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Role of toxicokinetics and alternative testing strategies in pyrrolizidine alkaloid toxicity and risk assessment; state-of-the-art and future perspectives

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ABSTRACT

Toxicokinetics influences the toxicity of chemicals. This also holds for 1,2-unsaturated pyrrolizidine alkaloids (PAs), which need bioactivation to become toxic. Given that only for a limited number of 1,2-unsaturated PAs in vivo toxicity data are available, alternative testing strategies including read-across and quantitative in vitro to in vivo extrapolation (QIVIVE) are important. This paper presents how physiologically-based kinetic (PBK) models for the PAs lasiocarpine and riddelliine were developed for rat and human, and used for conversion of in vitro data for toxicity in primary hepatocytes to quantitatively predict in vivo acute liver toxicity for both rat and human. Marked differences in toxicokinetics were observed between the two model PAs influencing the predicted in vivo toxicity. In a next step, in vitro toxicokinetic data that predicted relative bioactivation of the PAs, were shown to provide a possible basis for read-across from the $BMDL_{10}$ for tumor formation by riddelliine of 237 μg/kg bw per day to other PAs for which tumor data are lacking. It is concluded that when comparing toxicity of different PAs, or when extrapolating in vitro toxicity data for PAs to the in vivo situation, differences in toxicokinetics should be taken into account, while future challenges are also discussed.

1. Introduction

Toxicokinetics describes the absorption, distribution, metabolism and excretion (ADME) characteristics of chemicals. These ADME characteristics influence the bioavailability, the detoxification and bioactivation, and thus the toxicity of chemicals. This also holds for 1,2-unsaturated pyrrolizidine alkaloids (PAs), since they need bioactivation to dehydro-PAs to become toxic, while conversion to PA-N-oxides and hydrolysis to non-toxic necines and necic acids is considered to result in their (partial) detoxification (Edgar et al., 2011, 2015; Yang et al., 2017). Fig. 1 presents the structure of some relevant 1,2-unsaturated PAs referred to in the present paper. They include the heliotridine-type PA lasiocarpine and the retronecine-type PAs riddelliine, monocrotaline and retrorsine. Conversion of such PAs by cytochromes P450 (CYPs), in human especially CYP3A and CYP2B, results in dehydro-pyrrolizidine alkaloids (dehydro-PAs) which either directly or upon hydrolysis to dehydroretronecine-type metabolites or dehydroheliotridine-type metabolites (depending on the stereochemistry), also called (+/−)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP), may react with glutathione, proteins and/or DNA resulting in adduct formation and toxicity, including liver toxicity, genotoxicity and carcinogenicity (Chen et al., 2017; Knutsen et al., 2017). Fig. 2 presents an overview of the metabolic pathways of PAs using riddelliine as the model compound.

In 2017 the European Food Safety Authority (EFSA) concluded that, based on the available data on occurrence of PAs in food and feed, especially 17 PAs were considered relevant for monitoring in food and feed (Knutsen et al., 2017). These PAs include intermedine, lycopsamine, intermedine-N-oxide, lycopsamine-N-oxide, senecionine, senecivernine, senecionine-N-oxide, senecivernine-N-oxide, seneciphylline, seneciphylline-N-oxide, retrorsine, retrorsine-N-oxide, echimidine, echimidine-N-oxide, lasiocarpine, lasiocarpine-N-oxide and senkirkine (Knutsen et al., 2017). In spite of their relevance, in vivo toxicity data on most of these PAs are limited or even absent, hampering adequate risk assessment. For example, carcinogenicity data in experimental animals upon exposure to PAs are limited to oral studies in rats for lasiocarpine (NTP, 1978), riddelliine (NTP, 2003) and clivorine (Kuhara et al., 1980), an intraperitoneal injection study in rats with senkirkine and symphytine (Hirono et al., 1979) and a study in rats with subcutaneous injection of monocrotaline (Shumaker et al., 1976). Of all these data only the data on lasiocarpine and riddelliine appeared suitable for benchmark dose (BMD) modelling and definition of a so-called benchmark dose lower confidence limit for 10% extra tumor incidence ($BMDL_{10}$). Such $BMDL_{10}$ values are used in risk assessment of compounds that are both genotoxic and carcinogenic, such as these PAs, by the margin of exposure (MOE) approach (Chen et al., 2017;

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Abbreviations

$BMDL_{5/10/30}$	Benchmark dose lower confidence limit for 5%/10%/30% extra tumor incidence
$BMDU_{5/10/30}$	Benchmark dose upper confidence limit for 5%/10%/30% extra tumor incidence
QIVIVE	Quantitative In Vitro to In Vivo Extrapolation
LOAEL	Lowest Observed Adverse Effect Level
MOE	Margin of Exposure
NOAEL	No Observed Adverse Effect Level
PAs	Pyrrolizidine alkaloids
PBK	Physiologically based kinetic
PoD	Point of Departure

