247.
- Schoental R. 1975. Pancreatic islet-cell and other tumors in rats given heliotrine, a monoester pyrrolizidine alkaloid, and nicotinamide. *Cancer Res.* 35: 2020–2024.

- Schoental R, Cavanagh J. 1972. Brain and spinal cord tumors in rats treated with pyrrolizidine alkaloids. *J. Natl Cancer Inst.* 49: 665–671.
- Schoental R, Fowler M, 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.
- Shumaker RC, Robertson KA, Hsu IC, Allen JR. 1976. Neoplastic transformation in tissues of rats exposed to monocrotaline or dehydroretronecine. *J. Natl Cancer Inst.* 56: 787–790.
- Stalker M, Hayes M. 2007. Liver and biliary system. In Jubb, Kennedy, and Palmer's *Pathology of Domestic Animals*, 5th edn, Grant Maxie M (Ed.). Elsevier; 2: 297–388.
- Thoolen B, Maronpot RR, Harada T, Nyska A, Rousseaux C, Nolte T, Malarkey DE, Kaufmann W, Kuttler K, Deschl U, Nakae D, Gregson R, Vinlove MP, Brix AE, Singh B, Belpoggi F, Ward JM. 2010. Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. *Toxicol Pathol.* 38: 5s–81s.
- Vardiman P. 1952. Experimental feeding of *Senecio* silage to calves. *J. Am. Vet. Med. Assoc.* 121: 397.
- Venkatachalam S, Tyner SD, Pickering CR, Boley S, Recio L, French JE, Donehower LA. 2001. Is p53 haploinsufficient for tumor suppression? Implications for the p53<sup>+/-</sup> mouse model in carcinogenicity testing. *Toxicol Pathol.* 29(Suppl): 147–154.
- Wiedenfeld H, Roeder E, Bourauel T, Edgar J. 2008. *Pyrrolizidine Alkaloids Structure and Toxicity*. Bonn University Press: Gottingen.
- Yuzo H, Mariko S, Hiro K. 1977. Experimental insuloma in rats after a single administration of monocrotaline. *Toxicol. Lett.* 1: 41–44.

## CHAPTER VI

### CONCLUSIONS

DHPAs pose a risk to both humans and animals, and the extent of this risk is currently not completely understood. Grazing animals consume DHPA containing plants, particularly when other forage is not available. Penned animals, most prominently swine, and chickens can be exposed from contaminated feeds when plants or seeds are co-harvested with forage crops or grains.

Human exposure is often difficult to quantify. Major outbreaks have occurred as a result of grain contamination, typically in underdeveloped countries (Bras, *et al.* 1954; Mayer and Lüthy 1993; Mohabbat, *et al.* 1976). Such outbreaks result in overt symptoms of acute toxicity, and consequently the cause and results are therefore relatively straightforward. Other human exposure results from secondary exposure to DHPAs found as contaminants in foods such as honey, pollen, or milk. This is compounded by intentional intake of certain DHPA containing medicinal herbs.

The consequences of low/intermittent dose, chronic exposure to humans are unknown. It has been suggested they increased risk of chronic illnesses such as pulmonary hypertension (Gyorik and Stricker 2009), hepatic cirrhosis, or cancer (Edgar, *et al.* 2014). The risk for DHPA-induced neoplasia in humans is uncertain, although they are genotoxic and carcinogenic in other species.

It is clear that not all DHPAs are of equal potency with respect to acute toxicity. Because the mechanism for other chronic diseases such as cancer, is thought to be the same as for acute toxicity, it has been proposed that extrapolation of LD50 data might be

an appropriate method of risk evaluation. Because the majority of LD50 data were obtained using a single IP injection in a rat model it may not reflect most natural exposures. Oral exposure have been shown to have very different toxicity, especially for DHPA n-oxides. It is imperative that the data are compared to a more biologically relevant model (oral exposure model).

