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Abstract

Dehydropyrrolizidine alkaloid (DHPA) producing plants commonly poison livestock, wildlife and humans. Poisoning occurs when DHPAs are ingested as feed or food, or when they contaminate medicinal or herbal products. Direct toxicologic comparison of individual DHPAs is essential to estimate their actual health risks. This has been problematic due to varying models and difficulties in DHPA isolation or synthesis. In contrast, the macrocyclic DHPA riddelliine is readily isolated and it has been used as a benchmark to characterize different models of toxicity and carcinogenicity. Following earlier work with immortalized cell lines, the objective of this study was to characterize the effect of riddelliine on primary mouse, rat and chick hepatocyte cultures with the aim of developing a suitable, sensitive model for assessing DHPA-related cytotoxicity. After establishing viable cultures, the hepatocytes were exposed for 24 hours to riddelliine (from 0.1μM to 1.2mM) and cytotoxicity (CT_{50}) was estimated using a mitochondrial function assay (MTT). Despite a biphasic response, possibly attributable to a sub-population of resistant chick hepatocytes, chick hepatocyte cultures were highly sensitive (CT_{50} 0.9 μM) to riddelliine cytotoxicity relative to rat (CT_{50} 289 μM) and mouse (CT_{50} 627 μM) hepatocytes. Chick, mouse and rat hepatocyte cytochrome P450 3A4 activities did not correlate with riddelliine-induced cytotoxicity. With further development to utilize the highly sensitive primary chick hepatocytes, this model may be useful to directly compare panels of DHPAs, including rare or difficult to isolate alkaloids.

Keywords

dehydropyrrolizidine alkaloids; riddelliine; primary hepatocyte cultures; hepatocyte cytotoxicity, cytochrome P450 3A41

Introduction: Dehydropyrrolizidine alkaloid (DHPA) producing plants are well documented as causes of intoxication of livestock, wildlife and humans (Edgar *et al.* 2011; Stegelmeier *et al.* 1999; Beier 1990; and Mattocks 1986). Reported clinical effects and histopathologic lesions include hepatic degeneration and necrosis with less frequent vascular and veno-occlusive disease, portal hypertension, pulmonary hypertension or nephrosis. Most exposures are relatively acute or sudden since DHPA-producing plants, or parts there from, are ingested over several hours or days. This is often due to the invasive nature of many DHPA-producing plants allowing them to infest pastures, ranges, crops and contaminate feeds and foods. The DHPA-producing plants can also be included or inadvertently contaminate herbal products. However, poisoning is often inconsistent due to toxicity differences associated with DHPA structure (physical and chemical properties); environmental or geographic variations that alter plant toxins and concentrations; or species susceptibility and variation in response to poisoning (Molyneux *et al.* 2011). These inconsistencies often make it difficult to obtain a definitive poisoning diagnosis and, more importantly, when associated with different models of toxicity make it difficult to compare individual DHPA toxicity and predict risk. For example, *Cynoglossum officinale* (houndstongue) was originally reported not to poison aged ponies (Knight *et al.* 1984). However, additional studies found young animals are highly susceptible to houndstongue poisoning (Stegelmeier *et al.* 1996). Houndstongue major alkaloids include heliosupine, 3'-acetylheliosupine, echinatine and 7-angeloylheliotridine and the concentrations of these alkaloids varies in different plant phenological stages and different plant parts (El-Shazly *et al.* 1996). This results in plant toxicity variation from none to highly toxic and fatal. This impairs meaningful risk assessment suggesting that all exposures have to be considered potentially dangerous (El-Shazly *et al.* 1996; Stegelmeier *et al.* 1996). Similar to houndstongue, many other DHPA-producing plants have variations in alkaloids and concentrations resulting in a poor or unreliable understanding of the toxic potential of their individual and combined alkaloids.

In addition to these relatively acute exposures and their toxic sequelae, there is increasing worldwide concern about the potential health risks due to cumulative, intermittent or long-term, low-dose DHPA exposures. Such risks include an increased incidence of cancers and other chronic diseases (Brown *et al.* 2015; Edgar *et al.* 2015). Current indications suggest that bioactivation and metabolism of all DHPAs lead to the same toxic and potentially carcinogenic entities (Edgar *et al.* 2011). Consequently many existing regulations or recommendations regarding human exposure to DHPAs do not differentiate between the individual alkaloids. However, variation in alkaloid toxicity suggest this may not be accurate. Even though both clinical and mechanistic studies suggest many chronic DHPA exposures have carcinogenic potential, only two, lasiocarpine and riddelliine, have