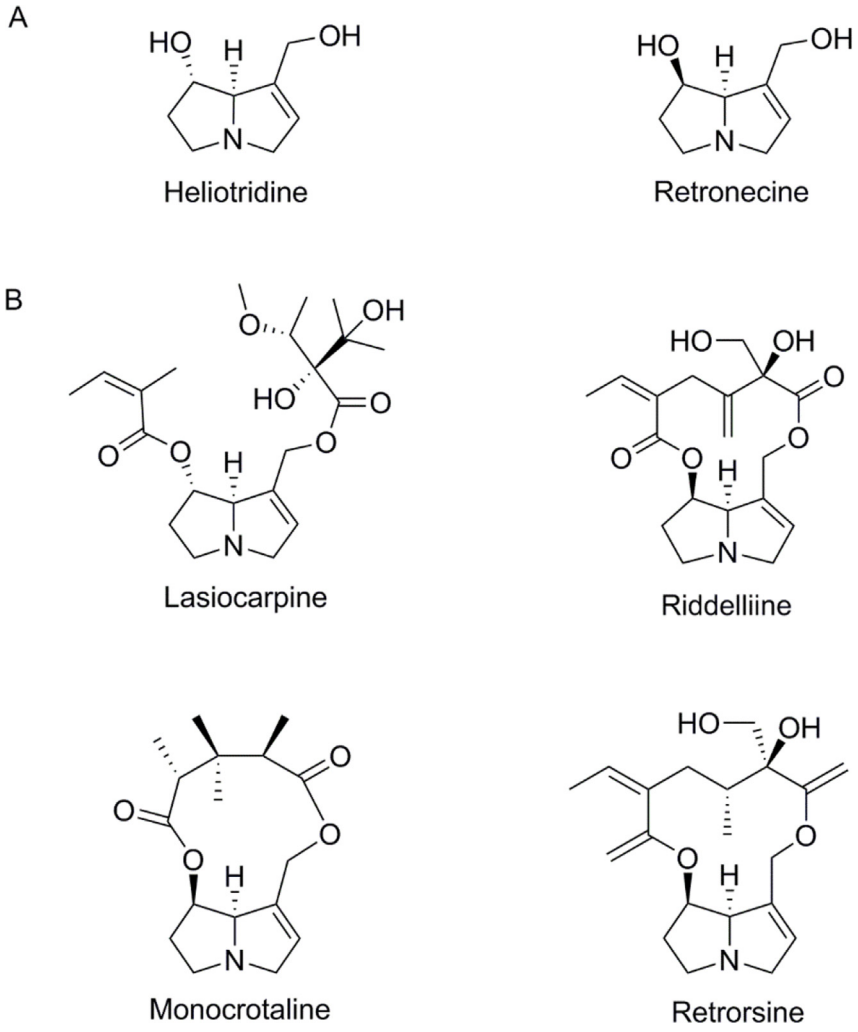

Fig. 1. Molecular structures of A) the necine bases) heliotridine and retronecine and of B) the PAs riddelliine, lasiocarpine, monocrotaline and retrorsine.

EFSA, 2011; Knutsen et al., 2017). The presence of toxicity data on carcinogenicity upon oral exposure suitable for dose-response modelling for only lasiocarpine and riddelliine, made regulatory bodies in first instance use the lowest $BMDL_{10}$ of 70 μg/kg bw per day for lasiocarpine for risk assessment of combined exposure to all PAs (BfR, 2013; EFSA, 2011). Later, however, EFSA considered that when taking differences in relative potency into consideration, use of lasiocarpine may be too worst-case and proposed use of the $BMDL_{10}$ value of 237 μg/kg bw per day for riddelliine (Knutsen et al., 2017). This $BMDL_{10}$ resulted from BMD model averaging using the data on formation of liver haemangiosarcomas upon oral exposure of female rats to riddelliine (NTP, 2003), as a more suitable point of departure (PoD) for risk assessment of combined exposure to PAs. EFSA also indicated that the currently available data are too limited to facilitate definition of reliable relative potency (REP) values, required for a combined risk assessment taking relative differences in potencies into account. Merz and Schrenk (2016) proposed interim REP values for PAs, based on available literature data on in vitro cytotoxicity, genotoxicity in *Drosophila*, and acute toxicity in rodents (LD50), showing an up to 100-fold difference in relative potencies. Chen et al. (2017) used the available data on tumor formation to compare, based on $BMDL_{10}$ and T_{10} values associated with an extra 10% tumor incidence in rodent bioassays, the relative potency of the six PAs for which such data from studies in rats were available, albeit not all using the oral route of administration. And although EFSA concluded that these interim REP values are not sufficiently robust for combined risk assessment of PAs, they also considered that the data supported that several of the PAs contributing to the dietary exposure are likely to be of substantially lower potency than the reference compounds so far used in risk assessment for PAs, riddelliine or lasiocarpine (Knutsen et al., 2017). Thus, the literature data and interim REP values supported a switch from using the $BMDL_{10}$ of lasiocarpine of 70 μg/kg bw per day to use of the less conservative $BMDL_{10}$ of riddelliine of 237 μg/kg bw per day for the risk assessment. In the risk assessment performed by EFSA combined exposure was assessed assuming dose addition without