Using a sensitive (male, California White chick) model seven purified DHPAs and three DHPA N-oxides comprising different structural classes, were tested. The results of this research are vitally important because multiple structurally different DHPAs were compared at biologically relevant doses and routes in the same model at the same molar doses. In this model, heliotrine appears to be at least as toxic as retronecine based macrocyclic diesters and heliotridine based diesters, and much more toxic than expected based on previously published IP exposure studies. The N-oxides used in this study were also more toxic than would be expected based on either IP exposure studies or cell based assays. The retronecine based diester, echimidine, was unexpectedly less toxic than heliotrine, the heliotridine based monoester. Lycopsamine was the least toxic of the compounds assessed in this study.

We identified tissue DHPA metabolites or “pyrrole” adducts in the livers from chicks exposed to all of the compounds. The adduct accumulation rate can be used as a toxicity indicator. The “pyrrole” accumulation rate was much higher in riddelliine exposed chicks than in any other group, as riddelliine is one of the few pure DHPAs that has been proven to be carcinogenic in several animal models, this may be biologically

relevant with respect to carcinogenicity. Future research on the relative carcinogenicity of structurally diverse DHPAs would be valuable.

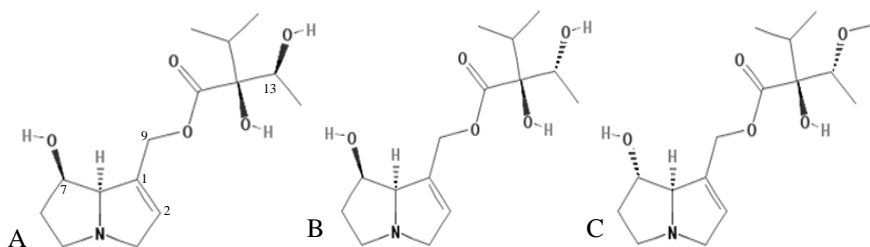
Extrahepatic lesions present in the highest dose of lasiocarpine exposed animals, in combination with results from other studies suggest that excreted conjugated pyrroles may be unstable in the intestine and may cause damage post excretion for some DHPAs. This adds credence to the concern with consumption of tissues such as liver from animals that have been exposed to DHPAs, and this potential instability should be evaluated in future studies.

Using oral doses in the chick model and based on weight, survival, serum biochemistry, and histopathology the most toxic group included the heliotrine, senecionine, seneciphylline, and lasiocarpine. The second group, of moderate toxicity, was composed of the three N-oxides (senecionine N-oxide, riddelliine N-oxide, and lasiocarpine N-oxide), which were more toxic than echimidine in virtually all parameters tested. Consistent with previous research, lycopsamine was the least toxic. Based on the model used it appears that in general, oral toxicity follows fairly close to what would be expected from IP LD50 studies; however, based on the marked difference in toxicity of heliotrine, it appears that there could be substantial differences in specific alkaloids. Consequently, historical IP LD50 data may markedly misrepresent the true risk of some compounds.

Results from in vitro assays using an immortalized chicken hepatocyte cell line were similar to those found in these chick toxicity studies (Field R 2013). In both cases heliotrine was found to be similar in toxicity to the retronecine based macrocyclic

diesters senecionine and seneciphylline as well as the heliotridine based open chain diester, lasiocarpine. There is one rather notable difference in which the present study excels. In the *in vitro* work, the same three N-oxides were studied. In that case they were found to be similar in toxicity to lycopsamine. In this live chick model they were consistently more toxic than lycopsamine. The difference is almost certainly due to microbial reduction of the N-oxides in the gastrointestinal tract, reducing the N-oxide to the free base form.

The vast majority of DHPA containing plants contain a mixture of DHPAs in a variety of forms (free base, N-oxide, acetylated etc.) as well as other compounds. If we are to use purified alkaloid toxicity studies to evaluate the risk posed by plants, we must presuppose that the toxicity of the plant will be equal to the sum of the toxic effects of the individual DHPAs found in that plant.



**Figure 6-1. Structural diagrams of lycopsamine (A), intermedine (B), and Heliotrine (C)**

Comfrey (*Symphytum officinale*) was used to test this hypothesis. The comfrey root extract used contained almost exclusively two DHPAs (lycopsamine and intermedine) and their acetylated derivatives. Furthermore, these two DHPAs are stereoisomers at C13. Heliotrine has a configuration around C13 similar to intermedine. Thus an evaluation could also be made as to the relative importance of this structural



characteristic. Using the same model as that used to test the purified individual DHPAs a three way comparison was made between purified lycopsamine and purified intermedine and a reduced crude comfrey root extract (containing lycopsamine, intermedine and trace amounts of their acetylated free bases).