been listed as Class 2B carcinogens (IARC 2017; Chan 1993). More work is needed to better characterize individual DHPA toxicity and to determine if all DHPAs have similar carcinogenicity. Consequently, research efforts have been expanded to include development of *in vivo* and *in vitro* models to directly compare the relative toxicity and carcinogenic potentials of purified DHPAs of both acute and chronic exposures (Brown *et al.* 2015; Brown *et al.* 2016; Field *et al.* 2015). These models have identified several DHPAs that are more toxic and probably more carcinogenic than riddelliine. However, a preliminary, highly sensitive screening of DHPAs is needed to better compare DHPAs that are available only in limited amounts. Such direct side by side comparison will enable rational selection of DHPAs for *in vivo* studies and to better predict the impact and risk of less toxic, rare DHPAs.

Cell culture models have been used to study both DHPA-induced toxicity and metabolism (Mattocks 1986). Directed by observations that chicks are highly sensitive to DHPA-induced toxicity (Brown *et al.* 2016), an *in vitro* chicken hepatocarcinoma (CLR-2118) model was developed and used to define a riddelliine CT_{50} (the concentration at which cell viability is 50% of the maximum response) of 192 µM compared to more than 100 mM for human HepG2 cells (Field *et al.* 2015). However, it has been suggested that primary, competent hepatocyte cultures (cells that are not dependent on co-culturing with other cells or microsome preparations to activate DHPAs) might be better models to study toxicity (Reindel and Roth 1991).

DHPA are bioactivated by liver mixed function oxidases. Of these, the cytochrome P450 (CYP) sub-families CYP3A and CYP2B have been reported to be the major hepatic CYPs that oxidize many DHPAs (Fu *et al.* 2004; Mattocks 1986). Recent primary human hepatocyte cultures identified a battery of CYP1A1, CYP1A2, CYP2A6, CYP3A4, CYP3A5, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 that could potentially metabolize DHPAs (Raun *et al.* 2014). Similar large batteries have also been identified in animals (Gordon *et al.* 2000). However, such lists are misleading as they indicate CYPs may metabolize DHPAs, but their relative importance has not been confirmed. Certainly CYP *in vivo* function is limited by alkaloid affinity and subsequent DHPA specificity and rates of metabolism. Consequently, most DHPAs are principally metabolized *in vivo* by only one or two CYPs (Ruan *et al.* 2014; Mattocks 1986). CYP3A4 has been identified as playing a major role in hepatic DHPA activation in man, mice, rats and chickens (Fashe *et al.* 2015; Haun *et al.* 1998a; Haun *et al.* 1998b). There are both species and individual variation in CYP activity and recently increased CYP3A4 activity has been linked to increased DHPA toxicity. A woman developed severe liver injury that was associated with increased CYP3A4 activation of echimidine from contaminated pollen while her similarly exposed husband with more normal CYP activities was unaffected (Rollason *et al.* 2016). As such

differences in CYP activities may also play a role in carcinogenicity of DHPAs, it would be beneficial to better correlate CYP expression relative to individual DHPA toxicity and later carcinogenicity.

The objectives of this present work were to: 1) determine the relative cytotoxicity of riddelliine in rat, mouse and chicken primary hepatocytes as measured by cell viability (MTT), 2) determine and compare the activity of hepatocyte cytochrome P450 3A4 (CYP3A4) in these species to determine if activity is related to cytotoxicity, and, 3) compare the *in vitro* cytotoxicity with available animal and clinical studies as described in the literature.

Materials and Methods: Riddelliine was accessed from the collection of pyrrolizidine alkaloids held by the Poisonous Plant Research Laboratory. It was initially isolated from *Senecio riddellii;* reduced to the free base; and verified chromatographically to be greater than 99% pure (Colegate *et al*. 2017; Brown *et al.* 2015). A stock solution and subsequent dilutions were made using absolute ethanol and checked for lack of hydrolytic ring-opening (Colegate *et al.* 2017). Hepatocytes were isolated from rat, mice and chick livers using modifications of techniques previously described by Ji et al. (2010). Briefly the entire rat (12-week-old, male, Sprague Dawley), mouse (12-week-old, male, BALB/c) and chick (3 to 6 -week-old, male, white California leghorn) livers were perfused and excised from euthanized animals (CO_2 narcosis followed by exsanguination following protocols approved by the Utah State University Institutional Animal Care and Use Committee (Protocols #2474 and 2643)). These ages were chosen as young weaning age animals are more sensitive to DHPA toxicity (Stegelmeier *et al.*1999; Mattocks 1986). Within minutes of euthanasia, the livers were perfused *in situ* via catheterization of the portal vein using perfusion HEPES buffer (Gibco ThermoFisher Scientific, Logan, UT) - pH 7.6 at 37°C at a flow rate of 10 mL/min until the liver blanched and then with perfusion buffer plus 0.05% collagenase type II (ThermoFisher Scientific, Logan, UT) at 25 mL/min for 6 min. The livers were placed in a sterile beaker with 20 mL William's complete media (ThermoFisher Scientific, Logan, UT) and gently broken into small 1 mm fragments using a sterile spatula. The mixture was strained through a 100 µm filter into a sterile conical tube. The cells within the filtrate were pelleted by spinning at 50 x g for 3 min at 4°C. The supernatant was removed and the pellet dispersed into 3°C William's complete media (20 mL). This washing was repeated 2 times. Finally the cell pellet was re-suspended in warm (37°C) William's complete medium (20 mL) and the cells were counted using a hemocytometer and examined microscopically identifying them as viable hepatocytes using trypan blue exclusion. The hepatocytes were then seeded into 96-well growth plates at $1.5X10^5$ cells/well (resulting in about 90% confluence). The cells were allowed to adhere by culturing (37°C with 5% CO_2) for 12 hours with a medium change at 4 hours. After 12 hours