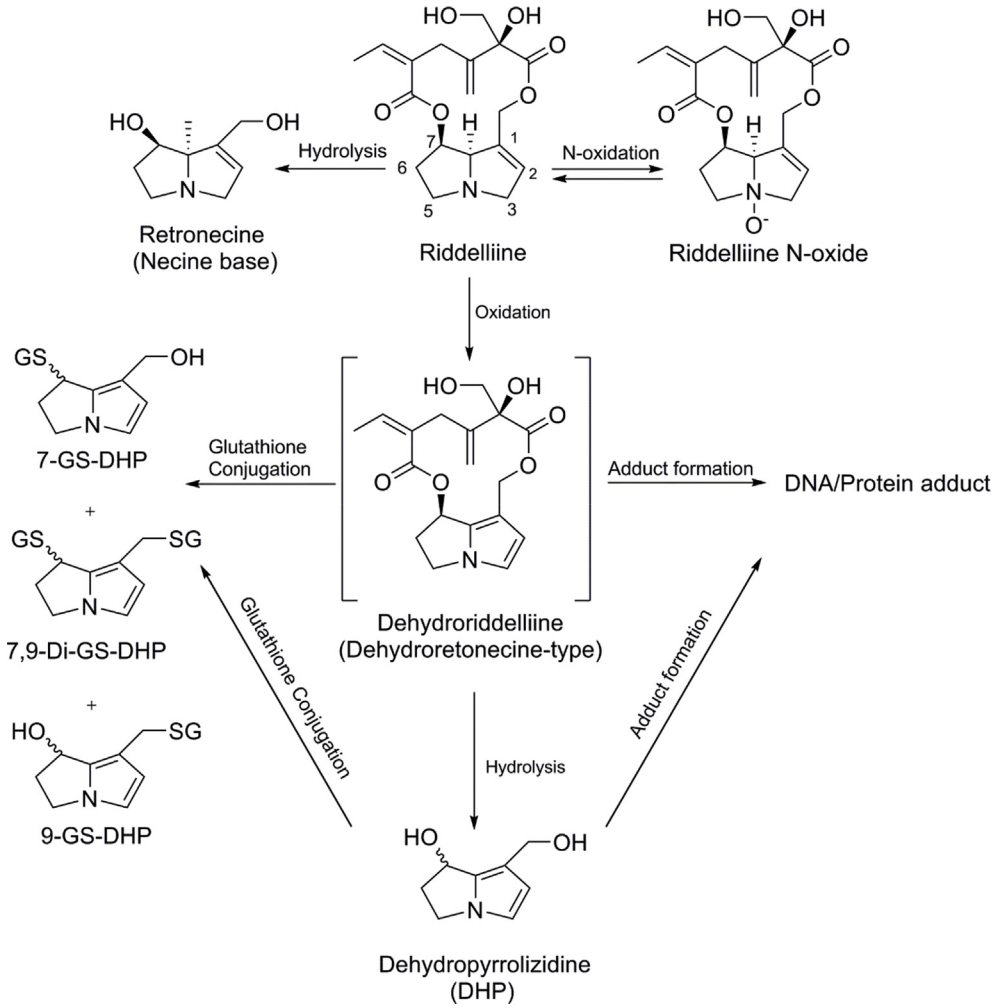

Fig. 2. Metabolic pathways for PAs presented for riddelliine as the model compound. Note that the dehydropyrrolizidine (DHP) reactive intermediate is similar for all retronecine-type PAs, whereas that for the heliotridine-type PAs has different stereochemistry at C7. Also the secondary GSH conjugates of DHP may result in DNA adduct formation (Xia et al., 2018).

taking differences in relative potency between the different PAs into account (Knutsen et al., 2017).

This current state-of-the-art reflects that there is a need for better data on the differences in relative potency of the different PAs to refine current risk assessment of combined exposure. Given that obtaining data from 2 year carcinogenicity studies for all PAs would be unrealistic, novel alternative strategies that enable prediction of their relative in vivo potencies are of interest. This is especially important given that only for a limited number of 1,2-unsaturated PAs that are considered relevant for food and feed, in vivo toxicity data are available, further supporting the need for read-across and alternative testing strategies.

Clearly such alternative testing strategies cannot rely on in vitro toxicity data alone, since differences in toxicokinetics should be taken into account when extrapolating in vitro toxicity data to the in vivo situation. This can be achieved using physiologically based kinetic (PBK) models. PBK models enable quantitative translation of in vitro concentration-response curves to in vivo dose-response curves using so-called reverse-dosimetry, thereby taking toxicokinetics into account. The next section describes examples of this quantitative in vitro to in vivo extrapolation (QIVIVE) for the PAs lasiocarpine and riddelliine.

2. Physiologically based kinetic (PBK) modelling facilitated prediction of in vivo acute liver toxicity of PAs

A PBK model is a set of mathematical equations that together describe the ADME characteristics of a compound within an organism. With a PBK model physiologically relevant concentrations of a compound in plasma or, when relevant, its active target organ of interest, can be modeled for a certain dose, time point and route of administration. Upon validation of the model against available in vivo data, the PBK model can be used to make predictions and to convert in vitro concentrations to relevant in vivo exposure levels, by so-called reverse dosimetry (Louisse et al., 2012, 2017). In this reverse dosimetry QIVIVE approach, in vitro concentrations of the concentration-response curve are set equal to plasma or tissue levels of the respective compound in the PBK model, following which the PBK model can calculate the corresponding in vivo dose level for any given route of administration. Subsequent BMD modelling can be applied on the predicted in vivo dose-response data, enabling definition of a PoD for risk assessment, like a $BMDL_{10}$. Proofs-of-principle that in vivo dose-response curves and PoDs for human risk assessment can be defined based on this in vitro PBK modelling facilitated QIVIVE approach have been described for the developmental toxicity of ethylene glycol monomethyl ether (Louisse et al., 2010), the developmental toxicity of phenols (Strikwold et al., 2013), the developmental toxicity of all-*trans*-retinoic acid (Louisse et al., 2015) and the kidney toxicity of aristolochic acid I (Abdullah

et al., 2016). Recently the approach was also used to predict the in vivo acute liver toxicity induced by riddelliine and lasiocarpine in rat (Chen et al., 2018), and human (Ning et al., 2019).

Development and use of a PBK model proceeds in several steps including; 1) definition of a conceptual model, 2) translation into a mathematical model, 3) defining parameter values, 4) solving the equations, 5) evaluation of model performance and 6) making predictions (Rietjens et al., 2011). Fig. 3 presents the conceptual model used for the PBK models for PAs (Chen et al., 2018; Ning et al., 2019). The model contains compartments for blood, fat, rapidly perfused tissue, slowly perfused tissue, liver, and intestine, that are mutually connected through the systemic circulation. Liver and intestine are included as separate compartments because metabolism of lasiocarpine and riddelliine occurs in liver and intestine and because liver is the target organ for toxicity. In a next step the model was converted into a mathematical model. The full model code can be found in literature (Chen et al., 2018; Ning et al., 2019). As an example the equations for the liver can be described as follows:

AL = Amount of the PA in liver tissue (in μmol)