Based on serum biochemistry, tissue pyrrole concentration and histopathological analysis, the comfrey extract was more toxic than an approximately molar equivalent dose of either pure intermedine or pure lycopsamine. Furthermore, the only potential difference between intermedine and lycopsamine was that lycopsamine tended to have a higher tissue pyrrole concentration than intermedine.

Possible reasons for the increased toxicity of the comfrey extract when compared to the two pure DHPAs include: a synergistic effect of the two alkaloids, a more potent toxicity of the acetylated forms, or effects from some other compound present in the extract. If the acetylated forms are more toxic it could be due to increased absorption. Presumably the acetylated forms could/would be de-acetylated and then exert their toxic effects identically to the free base forms. In reference to the third possibility, any compound present in the crude extract, which was undetected by HPLC, utilizing the same enzyme(s) for phase I or phase II metabolism, could alter the rate at which the DHPAs were bioactivated and/or conjugated. Elucidation of the actual mechanism for increased toxicity of the crude comfrey extract will require further investigation.

With respect to the carcinogenic risk of DHPAs, riddelliine has been more thoroughly investigated than any other DHPA. Presumably the mechanism for toxicity is the same as the mechanism for carcinogenicity. Consequently, it has been suggested that

LD50 data be used to estimate the risk of carcinogenicity by converting LD50s to riddelliine equivalents (Committee on Toxicity of Chemicals in Food 2008). The risk of utilizing IP derived LD50 data has already been discussed. Assuming the LD50 information used is accurate, the carcinogenic potential of multiple DHPAs would need to be evaluated in order to determine the utility of this method. Riddelliine is somewhat unique in that it is relatively easy to extract and isolate in large quantities, thus it has been the most thoroughly investigated. In order to conduct carcinogenicity studies on other, more difficult to obtain DHPAs, a more sensitive model is needed.

The male heterozygous p53 knockout mouse has been evaluated as a more sensitive model. The study was designed such that it could not only be compared to previously conducted chronic dosing studies, but also that it could evaluate the risk of short-term or intermittent exposure such as could occur with contaminated food. Multiple doses (5, 15 and 45 mg kg<sup>-1</sup> BW) were used and mice were exposed twice daily for two weeks. For the chronic low-dose exposure mice were exposed to 1 mg kg<sup>-1</sup> BW riddelliine mixed in pellets.

The significant results of this study were two-fold. First, the model behaved similar to those used in chronic dosing studies funded by the NTP (NTP 2003b), with the exception that this model required substantially less pure riddelliine. Thus the heterozygous p53 knockout mouse has potential utility as a more sensitive model for investigating other DHPAs that are more difficult to obtain in pure form. Secondly, there was a dose responsive increase in tumor incidence in those animals that were exposed to riddelliine for only two weeks. This supports the very low acceptable intakes

recommended by multiple regulatory and food safety agencies (Committee on Toxicity of Chemicals in Food 2008; EFSA 2011; RIVM 2005; WHO 2011).

Through this research, two new models for comparing toxicity and carcinogenicity of DHPAs have been introduced. These two models were controlled for age, gender, and strain such that further comparisons can be made with more relevance. In both the chick model for toxicity and the heterozygous p53 knockout mouse model for carcinogenicity, some findings were unexpected based on previous research. In the toxicity model, heliotrine was found to be much more compared to other DHPAs than expected. Using the carcinogenicity model the impact of low-dose prolonged exposure was more significant than may have been previously anticipated. In both cases a body of research already existed, and conclusions with expectations were made. Unfortunately, the existing research has been done with different routes, species/strains, ages, and genders of animals, making it very challenging to directly compare. This emphasizes the need for further research using the same model.