the medium was changed to include riddelliine. All cells were then incubated for 24 hours with 9, 18, 38, 75, 150, 300, 600, and 1200 µM riddelliine while the chick cells were also incubated with 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 3, 4, 6 and 8 µM riddelliine in William's complete media.

The hepatocytes were evaluated microscopically to evaluate cellular integrity and then cytotoxicity was quantitated using a mitochondrial function assay (colorimetric measurement of formation of formazan from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) following the manufacturer's suggested protocol (CellTiter 96^R non-radioactive Cell Proliferation Assay, Promega, Madison WI- MTT). All assays were done in triplicate (hepatocytes harvested from 3 different animals) with 2 replicates or plates for each animal. The cytotoxicity 50 (CT_{50}) was estimated by determining the riddelliine concentration that produces ½ the maximal response (total SDS-induced cellular lysis following the CellTiter suggested protocol).

To compare the hepatocellular cytochrome P450 3A4 (CYP3A4) activities, microsome preparations from the livers of 6 mice, rats and chicks were separately processed as described by Klein et al. (2000). The CYP3A4 activities were then measured using a luciferin-based assay also following the manufacturer's suggested protocol (P450-Glo™ CYP3A4 Assay with Luciferin-IPA, Promega, Madison WI) using a Molecular Devices Flexstation 2 (Sunnyvale, CA USA) measuring absorbance at 570 nm. The MTT means, CT_{50} and CYP3A4 means were compared using analysis of variance to identify significant difference ($P<0.05$) and the means were separated using Duncan's procedure (Proc GLM SAS 9.3 Cary NC).

Results: The hepatocyte cultures from the three species were morphologically identified as predominately hepatocytes and nearly all cells had adhered in the wells with about 90% confluence. After the washes and media changes the tested cells were composed of approximately 95% hepatocytes with fewer numbers of mononuclear cells, fibroblasts and rare erythrocytes (Figure 1A). When incubated with riddelliine the cells became swollen and often detached from the surface of the well to float in the medium (Figure 1B). Severely damaged cells had nuclear pyknosis, karyorrhexis and cytoplasmic blebs, the latter leading to rupture and consequent fragments of cellular debris in the medium (Figure 1C). The morphologic cellular degeneration and necrosis was reflected metabolically with decreased MTT metabolism (Figure 2 and Table 1).