$AL' = QL \times CB + QSi \times CVSi - (QL + QSi) \times CVL - AMLM'$

Init AL = 0

$AMLM' = V_{max}LM \times CVL/(K_mLM + CVL)$

Init AMLM = 0

In these equations AL presents the amount of PA in the liver (in μmol) and AL' the change in this amount in μmol/hr. The initial amount of the PA in the liver at time zero is zero (Init AL = 0). QL × CB is the amount of PA entering the liver from the arterial blood, described by the blood flow to the liver (QL in L/hr) times the concentration of the PA in this arterial blood (in μmol/L) so that this represents the amount of PA entering the liver per hour via the arterial blood flow (in μmol/hr). In the same way QSi × CVSi reflects the amount of PA entering the liver per hour via the portal vein from the intestinal tissue, minus (QL + QSi) × CVL represents the amount per hour leaving the liver via the liver veins, and minus AMLM′ is the total amount of PA metabolized in the liver in μmol/hr. This amount of PA metabolized in the liver (AMLM') is zero at time zero (InitAMLM = 0) and can be described by the Michaelis Menten equation for conversion of the parent PA with V_{max} being the maximum rate of conversion of the PA expressed in μmol/hr, K_mLM being the Michaelis Menten constant for this conversion in μmol/L, and CVL being the concentration of the PA in the liver veins (in μmol/L).

The third step in developing the model consists of definition of the parameter values, with many physico-chemical and physiological parameters already described in literature, such as for example log Koctanol/water partition coefficients used to calculate tissue/blood partition coefficients, and tissue volumes and blood flow to the various tissues. Compound specific parameters like especially the V_{max} and K_m parameters for metabolic conversion need to be defined in suitable in vitro models. As an example Fig. 4 presents the PA concentration dependent metabolic conversion of riddelliine and lasiocarpine in liver incubations with rat (Fig. 4A) and human (Fig. 4B) microsomes. Table 1 presents the V_{max} values derived from these curves and also indicates how these are scaled from a V_{max} expressed in nmol/minute/mg microsomal protein to a V_{max} expressed in μmol/hr using a conversion factor of 35 mg microsomal protein/g liver, and the bodyweight and liver fraction for rat and human. Table 1 also presents the K_m values as well as the catalytic efficiencies (defined as V_{max}/K_m) derived from these data. These results reveal that the catalytic efficiency for clearance of lasiocarpine is 10-fold and 27-fold higher than that for riddelliine in rat and human, respectively. This indicates a marked difference in toxicokinetics between the two PAs.

Including all parameters in the model and solving the equations generates predictions on the toxicokinetics of the PAs. The models can predict for example the time dependent plasma concentrations at a certain dose level (Fig. 5A), or the dose dependent C_{max} values in the liver (Fig. 5B). Evaluation of the performance of the models can be based on only a limited set of toxicokinetic data available for these PAs. Based on data on the blood concentrations of riddelliine upon dosing 10 mg/kg bw of this PA to rats or mice it could be demonstrated that predicted values for C_{max} (the maximum concentration of riddelliine in serum) are respectively 2- and 9.5- fold higher than the reported concentrations for mouse and rat (Chen et al., 2018; Williams et al., 2002). Why the mouse model performed better than the rat model remains unclear and it may require further in vivo kinetic data to solve this matter, although the discrepancy may also be due to the fact that the in vivo rat plasma data may not be fully adequate since they do not show a clear C_{max}.

In a next step the model was used to predict the acute in vivo liver toxicity of riddelliine and lasiocarpine from in vitro data on cytotoxicity towards primary hepatocytes. Fig. 6 presents the in vitro toxicity of both PAs in primary rat hepatocytes and the conversion of these data to predicted in vivo dose-response curves for acute liver toxicity in rat as obtained by reverse dosimetry using a rat PBK model (Chen et al., 2018). Recently a similar study was performed for human, predicting acute liver toxicity in human based on cytotoxicity towards human hepatocytes and reverse dosimetry using human PBK models for lasiocarpine and riddelliine (Ning et al., 2019).

The results obtained for both rat and human reveal that the difference in in vitro toxicity between riddelliine and lasiocarpine is limited with riddelliine being 1.7-fold more toxic in rat hepatocytes and 2.1-fold less toxic in human hepatocytes. The predicted in vivo difference in acute liver toxicity is more pronounced, riddelliine being predicted to be to 4- to 5- fold more toxic for rat and 7- to 10- fold more toxic for human than lasiocarpine (Chen et al., 2018; Ning et al., 2019). This reflects the importance of toxicokinetics in determining the ultimate in vivo toxicity, with the slower clearance of riddelliine, as already reflected by the lower catalytic efficiency for its in vitro clearance (Table 1), resulting in relatively higher C_{max} values and relatively higher toxicity at a comparable dose level, than what would be predicted based on the in vitro toxicity data alone. For lasiocarpine the predictions can also be compared to reported toxicity data in rat reported in the literature (Nolan et al., 1966). The in vitro PBK modelling based predicted dose-response curve resulted in a $BMDL_5$-$BMDU_5$ (Benchmark dose lower and upper confidence limits for 5% effect) value of 23.0–34.4 mg/kg bw per day (Chen et al., 2018). This

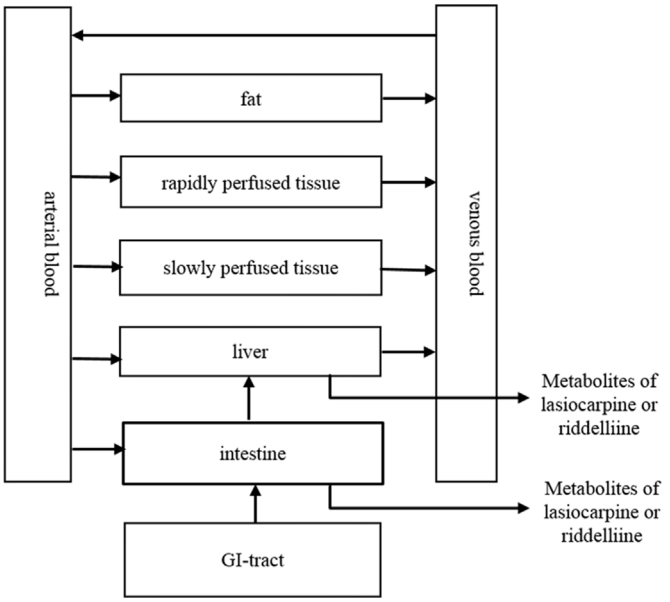

Fig. 3. Conceptual model for definition of the PBK model for PAs (Chen et al., 2018; Ning et al., 2019).