In an effort to explain differences in toxicity between DHPAs, much effort has been given to understanding the chemistry behind bioactivation (Mattocks 1986). It is surprising that through all of the research that has been done, there is a relative paucity of understanding with respect to kinetics of the different compounds. How much of the difference in toxicity is actually due to differences in absorption or some other biologically relevant, but unexplored factor? This is particularly important with respect to biologically active metabolites. Little, if any data are available with respect to how

long adducts are present in tissues such as liver. Such information would be invaluable with respect to food safety.

Comparing carcinogenicity studies in an effort to understand the relative carcinogenicity of different DHPAs is even more challenging than comparing acute toxicity studies. Studies have been done with a wide variety of species/strains, routes of exposure, ages, genders, length of exposure, and time from exposure to euthanization. In order to understand the relative carcinogenic potential of different DHPAs it is critical to control for all of these variables. This p53 knockout mouse model allows for such comparisons, and a study using multiple structurally different DHPAs would be very beneficial to the current body of literature.

## References

- Bras G, Jelliffe DB, Stuart KL. 1954. Veno-occlusive disease of liver with nonportal type of cirrhosis, occurring in Jamaica. *AMA Arch Pathol.* 57:285-300.
- Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT). 2008. COT statement on pyrrolizidine alkaloids in food. Available: <http://cot.food.gov.uk/pdfs/cotstatementpa200806.pdf> [Accessed 6 June 2015].
- Edgar JA, Molyneux RJ, Colegate SM. 2014. Pyrrolizidine Alkaloids: Potential Role in the Etiology of Cancers, Pulmonary Hypertension, Congenital Anomalies and Liver Disease. *Chem Res Toxicol* 28: 4-20.
- EFSA. 2011. EFSA Panel on Contaminants in the Food Chain (CONTAM). Scientific Opinion on Pyrrolizidine alkaloids in food and feed. *EFSA Journal* 9:1-134. DOI: 10.2903.
- Field R, Colegate SM, Brown AW, McCollum I, Knoppel E. 2013. Comparative Cytotoxicity of Dehydropyrrolizidine *In vitro*. In International Symposium on Poisonous Plants. HoHot China.
- Gyorik S, Stricker H. 2009. Severe pulmonary hypertension possibly due to pyrrolizidine alkaloids in polyphytotherapy. *Swiss Med Wkly.* 139:210-211.
- Mattocks A. 1986. Chemistry and toxicology of pyrrolizidine alkaloids. Academic Press London.
- Mayer F, Lüthy J. 1993. Heliotrope poisoning in Tadjikistan. *Lancet.* 342:246-247.
- Mohabbat O, Shafiq Younos M, Merzad A, Srivastava R, Ghaos Sediq G, Aram G. 1976. An outbreak of hepatic veno-occlusive disease in north-western Afghanistan. *Lancet.* 308:269-271.
- NTP. 2003. NTP Technical report on the toxicology and carcinogenesis studies of riddelliine (CAS NO. 23246-96-0) IN F344/N rats and B6C3F1 mice(gavage studies). Program NT (Ed.). US Department of Health and Human Services: North Carolina.
- RIVM. 2005. Advisory Report on Pyrrolizidine Alkaloids in Herb Preparations. Available:[http://www.rivm.nl/en/Documents\\_and\\_publications/Scientific/Reports/2015/april/Pyrrolizidine\\_alkaloids\\_in\\_herbal\\_preparations](http://www.rivm.nl/en/Documents_and_publications/Scientific/Reports/2015/april/Pyrrolizidine_alkaloids_in_herbal_preparations) [Accessed 8 April 2015].
- WHO. 2011. Discussion paper on pyrrolizidine alkaloids, Joint FAO/WHO food standards programme, Codex committee on contaminants in foods. The Hague, The Netherlands.