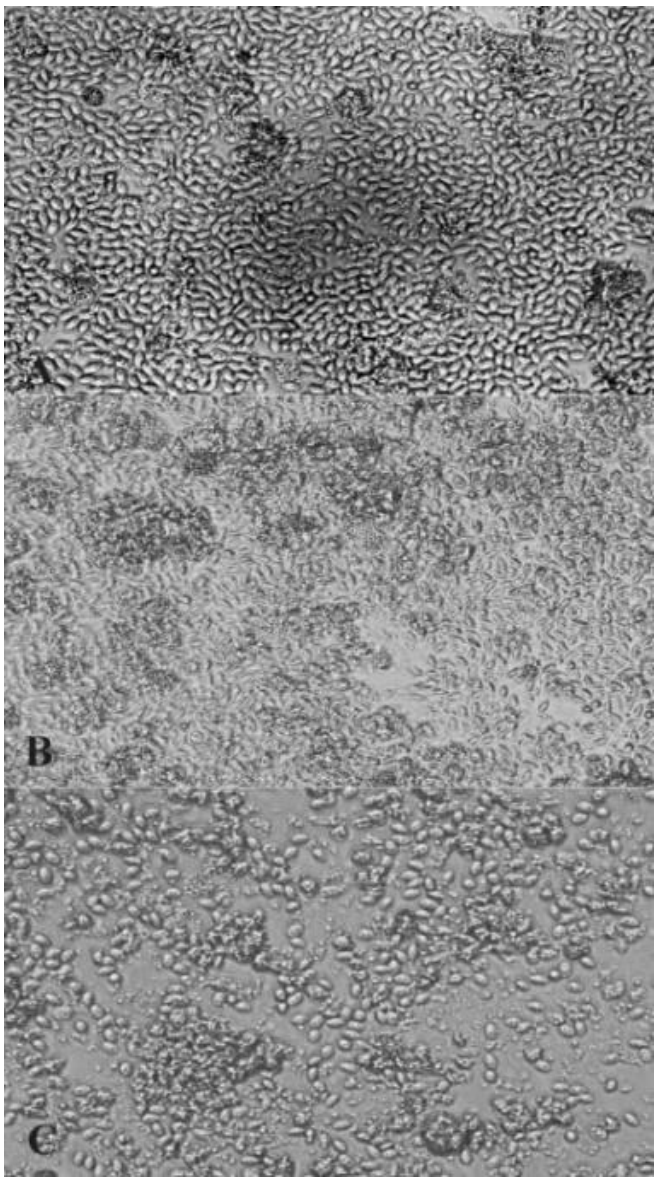

Figure 1: Incubating primary chicken hepatocytes that were incubated for 24 hours with 0(A), 5(B), and 600 µM riddelliine (C). These photographs were created in the plates with an inverted microscope using phase contrast resulting in limited resolution. The chick hepatocytes are morphologically different than other species. Notice the mostly normal chick hepatocytes (A) have smooth cell margins and though they are attached to the well bottom they do not spread out on the plate like mouse or rat hepatocytes (A). Degenerative hepatocytes incubated with riddelliine have rough cell margins (B and C), they often detach and rupture resulting in debris and cellular fragments floating in the media. Notice 30-40% of the cells appear more normal (C). This is reflected in the MTT results as some cells appear to be resistant to riddelliine cytotoxicity.

Table 1: Comparison of MTT metabolism (expressed as percent of control) in primary hepatocytes from chicks, rats and mice incubated with increasing riddelliine concentrations (µM). The means were produced from three replicates (plates) with each riddelliine concentration ran in duplicate. The significantly different means ($P<0.05$) within the species for different riddelliine concentrations are indicated with capital superscripts. Significantly different means ($P<0.05$) between species at the same riddelliine concentration are indicated with small letter superscripts.

Riddelliine Concentration µM	Chick Hepatocytes	Rat Hepatocytes	Mouse Hepatocytes
0.1	99.8 ± 4.8^{A}		
0.2	99.0 ± 5.5^{A}		
0.3	94.7 ± 2.6^{A}		
0.4	61.6 ± 2.4^{B}		
0.5	56.6 ± 3.3^{BC}		
1.0	52.3 ± 2.8^{CDE}		
2.0	54.3 ± 4.2^{BC}		
3.0	49.6 ± 3.5^{CDE}		
4.0	52.0 ± 2.3^{CDE}		
6.0	47.1 ± 1.7^{DEF}		
8.0	52.3 ± 3.9^{CDE}		
9.0	$51.1\pm13.0^{CDE\ a}$	$84.3\pm11.0^{A\ b}$	$98.7\pm5.4^{AB\ c}$
10	51.8 ± 4.3^{CDE}		
18	$62.2\pm13.6^{B\ a}$	$67.2\pm14.1^{B\ a}$	$93.6\pm7.8^{ABC\ b}$
38	$43.5\pm8.5^{EF\ a}$	$58.3\pm10.3^{B\ b}$	$70.0\pm13.2^{BC\ b}$
75	$52.8\pm12.2^{CDE\ a}$	$69.7\pm16.8^{AB\ ab}$	$78.3\pm13.9^{ABC\ b}$
150	$47.3\pm11.3^{CDEF\ a}$	$40.9\pm20.6^{C\ a}$	$84.7\pm14.3^{ABC\ b}$
300	$46.1\pm11.3^{DEF\ a}$	$43.1\pm11.0^{C\ b}$	$107\pm10.2^{AB\ b}$
600	$39.5\pm8.1^{FG\ a}$	$3.4\pm1.4^{D\ b}$	$55.5\pm1.7^{C\ c}$
1200	$34.3\pm4.0^{G\ a}$	$11.8\pm3.5^{D\ b}$	$23.7\pm15.7^{D\ ab}$