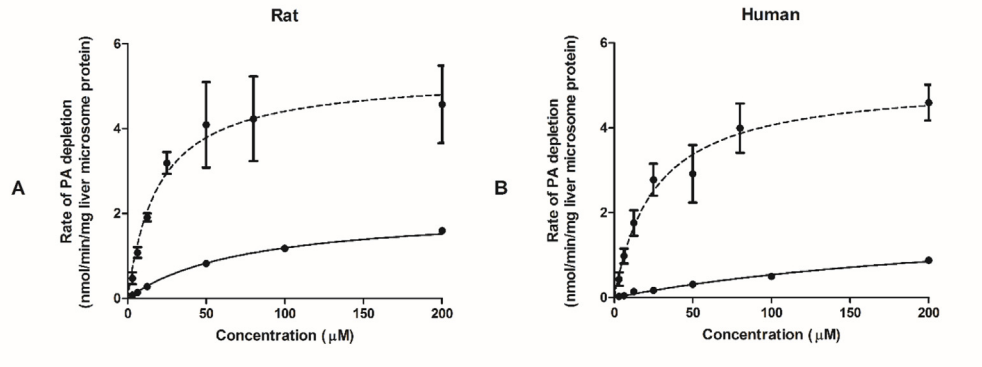

Fig. 4. PA concentration dependent conversion of riddelliine (solid line) and lasiocarpine (dash line) in incubations with liver microsomes from A) rat and B) human.

compares relatively well to the fact that 3- to 4- fold higher single oral dose levels of 80 and 120 mg/kg bw represent LOAELs (Lowest Observed Adverse Effect Levels) for oral toxicity of lasiocarpine causing increased incidence of acute zonal hepatic necrosis and megalocytosis (Nolan et al., 1966). Using an uncertainty factor of 3 to convert these LOAELs for oral toxicity data on lasiocarpine to PoDs for risk assessment would result in a PoD of 27–40 mg/kg bw per day, a value similar to the PoD of 23.0–34.4 mg/kg bw per day predicted by the PBK model facilitated reverse dosimetry QIVIVE approach (Chen et al., 2018). The fact that the acute liver toxicity of lasiocarpine was relatively well predicted further validated the PBK models developed.

Furthermore, it is of interest to note that in a 28 day study using daily repeated oral exposure to lasiocarpine a NOAEL (No Observed Adverse Effect Level) of 0.6 mg/kg bw per day was reported (Dalefield et al., 2016). This is substantially lower than the PoDs presented above for acute liver toxicity. Available experimental data as well as PBK model based predicted plasma profiles reveal that clearance of the PAs upon an oral dose of 10 mg/kg bw is expected to be complete within 10 h (Fig. 5A). This suggests that the PA itself will not be accumulating upon repeated dosing. However, repeated dosing may result in accumulation of liver damage that may not be repaired within the 24 h between subsequent doses. This would explain the lower NOAEL values for repeated dose exposure as compared to single exposure regimens. It would be of interest to validate this in a HepaRG model system since these cells, in contrast to the primary hepatocytes, can be cultivated for longer time periods and thus be exposed daily for longer periods of time, while they appear to be only somewhat less sensitive towards the toxicity of PAs (Fig. 7).

3. Use of PBK models to investigate inter-ethnic differences

The PBK models now developed for PAs also allow studying inter-individual and inter-ethnic differences. As an example the in vivo dose-response curves for acute liver toxicity of riddelliine and lasiocarpine in the Chinese as compared to the Caucasian population were predicted (Ning et al., 2019). This prediction was based on human PBK models developed for both the Caucasian and Chinese population using physiological and kinetic parameters for both ethnic groups. In addition to the inter-ethnic differences in toxicokinetics also the inter-ethnic differences in toxicodynamics were taken into account, defining the in vitro concentration-response curves for lasiocarpine and riddelliine in liver hepatocytes from Caucasian donors and defining the corresponding data for Chinese donors by correcting the curve for the Caucasian donors by the relative differences in bioactivation of the PAs in incubations with liver microsomes from Caucasian and Chinese donors (Ning et al., 2019). This relative difference in bioactivation was characterised in in vitro microsomal incubations in the presence of GSH used to scavenge and quantify the relative bioactivation on the basis of formation of the 7-GSH-DHP adduct (Fig. 2). Since the bioactivation of riddelliine and lasiocarpine are expected to result in similar GSH-DHP adducts these incubations allow quantification of the relative difference in bioactivation of the PAs by the Chinese and Caucasian population, and also of the relative differences in bioactivation of the different PAs.

4. Quantifying the relative differences in bioactivation of PAs

Given that PAs need bioactivation to reactive dehydro-PAs to exert their toxicity, methods able to quantify relative differences in bioactivation may provide surrogate endpoints to define the REP values for PAs. To quantify relative differences in toxic potency one can use cell based assays such as cytotoxicity towards hepatocytes in vitro, combined with translation of these data to the in vivo situation using PBK model based reverse dosimetry, as illustrated in the previous sections. However, one could also consider to use methods to detect and quantify formation of the reactive dehydro-PA metabolites. Given the unstable nature of these metabolites such methods should be based on

Table 1
Kinetic constants for clearance of lasiocarpine (L) and riddelliine (R) in incubations with rat and human liver microsomes as reported (Chen et al., 2018; Ning et al., 2019).