APPENDICES

Appendix A. Curriculum Vitae

## CURRICULUM VITAE

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**EDUCATION**

<b>Utah State University</b> PhD candidate, Toxicology	Logan, UT August 2012 - Present
<b>Veterinary Pathology Resident</b> ACVP Board certification – September 2007	Armed Forces Institute of Pathology Washington, DC 20306 July 2004 – October 2007
<b>Washington State University</b> Doctor of Veterinary Medicine	Pullman, Washington September 1995 – May 1999
<b>Utah State University</b> BS, Bioveterinary Science	Logan, UT September 1992 – May 1995
<b>College of Eastern Utah San Juan Campus</b>	Blanding, UT October 1989 – May 1990
 <b><u>EXPERIENCE</u></b>	
<b>Chief Pathobiology</b> July 2010 – July 2012	59 <sup>th</sup> Clinical Research Division Lackland Air Force Base 2200 Bergquist Dr., Bldg 4430 San Antonio, TX 78236
<b>Officer In Charge (OIC) Clinicopathology Laboratory</b> June 2008- July 2010	Walter Reed Institute of Research 503 Robert Grant Ave. Silver Spring, MD 20910
<b>OIC, Necropsy Laboratory</b> November 2007 – May 2008	Walter Reed Institute of Research 503 Robert Grant Ave. Silver Spring, MD 20910
<b>Chief, Rota Branch Veterinary Services</b> July 2001 - July 2004	Southern Europe Veterinary Detachment; Rota Spain
<b>OIC, Twentynine Palms Veterinary Services</b> June 1999- July 2001	Southern California District Veterinary Command Marine Corps Air Ground Combat Center Twentynine Palms, CA 92278



**PROFESSIONAL SOCIETY MEMBERSHIPS**

American Veterinary Medical Association  
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**SPECIALIZED TRAINING**

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**JOURNAL PUBLICATIONS**

1. Brown, A. W., Stegelmeier, B. L., Colegate, S. M., Panter, K. E., Knoppel, E. L., & Hall, J. O. (2015). Heterozygous p53 knockout mouse model for dehydropyrrolizidine alkaloid-induced carcinogenesis. *J Appl Toxicol.*
2. Brown, A. W., Stegelmeier, B. L., Colegate, Gardner D.R., S. M., Panter, K. E., Knoppel, E. L., & Hall, J. O. (2015). The Comparative Toxicity of a Reduced, Crude Comfrey (*Symphytum officinale*) alkaloid extract and the Pure, Comfrey-Derived Pyrrolizidine Alkaloids, Lycopsamine and Intermedine in Chicks (*Gallus gallus domesticus*). *J Appl Toxicol.*
3. Stegelmeier, B. L., Brown, A. W., & Welch, K. D. (2015). Safety concerns of herbal products and traditional Chinese herbal medicines: dehydropyrrolizidine alkaloids and aristolochic acid. *J Appl Toxicol.*
4. Field, R. A., Stegelmeier, B. L., Colegate, S. M., Brown, A. W., & Green, B. T. (2015). An *in vitro* comparison of the cytotoxic potential of selected dehydropyrrolizidine alkaloids and some N-oxides. *Toxicol*, 97, 36-45.
5. Connor, M. P., Brown, A., Palazzolo, M., Kunkel, J., Eller, R., Hartnick, C., & Mauro, S. (2014). Effect of Vocal Fold Injection of Cidofovir and Bevacizumab in a Porcine Model. *JAMA Otolaryngology–Head & Neck Surgery*, 140(2), 155-159.
6. Wang, Y., Oguntayo, S., Wei, Y., Wood, E., Brown, A., Jensen, N., ... & Nambiar, M. P. (2012). Neuroprotective effects of imidazenil against chemical warfare nerve agent soman toxicity in guinea pigs. *Neurotoxicology*, 33(2), 169-177.
7. Moon-Massat, P., Scultetus, A., Arnaud, F., Brown, A., Haque, A., Saha, B., ... & Freilich, D. (2012). The effect HBOC-201 and sodium nitrite resuscitation after uncontrolled haemorrhagic shock in swine. *Injury*, 43(5), 638-647.
8. Gao, X., Anderson, D. R., Brown, A. W., Lin, H., Amnuaysirikul, J., Chua, A. L., ... & Ray, P. (2011). Pathological studies on the protective effect of a macrolide antibiotic, roxithromycin, against sulfur mustard inhalation toxicity in a rat model. *Toxicol Pathol.*, 39(7), 1056-1064.
9. Anderson, D. R., Brown, A. W., Amnuaysirikul, J., Chua, A. L., Lin, H., Ishida, H., ... & Gao, X. (2011). Macrolide Antibiotics Protect Against Lung Damage Caused By Sulfur Mustard Inhalation In Rat. *Am J Respir Crit Care Med*, 183, A2901.