The mean calculated CT_{50}s for the rat and mouse hepatocytes were 289±101 µM and 627±88 µM riddelliine, respectively (Table 2). *In vivo* studies in both mouse and rat suggest that morphological changes were evident at riddelliine doses between 600 to 900 µM which are similar to these CT_{50}'s. By contrast, chick hepatocytes were much more sensitive with an estimated CT_{50} of 0.9±0.04 µM, statistically different to the rat and mouse hepatocyte cultures (Table 2). Morphologic changes in chick hepatocytes were evident at riddelliine doses between 6 to 10 µM. The mouse and rat MTT responses were variable with relatively high standard deviations such that only the relatively high concentrations of 600 and 1200 µM were significantly different than the lower exposures. In contrast, the consistent response (low variability) of chick hepatocytes between 0.1 and 1 µM and again between 1 and 10 µM demonstrate a significant initial reduction of about 40% in MTT metabolism (cytotoxicity) followed by similar responses between 40 and 50% suggesting a short plateau in which additional cells

were not damaged (Figure 2). At higher concentrations between 18 and 1200 µM the MTT response had increased variability with gradual decreases to a maximal response of about 30% of control. This high concentration response with increased variability was similar to that seen in both rat and mouse hepatocytes exposed to similar riddelliine concentrations (Figure 2, Table 1). The rat MTT response was lower nearly to zero at high riddelliine concentrations. Chick hepatocyte MTT metabolism at 600 and 1200 µM concentrations was significantly less than that seen at the lower exposures. This bi-phasic response of the chick hepatocyte culture was clearly different to the response of the mouse and rat hepatocyte cultures (Figure 2, Table 2).

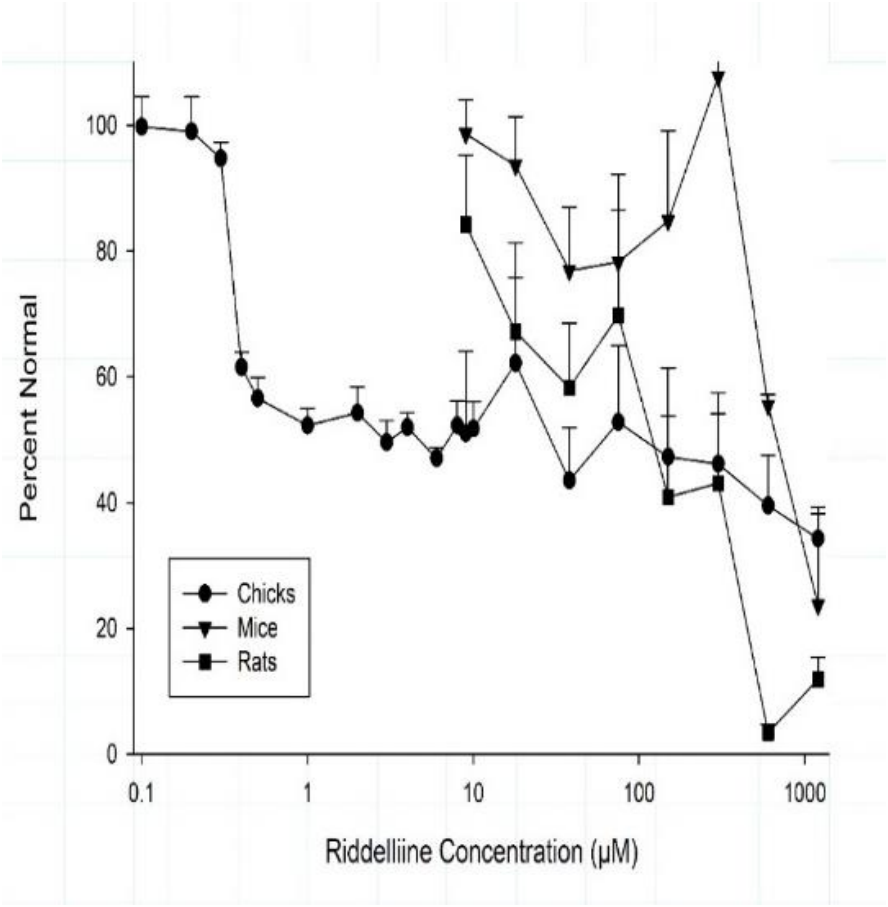

Figure 2: Cytotoxicity of primary chicken, mouse and rat hepatocytes exposed to riddelliine. Cytotoxicity was estimated using MTT (a mitochondrial function assay). Notice the biphasic response of chicken hepatocytes as about half the MTT activity lost between 0.1 and 0.6 µM. The response then plateaus between 1 and 10 µM and gradually decreases at higher doses between 18 and 1200 µm. The rat and mouse response was similar to chick hepatocytes with decreases in MTT starting at about 50 µM with most cytotoxicity occurring between 200 and 800 µM.