PA	Species	V_{max} [a]	K_m [a]	Catalytic efficiency [a]	Scaled V_{max} [b]	Scaled Catalytic efficiency [b]
Lasiocarpine	rat	5.3 ± 0.6	19.5 ± 7.7	0.27	94.6	4.9
	human	5.1 ± 0.5	25.8 ± 7.5	0.20	19492	756
Riddelliine	rat	2.1 ± 0.1	75.7 ± 7.4	0.028	37.5	0.49
	human	2.0 ± 0.3	274.1 ± 87.1	0.0073	7720	28.2
Ratio L/R	rat	2.5	0.26	10	2.5	10
	human	2.6	0.09	27	2.6	27

[a] In vitro V_{max} in nmol/min/mg microsomal protein, K_m in μM, catalytic efficiency in ml/min/mg microsomal protein (in vitro V_{max}/K_m). The values are mean ± SEM of three independent experiments

[b] In vivo scaled V_{max} in μmol/h, calculated from the in vitro V_{max} based on a microsomal protein yield of 35 mg/g tissue for liver, bodyweight and liver fraction of rat and human, and scaled catalytic efficiency in L/h (scaled V_{max}/K_m)

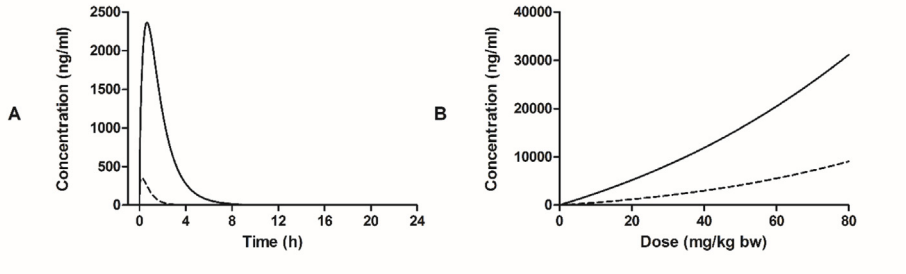

Fig. 5. PBK model predicted time dependent plasma concentration of riddelliine (solid line) and lasiocarpine (dash line) at a dose of 10 mg/kg bw for rat (Fig. 5A), and dose dependent C_{max} values in the liver blood for both compounds also in rat (Fig. 5B).

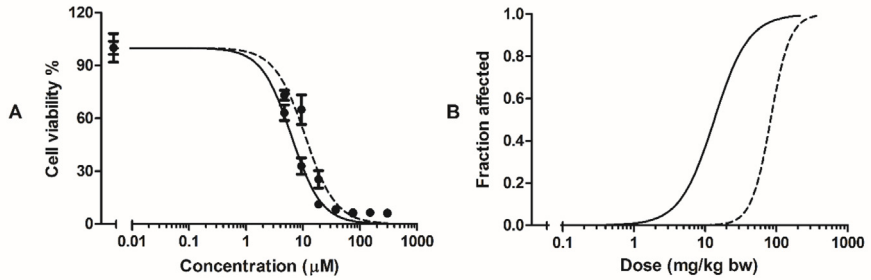

Fig. 6. A) In vitro toxicity of riddelliine (solid line) and lasiocarpine (dash line) in primary rat hepatocytes and B) their conversion to predicted in vivo dose-response curves for acute liver toxicity, as obtained by reverse dosimetry using a rat PBK model (Chen et al., 2018).

scavenging these metabolites by for example thiol reagents like GSH or cysteine. Cysteine-DHP adducts in hepatic proteins have been isolated from PA-exposed perfused livers (Yan and Huxtable, 1995) and experimental animals (Mattocks and White, 1970). In addition, DHP adducts of blood proteins have been observed in patients who were accidently exposed to PAs (Lin et al., 2011; Ruan et al., 2015). Bioactivation resulting in formation of protein, GSH or DNA adducts is likely involved in the mode of action underlying the toxicity of PAs (Xia et al., 2013, 2015, 2018), so relative differences in these surrogate endpoints may reflect differences in toxicity. This consideration is in line with data from a comparative study on the hepatotoxicity of retrorsine and monocrotaline (Fig. 1) (Yang et al., 2017). Retrorsine, is known to be more hepatotoxic than monocrotaline although they have the same basic retronecine core structure. The study showed that the in vitro catalytic efficiency (V_{max}/K_m) for conversion of retrorsine to the corresponding pyrrole-GSH conjugates in incubations with mouse liver microsomes, was 5.5-fold higher than that for monocrotaline. Furthermore, upon dosing male Kunming mice intraperitoneally with an equimolar dose of 0.02 mmol/kg bw of either of the two PAs, retrorsine produced higher levels of liver toxicity, pyrrole-GSH conjugates and pyrrole-protein binding. The study allows comparison of the relative potency differences between the two PAs for their in vitro bioactivation, and the relative differences in in vivo parameters for toxicity, measured at an equimolar dose level (0.2 mmol/kg bw dosed intraperitoneally) in the same mouse model. Compared to monocrotaline, in the in vivo mouse model retrorsine induced 10.3- fold higher serum alanine transaminase (ALT) levels, 3.6-fold higher serum aspartate transaminase (AST) levels, 2.9-fold higher levels of plasma pyrrole-GSH adducts, 2.7-fold higher levels of liver pyrrole-GSH adducts and 3.8-fold higher levels of liver pyrrole-protein adducts (Yang et al., 2017). In contrast, the AUC values for the two compounds were similar while the C_{max} for retrorsine was only 1.67-fold higher than that for monocrotaline. The authors argued that not the C_{max} as such but the formation of the dehydro-PAs from the PAs and their chemical reactivity (electrophilicity) may be the critical factors underlying the different toxic potential. The data reveal that the difference in catalytic efficiency for bioactivation in the in vitro microsomal incubation (5.5-fold) is in line with the fold difference in ALT (10.3-fold), AST (3.6-fold), plasma