10. Nemelka, K. W., Brown, A. W., Wallace, S. M., Jones, E., Asher, L. V., Pattarini, D., ... & Baqar, S. (2009). Immune response to and histopathology of *Campylobacter jejuni* infection in ferrets (*Mustela putorius furo*). *Comparative medicine*, 59(4), 363.

### **POSTERS / PRESENTATIONS**

1. Brown A.W., Stegelmeier, B.L., Colegate, S.M., Panter, K.E., Knoppel, E.L., Field, R.A., Hall, J.O.: Carcinogenic Effects of Riddelliine on P53 Knockout Mice. International Society on Poisonous Plants, July 2014
2. Ying Wang<sup>1</sup>, Elisa Tracy<sup>1</sup>, Samuel Oguntayo<sup>1</sup>, Ammon Brown<sup>1</sup>, James Auta<sup>2</sup>, Alessandro Guidotti<sup>2</sup>, Neil Jensen<sup>3</sup>, Madhusoodana P. Nambiar: Protective Efficacy of Imidazenil Against Soman Induced Seizure and Neuropathology in Guinea Pigs. Bioscience Review, May 2010
3. Xiugong Gao, Dana R. Anderson, Ammon W. Brown, Hiroshi Ishida, Aileen L. Chua, Wesley W. Holmes, Michele L. Conti, Prabhathi Ray: Pathological Study of the Protective Effect of Macrolide Antibiotics on Sulfur Mustard Inhalation Toxicity in a Rat Model. Bioscience Review, May 2010
4. Prabhathi Ray, Peng Zhang, Xiugong Gao, Hiroshi Ishida, Aileen Chua, Jack Amnuaysirikul, Peter J Weina, Max Grogl, Mark Hickman, Tom Hudson, Colin Ohrt, Aruna Sampath, Ammon Brown, and, Alan J Magill: A Glucose-6-Phosphate Dehydrogenase (G6PD)-Deficient (G6PDD) Mouse Model to Study Hemolytic Toxicity of 8-aminoquinolines (8-AQ). Apr 2010
5. Ying Wang, Elisa Tracy, Samuel Oguntayo, Ammon Brown, Neil Jensen, James Auta, Alessandro Guidotti, Madhusoodana P. Nambiar: Anticonvulsant, Imidazenil Protects Against Chemical Warfare Nerve Agent Soman Induced Seizure and Neuropathology in Guinea Pigs. 49<sup>th</sup> Annual Meeting, Society of Toxicology, Salt Lake City Utah, Mar 2010

Appendix B. Co-Author Permission Forms

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To Co-Authors:

I am preparing my dissertation in the Animal, Dairy and Veterinary Science Department at Utah State University. I hope to complete me degree in the summer of 2015.

An article, The Comparative Toxicity of a Reduced, Crude Comfrey (*Symphytum officinale*) alkaloid extract and the Pure, Comfrey-Derived Pyrrolizidine Alkaloids, Lycopsamine and Intermedine in Chicks (*Gallus gallus domesticus*), of which I am first author, and you are all co-authors, was recently submitted to the Journal of Applied Toxicology. This article reports an essential part of my dissertation research. I would like permission to reprint it as a chapter in my dissertation (Reprinting the chapter may necessitate some revision.) Please note that USU sends dissertations to ProQuest Dissertation Services to be made available for reproduction.

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I am preparing my dissertation in the Animal, Dairy and Veterinary Science Department at Utah State University. I hope to complete me degree in the summer of 2015.

An article, Heterozygous p53 knockout mouse model for dehydropyrrolizidine alkaloid-induced carcinogenesis, of which I am first author, and you are all co-authors, was published in the Journal of Applied Toxicology. This article reports an essential part of my dissertation research. I would like permission to reprint it as a chapter in my dissertation (Reprinting the chapter may necessitate some revision.) Please note that USU sends dissertations to ProQuest Dissertation Services to be made available for reproduction.

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Kip Panter	_____	_____
Edward Knoppel	_____	_____