Table 2: Cytochrome P450 3A4 activities and riddelliine-induced 50% cytotoxic doses (CT_{50}) in rat, mouse and chick primary hepatocytes. Data are presented as means ± standard deviation. Significantly different means between species are indicated by superscript letters.

Species	CT50 µM Riddelliine	CYP3A4 µM d Luciferin
Chick	0.9±0.04 ^{A}	17.3±7.7 ^{A}
Rat	289±101 ^{B}	252.5±87.0 ^{B}

The mean microsomal CYP3A4 activities (µM D-luciferin produced in 30 min) were similar for chicks (17.3±7.7) and mice (11.4±4.4), but significantly less (P = 0.001) than rats (252±87) (Table 1).

Discussion: The purpose for developing rapid and sensitive *in vitro* DHPA bioassays is to offer a direct, side by side comparison of alkaloid toxicity, especially those alkaloids that are rare, or difficult to isolate or synthesize. Benchmark alkaloids such as riddelliine allow meaningful risk estimation. Ideally, the assays should be biologically relevant in terms of approximating natural exposures and they should allow extrapolation of *in vitro* observations to *in vivo* predictions for specific livestock species and especially humans. Ideal assays would also provide a useful preliminary screening bioassay for selecting specific alkaloids for more in-depth studies of their genotoxic and/or carcinogenic potential.

Animal toxicity studies with *Senecio riddellii* have shown that doses of 15 mg "total pyrrolizidine alkaloid/kg bodyweight per day for 20 days are lethal to calves" (Johnson *et al.* 1985). *S. riddellii* contains primarily riddelliine *N*-oxide with a small amount of free base; however, it has been shown that orally administered riddelliine *N*-oxide is as toxic as its free base riddelliine. This is presumably due to reduction in the digestive tract prior to absorption (Molyneux *et al.* 1991). Assuming complete reduction of the *N*-oxide and complete absorption of riddelliine from the digestive tract, both of which are unlikely, the daily body riddelliine concentration would be about 40 µM. This crude estimate does not account for complicating factors such as absorption differences, portal circulation, first pass hepatic metabolism and potential accumulation in some tissues (Mattocks 1986), but the implication is that suitable *in vitro* DHPA bioassays probably need to be sensitive in the low µM range. This assumption is supported by toxicokinetic studies of riddelliine, in which serum riddelliine concentrations of 0.7 µM in rats and 1.4 µM in mice were measured following a single, oral gavage of 10 mg riddelliine/kg bodyweight (Williams *et al.* 2002). In this primary cell culture study the CT_{50}s were estimated to be 627 and 289 µM riddelliine for mouse and rat hepatocytes respectively (see Table 2). This is similar to other rodent primary

hepatocyte DHPA studies (Ji *et al.* 2010; Williams and Mori 1980). These cytotoxic concentrations are much higher than the observed (or conservatively estimated) *in vivo* concentrations following oral dosing with riddelliine. As mentioned previously, the CT_{50} of riddelliine was estimated to be 192 uM in immortalized chick hepatocarcinoma CLR-2118 cells (Field *et al.* 2015), again higher than the target *in vivo* concentrations. While this assay is adequate for relatively toxic alkaloids such as senecionine, seneciophylline, or lasiocarpine, it may lack the sensitivity needed to compare some rare or less toxic alkaloids. By comparison, the chick primary hepatocyte culture, despite the biphasic nature of the response, defined cytotoxicity of riddelliine with a $CT_{50} \sim 0.9$ µM. This estimate of cytotoxicity was mathematically obtained and replicated with standard deviation of 0.4 µM. However, Figure 2 indicates this CT_{50} is on the central flat portion of the response curve where there is little difference in response with adjacent concentrations (0.8 to 10 µM). While more work is needed to better understand this response, the conclusion that primary chick hepatocytes are more sensitive to riddelliine cytotoxicity appears valid. With, or without further development to identify and address the reasons for the biphasic response, this additional sensitivity might enable the determination of physiologically relevant *in vitro* CT_{50}s for a wide range of DHPAs. For example, Field *et al*. (2015) defined a $CT_{50} > 300$ µM for the retronecine monoester dehydropyrrolizidine alkaloids lycopsamine and intermedine, and 256 µM for the macrocyclic diester monocrotaline. This essentially ranks them as equally toxic, even though clinical exposures suggest they have different toxicities and associated clinical diseases (Mattocks 1986). Comparing their cytotoxicity against riddelliine in the chick primary hepatocyte culture may provide a more physiologically relevant assessment of their actual cytotoxic and clinical toxic potential.