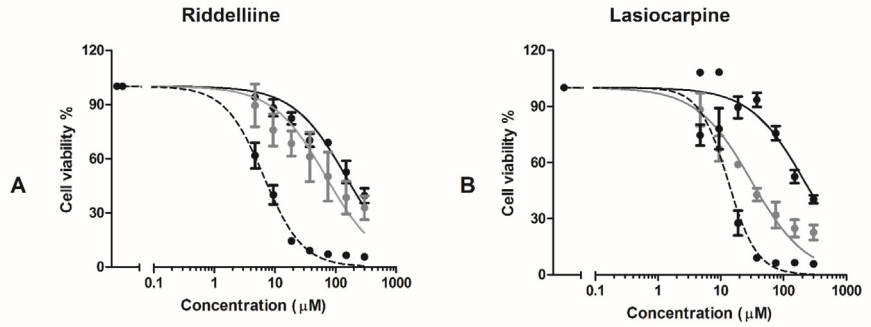

Fig. 7. Toxicity of A) riddelliine and B) lasiocarpine in HepaRG cells (black solid line) as compared to primary rat (black dash line) and human hepatocytes (grey solid line). The values are mean ± SEM of three independent experiments.

pyrrole-GSH adducts (2.9-fold), liver pyrrole-GSH adducts (2.7-fold) and liver pyrrole-protein adducts (3.8-fold). This indicates that catalytic efficiencies for formation of pyrrole-GSH adducts in liver microsomal incubations may provide a surrogate endpoint for defining relative potencies in liver toxicity between different PAs.

It remains to be established whether the in vitro surrogate endpoint of catalytic efficiency for pyrrole-GSH adduct formation may even provide a first tier estimate of relative potency differences in formation of DNA damage, genotoxicity and perhaps even carcinogenicity of the PAs.

For lasiocarpine and riddelliine the relative difference in catalytic efficiency for formation of the pyrrole-GSH adduct in human liver microsomal incubations was determined to be 7.2-fold with bioactivation of lasiocarpine being more efficient (Ning et al., 2019). When this value would be used for a read-across from riddelliine to convert the $BMDL_{10}$ for tumor formation of 237 μg/kg bw per day to a $BMDL_{10}$ for lasiocarpine this would result in a value of 237/7.2 = 33 μg/kg bw per day which is quite comparable to the value of 70 μg/kg bw per day derived from the data on induction of liver haemoangiosarcomas by lasiocarpine in male rats (EFSA, 2011; NTP, 1978). Given the higher accuracy in the $BMDL_{30}$ than in the $BMDL_{10}$ value derived from the tumor data for lasiocarpine and riddelliine one could also make this comparison based on the $BMDL_{30}$. The $BMDL_{30}$ for tumor formation of riddelliine amounts to 373 μg/kg bw per day (Knutsen et al., 2017). Using the factor 7.2 the predicted $BMDL_{30}$ for lasiocarpine would be 52 μg/kg bw per day, as compared to a value of 211 μg/kg bw per day derived from the actual tumor data (Knutsen et al., 2017). Given the fact that these predictions are not yet corrected for the higher level of clearance of lasiocarpine as compared to riddelliine, it can be expected that taking the kinetics for clearance into account the predicted BMDL values for lasiocarpine based on read across from riddelliine may further increase.

It would be of interest to extend this in vitro approach to other PAs but also to genotoxicity and/or formation of adducts with DNA, and compare the relative potencies obtained from for example in vitro microsomal incubations with DNA bases as the scavenger of the reactive dehydro-PA metabolites. Alternative endpoints may be found in relative potency differences in in vitro assays for genotoxicity such as the Ames test or the γH2AX assay, or data already available on in vivo DNA adduct formation in the liver of rats exposed to 24 μmol/kg bw/day of lasiocarpine and 4.5 μmol/kg bw/day of riddelliine for three days (Xia et al., 2013).

5. Discussion

PAs form a group of food-borne contaminants of increasing interest, especially given their occurrence in the modern food chain, their toxicity and the limited toxicological data base, hampering accurate risk and safety assessment (Knutsen et al., 2017). Clearly, developing insight into their mode of action for liver toxicity and liver carcinogenicity could facilitate the use of read-across from PAs for which toxicity data are available to PAs for which such data are lacking. In addition, defining ways to quantify the relative potencies of the various PAs would facilitate this read-across and also a combined risk assessment taking the relative potencies of the different PAs into account. Given the large number of PAs for which data are limited or even completely absent it is of importance to consider alternative testing strategies to obtain a first estimate of these relative differences in potency.

When considering the use of such alternative testing strategies, selection of an appropriate in vitro system is of importance. Ideally, a reliable in vitro hepatotoxicity model should resemble the phenotype observed in vivo and be able to conduct long-term studies and high-throughput applications (Lauschke et al., 2016). Use of in vitro assays such as primary hepatocytes are of value but they also come with limitations, such as their limited lifespan and loss of biotransformation capacity upon prolonged incubations, hampering repeated dose exposures. Given the fact that PA exposure may be chronic rather than acute, and that PA dose levels causing chronic liver toxicity tend to be lower than those causing acute toxicity (Ning et al., 2019), future efforts should be directed at taking repeated dose exposure into account. This requires the development of in vitro liver model systems that enable repeated long term studies, a strategy not possible when using primary hepatocytes. For repeated exposure the HepaRG hepatoma cell line could be considered, which represents a promising in vitro surrogate of primary human hepatocytes, especially because cells of this cell line display relatively high levels of metabolic enzymes and are able to retain metabolic activity for about several weeks (Klein et al., 2014). Another drawback of primary human hepatocytes is the donor-to-donor variability which may hamper the reproducibility, which may be substantial. Therefore at the current state-of-the art it is best to use pooled human hepatocytes. Alternatively human hepatocytes derived from iPSCs generated from multiple individuals with different polymorphic characteristics may provide a supply of hepatocytes for high-throughput screening with minor batch variability to improve reproducibility (Gomez-Lechon et al., 2014).

