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An *in vitro* comparison of the cytotoxic potential of selected dehydropyrrolizidine alkaloids and some *N*-oxides

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ABSTRACT

Plants producing dehydropyrrolizidine alkaloids (DHPAs) are found throughout the world and they are dangerous to human and animal health. Several DHPAs are carcinogenic but only riddelliine has been classified as a potential human carcinogen by the National Toxicology Program. As DHPA-related carcinogenicity is probably linked to cytotoxicity, a model of CRL-2118 chicken hepatocyte cytotoxicity was developed to compare equimolar DHPA exposures between 19 and 300 μM. Alkaloid-related cytotoxicity was estimated using cytomorphology, cell viability reflected by mitochondrial function and cellular degeneration reflected by media lactate dehydrogenase activity. Lasiocarpine induced cytotoxicity and decreased cell viability in a concentration dependent manner at 24 h. At similar concentrations and exposures of 48 and 72 h, seneciphylline, senecionine, monocrotaline and riddelliine were cytotoxic. None of the DHPA-*N*-oxides were significantly cytotoxic at these concentrations. Using graphic analyses the median cytotoxic concentration (DHPA concentration that produced ½ the maximum response) were estimated. The estimated descending order of cytotoxicity was lasiocarpine, seneciphylline, senecionine, heliotrine, riddelliine, monocrotaline, riddelliine-*N*-oxide, lycopsamine, intermedine, lasiocarpine-*N*-oxide and senecionine-*N*-oxide. This comparison identifies DHPAs that were more cytotoxic than carcinogenic riddelliine. Additional studies to better characterize the carcinogenic potential of these alkaloids are essential to better determine the risk they each may pose for human and animal health.

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1. Introduction

Toxic dehydropyrrolizidine alkaloids (DHPAs) are mainly mono- or diesters (open chain or macrocyclic) of the necine base 1-hydroxymethyl-7-hydroxy- 1,2-dehydropyrrolizidine (heliotridine or retronecine), or of the *seco* form of the necine base (otonecine), with various necic acids differing in 2D structure or stereochemistry (see Fig. 1 for examples) (Bull et al., 1968; Mattocks, 1986). With a worldwide distribution, the DHPA-producing plants are estimated to compose more than 3% of the world's flowering plants. Due to the weedy nature of some of these plants, DHPAs often contaminate feeds and food thereby poisoning or threatening livestock, wildlife and humans.

Toxicity is dependent on *in vivo* bioactivation of the DHPAs by cytochrome P450 monooxygenases leading to highly reactive didehydropyrrolizidine alkaloids referred to as “pyrroles”. These electrophilic pyrroles are potent bioalkylating agents that react with cellular proteins and nucleic acids resulting in cellular degeneration and necrosis and, under some conditions, neoplastic transformation. As bioactivation occurs largely in the liver, most DHPAs are potent hepatotoxins. Several DHPAs have also been shown to be carcinogenic; however, only riddelliine has been listed as reasonably anticipated to be a human carcinogen (United States National Toxicology Program) (Chan et al., 2003; Mattocks, 1986).

Human DHPA poisoning generally results from contamination of food or intentional use of DHPA-producing plants as herbal supplements or food additives (Edgar et al., 2011). Several DHPA-producing plants and a few purified alkaloids have been shown to be carcinogenic in animals, but these studies are in biologically diverse models with little opportunity for direct comparison (Chan et al., 1994; Hirono, 1986, 1978, 1983; Kuhara et al., 1980; Mattocks, 1986; Mattocks and Cabral, 1982; Newberne and Rogers, 1973;

Abbreviations: DHPA, dehydropyrrolizidine alkaloid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; LDH, lactate dehydrogenase.