Riddelliine cytotoxicity increased between concentrations of 9 and 600 µM with a trending, gradual increase in cytotoxicity in rat hepatocytes. In mouse hepatocytes there was little riddelliine-induced cytotoxicity at 9 and 18 µM while cytotoxicity increased with the maximum effect seen at 1600 µM. Riddelliine cytotoxicity in both rat and mouse hepatocytes, especially at higher concentrations, are indicative of a variable dose-associated response of resistant, homogeneous cell populations (Figure 2, Table 1). By contrast, the apparent biphasic response of the chick hepatocytes indicates a more complex response. It may be that the outwardly homogenous population of cells is in fact comprised of two sub-populations, one more resistant to riddelliine than the other. Similar cellular differences are often seen in histologic studies as some toxins affect only certain morphologic zones within the hepatic lobule (Allen *et al.* 2005). Alternatively, it may be that the metabolic activation of riddelliine by the chick hepatocytes is different than that of the mouse and rat hepatocytes. Activating metabolic enzymes in chick hepatocytes might become overwhelmed, seemingly causing no increase in toxicity with

increased riddelliine concentrations. Similar impressions of reduced toxicity in the face of higher doses have also been postulated in studies using intra-ruminal infusion of riddelliine or its *N*-oxide in livestock (Molyneux *et al.* 1991). It has also been suggested that the initial DHPA exposure may saturate or temporarily inhibit the synthesis of oxidative enzymes required for activation (Shull *et al.* 1976). Biphasic MTT responses have also been described when immortalized human fibroblast cultures were incubated with ciprofloxacin. Concentrations between 50-75 mg/L produced dose responsive cytotoxicity while doses higher than 75 mg/L were not cytotoxic. The authors suggested that higher doses upregulated intracellular glutathione, catalase, superoxide dismutase and glutathione peroxidase resulting in decreased oxidative damage (Hincal *et al.* 2003). Biphasic cytotoxic responses have also been attributed to toxins that alter apoptosis. Lithium chloride alters apoptosis-regulating glycogen synthase kinase 3ß and other caspases activities in human breast adenocarcinoma cells (MCF-7) thereby changing dose-related cytotoxicity (Suganthi *et al.* 2012). It is not presently known if similar metabolic changes contribute to the DHPA-induced chick hepatocyte response. The initial response seen in primary chick hepatocyte cultures might be used to develop a sensitive cytotoxicity assessment tool. Future development and investigation may determine the mechanism of the biphasic response and allow the sensitive cells to be isolated or enhanced so that all respond uniformly, avoiding the biphasic response.

The CT_{50}s estimated for the rat hepatocytes (289 µM riddelliine) and mouse hepatocytes (627 µM) indicated that the rat hepatocytes were more susceptible to the cytotoxic effects of riddelliine. This is in agreement with the reported higher susceptibility of rats to riddelliine-induced carcinomas relative to mice (IARC 2017; Chan 1993). Both the initial dose-related response of the chick hepatocytes between 0.1 and 0.8 µM and the relatively stable plateau between 1 and 10 µM have minimal variation. At increasing concentrations between 150 and 1200 µM there was increased cytotoxicity that was similar in both response and variability to mouse and rat hepatocytes. At these concentrations there was no statistical difference in mean MTT metabolism between these species (Figure 2 and Table 1). However, at 1200 µM riddelliine the rat hepatocytes were nearly all necrotic and MTT metabolism was lower than, and significantly different to the rates for the mouse and chick hepatocytes. Higher concentrations (data not shown) did not further damage any of the surviving cells. At 1200 µM, the metabolism of MTT by the chick and mouse hepatocytes was about 30% of control MTT metabolism suggesting that, for presently unknown reasons, a small number of hepatocytes are resistant to riddelliine cytotoxicity.

The CYP3A4 assays (Table 2) are estimates as specificity has not been verified in all species; however, they are often useful for comparison. These results suggest that the rat hepatocytes have significantly higher activities than mice or

chicks. Since this is not reflected in the hepatocyte response to riddelliine, it also suggests that CYP3A4 activity is not a major factor in riddelliine toxicity in these species. However, it may be an essential component of other DHPA toxicity. The various CYP activities are toxin specific and it has been suggested that for some DHPAs other unidentified esterases may be more important in tipping the balance between toxicity and detoxification (Tang *et al.* 2007). Nonetheless, CYP3A4 has been shown to be important in DHPA activation in many species (Fu *et al.* 2004). For example, lasiocarpine is primarily activated by CYP3A4 in human hepatocytes (Fashe *et al.* 2015) and the CYP3A family activates senecionine in sheep and hamsters (Huan *et al.* 1998a). These suggest the possibility that these results are riddelliine-specific and that other DHPA cytotoxicity may be more dependent on CYP3A4 activation. It is unknown whether CYP3A4 expression impacts the response of other species to riddelliine or whether upregulated expression of other specific CYPs affects species responses to other individual DHPAs.