Whatever in vitro model applied, for reliable read-across of prediction of in vivo toxicity, differences in toxicokinetics should be taken into account. This can be achieved using PBK modelling. PBK models, defined using parameters that can be established without a need for animal experimentation, provide a way to include differences in kinetics into a combined testing strategy. First proofs of principle are available showing that PBK model facilitated reverse dosimetry can be used to translate in vitro concentration-response curves for toxicity of PAs towards primary hepatocytes from both rat and human into in vivo dose-response curves for acute liver toxicity (Chen et al., 2018; Ning et al., 2019). In this way the QIVIVE strategy does not only provide insight in differences in relative potency, but even provides a way to define PoDs for risk assessment. Additional results obtained so far indicated that the dose-response curves for liver toxicity do match experimental data for acute liver toxicity, but the related $BMDL_{10}$ values are higher than the $BMDL_{10}$ values obtained in experimental animal studies with repeated dose regimens for chronic liver toxicity and carcinogenicity. It is of importance to note that development of the PBK models and also the subsequent prediction of in vivo toxicity using PBK model facilitated reverse dosimetry to define PoDs for human risk assessment for PAs, requires evaluation of the models and predictions made. This turned out possible for the PBK models and the prediction of acute liver toxicity of riddelliine and lasiocarpine, for which in vivo data were available. However, the lack of in vivo data for the other PAs may turn out a serious bottle neck for further development of this alternative testing strategy for prediction of PA toxicity by such an alternative testing strategy. This means that extending the approach presented for lasiocarpine and riddelliine to other PAs, as well as to repeated dose exposure and/or to other endpoints relevant for PA risk assessment including genotoxicity and carcinogenicity remains a topic for future work.

At the current state-of-the-art estimates for the differences in relative potency between the carcinogenicity of different PAs have to be based on read-across from data for riddelliine and/or lasiocarpine for which $BMDL_{10}$ values for tumor formation upon oral exposure have been defined (Chen et al., 2017; Knutsen et al., 2017).

Such a read-across from the tumor data for riddelliine and lasiocarpine requires definition of the relative difference in potency, taking also differences in ADME characteristics into account. In a first approximation this read-across could be based on the catalytic efficiency for bioactivation of the PAs in incubations with liver microsomes, in which the reactive dehydro-PA metabolites were scavenged by GSH. This provided a way to quantify the relative bioactivation based on quantification of the relative differences in catalytic efficiency for formation of the respective pyrrole-GSH adducts. Application of this approach to define a $BMDL_{10}$ value for tumor formation by lasiocarpine based on read-across from the $BMDL_{10}$ value for riddelliine, provided a value that was only 2-fold lower than the $BMDL_{10}$ value derived from

the data on induction of liver haemoangiosarcomas by lasiocarpine in male rats (EFSA, 2011). Extending this approach taking kinetic clearance of the parent PA into account, may further improve the prediction. It is important to realise that for PBK model facilitated reverse dosimetry based prediction of acute liver toxicity, C_{max} may be the relevant parameter, while for tumor formation the AUC might be the parameter of choice. The choice of the dose metric used for the reverse dosimetry depends on the mode of action of the compound and the toxic endpoint of interest. When predicting toxicity for which a threshold exists and thus a concentration below which there will be no effect, C_{max} may be the selected dose metric. The AUC could be used when testing and endpoint representing cumulative damage, such as genotoxicity or carcinogenicity (Groothuis et al., 2015). Other issues to consider are whether to use total or unbound concentrations and/or whether to use intracellular or extracellular concentrations (Rietjens et al., 2019). The unbound extracellular concentration was the metric of choice for the reverse dosimetry performed for lasiocarpine and riddelliine because it was considered that this free concentration represents best what the cells in the monolayer in the in vitro cell model and also in the tissue experience as the fraction available for cellular interaction. The metric to be selected may vary on a case by case basis, depending on the mode of action and the level of protein binding. Whatever the choices and assumptions made they need mechanistic considerations and also ultimate validation of the predictions made.

With respect to predicting the genotoxicity of PAs, it would be of interest to investigate whether catalytic efficiencies for formation of DNA adducts would result in similar relative potencies as predictions for acute liver toxicity. Given that the bioactive metabolite formed from the different PAs is similar, i.e. the dehydroretronecine-type/dehydroheliotridine-type/dehydropyrrolizidine (DHP) and its secondary metabolites (Xia et al., 2018), it can be expected that relative potencies for formation of adducts with DNA bases measured in vitro will reflect the relative potencies for formation of the respective DNA adducts in vivo. It would also be of interest to investigate whether the PBK model facilitated reverse dosimetry of in vitro data for genotoxicity of PAs would adequately predict their in vivo genotoxicity. This would provide a novel approach to defining REP values for the different PAs. Finally, given the fact that development of PBK models for all relevant PAs may be time consuming, it is of interest to work towards generic models that require a limited number of experimental parameters. The PBK models defined for lasiocarpine and riddelliine that require experimental data on kinetics for clearance while other compound specific model parameters may be obtained by quantitative structure activity approaches, may provide a basis to work towards this set of PBK models for at least the 17 PAs considered by EFSA to relevant in food and feed (Knutsen et al., 2017).

Taking all together it is concluded that use of alternative testing strategies are important to fill the data gaps that currently exist in the data base on the different PAs relevant in our food and feed. Whatever the alternative strategy applied, being read-across and/or QIVIVE predicting PoDs for human risk assessment, it is important to take into account that differences in toxicity between the different PAs may originate from differences in both toxicokinetics and toxicodynamics. And while in vitro toxicity test may reveal differences in relative potencies and thus in toxicodynamics, the role of toxicokinetics in pyrrolizidine alkaloid toxicity and risk assessment should not be ignored.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110572.

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