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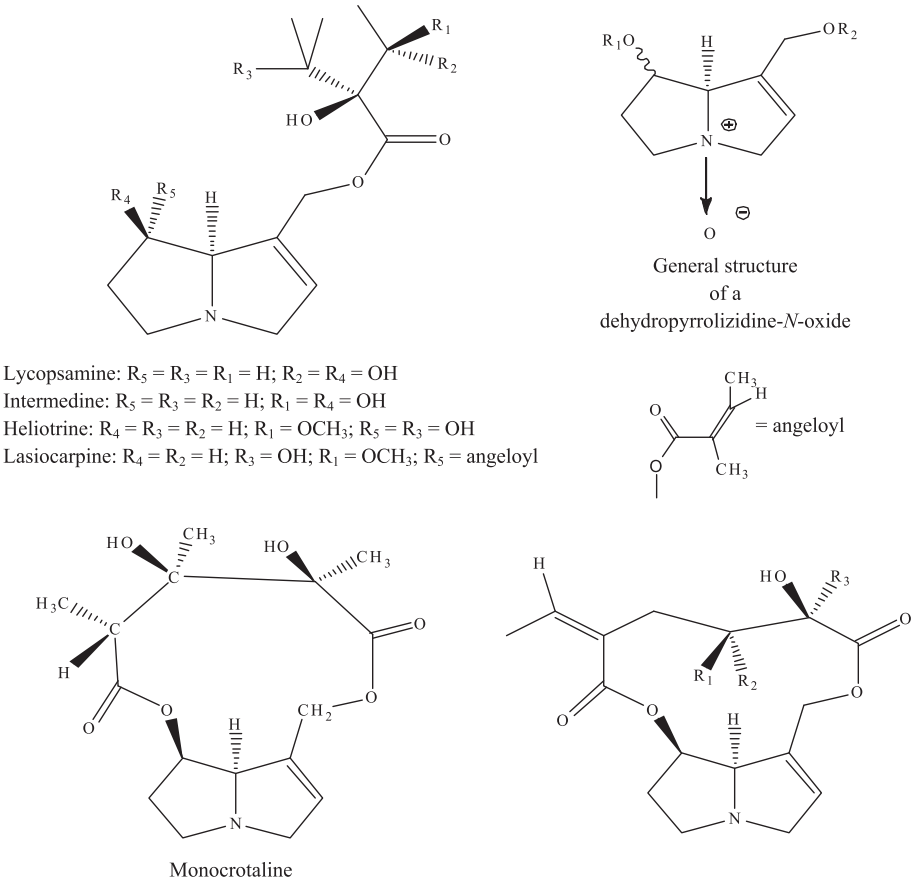

Fig. 1. Structures of dehydropyrrolizidine alkaloids (DHPAs) and *N*-oxides investigated in this *in vitro* cytotoxicity study. DHPAs consist of a necine base and one or more necic acids. Retronecine and heliotridine (isomers with a single stereochemical difference at the C7 position) are necine bases commonly found in DHPAs (Mattocks, 1986; Prakash et al., 1999).

Schoental, 1975; Schoental et al., 1954; Svoboda and Reddy, 1972). The lack of direct comparison makes predicting risk and carcinogenic potential difficult for individual alkaloids.

There are marked species, gender, age and nutritional status differences in susceptibility to DHPA intoxication. For example, *Cynoglossum officinale* was studied in aged ponies and determined not to be toxic at doses as high as 300 mg total DHPAs/kg bw/day for 20 days (Knight et al., 1984). However, yearling horses were severely poisoned when exposed to 15 mg total DHPAs/kg bw/day for 7 days (Stegelmeier et al., 1996). There is also huge variation in concentrations of DHPAs in the plants. For example riddelliine concentration in the same *Senecio riddellii* populations and phenotypes has been documented to range from nearly 18% to less than 0.2% in the next year (Johnson et al., 1989). This animal and plant variability impairs the ability to predict risk and to compare relative toxicity and subsequent carcinogenicity of DHPA-producing plants.

In vitro models using primary and transformed cell cultures have been used to document DHPA cytotoxicity. Some cells require DHPA preactivation while others readily metabolize DHPAs and develop cytotoxicity at concentrations similar to those projected from animal studies. In primary hepatocyte cultures Williams and Mori (1980) reported that monocrotaline and petasitenine damaged more DNA than lasiocarpine, senkirkine or clivorine. In a similar model Green et al. (1981) showed that senecionine is cytotoxic following a rapid uptake from the media and subsequent cellular metabolism leading to soluble metabolites and cellular adducts. They also found that concentrations above 16 nM/10^6 were cytotoxic as indicated by induction of DNA repair, release of LDH into media and morphological cellular swelling and degeneration. Non-mammalian models have also been developed. For example, *Drosophilia melangaster* wing cells were used to monitor DHPA somatic mutation and genotoxicity (Frei et al., 1992). More recent studies of DHPA-induced cellular degeneration and reduced cellular viability using immortalized cells such as human hepatocellular carcinoma (HepG2) or human embryonic kidney (ERK293). These models were relatively less sensitive as they required higher DHPA concentrations to alter cellular viability or induce cellular degeneration (Ji et al., 2008; Li et al., 2013). As this work was done in very different cell models with varying DHPA purification, chemical characterization and exposures, direct comparison is difficult. Additional work is needed to develop a sensitive model that does not require DHPA preactivation to do side by side comparisons of relatively small amounts of purified DHPAs and their metabolites.

The objective of this study was to develop a sensitive *in vitro* cell model that can uptake and metabolically activate media DHPAs and their *N*-oxides. If DHPA media concentrations were similar to those thought to develop *in vivo* (~20 μM, Mattocks, 1986), the results might be more easily compared with reported toxicity data. Such a sensitive model will also allow ranking the cytotoxicity of relatively small amounts of purified DHPAs and/or their *N*-oxides which will be useful in assessing the risk each pose for human and animal health.

2. Materials and methods

2.1. Dehydropyrrolizidine alkaloids

Authenticated (NMR, HPLC-esiMS and MS/MS) lycopsamine, intermedine, heliotrine, senecionine, senecionine-*N*-oxide,