Although the CYPs that activate the different DHPAs in chickens have not been definitively identified, chicken CYP activity and adduct production has been documented and linked to DHPA toxicity in chickens (Fashe *et al.* 2015; Haun *et al.* 1998b). Since the data reported in this present study indicate that CYP3A4 activity is not correlated to *in vitro* cytotoxicity of the chick, rat and mouse primary hepatocytes, further investigations are required. These will include testing of a wider range of DHPAs to determine whether the primary chick hepatocytes are universally, or generally more sensitive than the rat and mouse hepatocyte cultures. There is also a need to assess the relative activities of various CYPs in the three cell lines to determine whether the increased cytotoxicity in the chick hepatocytes can be attributed to an increased activity of a specific CYP in the chick cells, relative to the rat and mouse cells.

Toxin activation and detoxification, especially oxidative status, determines DHPA toxicity. It has been suggested that DHPA-related disease occurs when pyrrole production overwhelms detoxification. Many pyrroles are conjugated with glutathione and excreted in the bile. Increasing cellular oxidative stress can overwhelm this detoxification mechanism. DHPA cytotoxicity increases lipid peroxidation and overwhelms glutathione peroxidase, glutathione S-transferase, and glutathione reductase. Low doses of DHPAs often induce increases in catalase while all DHPA doses reduce superoxide dismutase activities. These changes indicate oxidative stress and damage to cellular oxidative protection mechanisms (Ji *et al.* 2010). Differences in the glutathione antioxidant system have also been associated with differences in DHPA susceptibility related to age and gender in mice (Liang *et al.* 2011a; Liang *et al.* 2011b). Additionally antioxidants such as α-tocopherol or quercetin can be protective against DHPA-induced damage (Ji *et al.* 2014; Molteni *et al.* 2004). The effect is also apparent clinically as animals under oxidative stress are more susceptible to DHPA-induced disease. For example,

Amsinckia intermedia Lehmann or fiddleneck contains DHPAs but poisoning in livestock is rare. In some areas there is little other forage and apparently resistant animals eat nothing but fiddleneck without obvious poisoning. However, in cattle concurrently poisoned with *Isocoma plurifora* (burroweed) the combined oxidative stress with fiddleneck produces both muscular necrosis characteristic of burroweed poisoning and fiddleneck-induced liver disease (Panter *et al.* 2017). Similar intermittent poisonings have been associated with *Echium* spp. and other DHPA-containing plants (Betteridge *et al.* 2005; Culvenor *et al.* 1981).

Despite the increased sensitivity of the chick primary hepatocyte cultures, the chicken hepatocellular carcinoma model (CLR-2118) previously described does have several advantages (Field *et al.* 2015). Since it is an immortalized cell line propagation is relatively easy and there is little inter passage variation. This lends confidence when comparing different experiments as genetic and metabolic change is minimal. However, CLR-2118 cells still lack sufficient sensitivity since the CT_{50} is close to estimates of tissue DHPA concentrations and, for less toxic or difficult to isolate DHPAs it may not be possible to obtain enough alkaloid to thoroughly compare. Further development of the chick primary hepatocyte cultures described herein may be warranted to further characterize the biphasic response and determine if the putative population of sensitive cells exists and can be isolated and cultured.

Conclusions: These findings suggest chick primary hepatocytes cultures are highly sensitive to riddelliine cytotoxicity. Primary chick hepatocyte cytotoxicity does not appear to be related to microsomal CYP3A4 activity. However, some chick hepatocytes appeared to be resistant to riddelliine toxicity, or there occurs an inhibiting effect on riddelliine bioactivation, resulting in a biphasic response with sensitive cells damaged between 0.1 and 20 µM and more resistant cells requiring doses similar to rat and mouse hepatocytes. More work is needed to better characterize these differences. These findings also suggest that with improved isolation some highly sensitive primary chick hepatocytes may be useful in the direct comparison of a wide range of DHPAs, including those that are less toxic or difficult to isolate in larger quantities, as a preliminary screening of the cytotoxic potential at physiologically relevant concentrations.

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