In vitro investigations on the hepatotoxicity and genotoxicity of food-relevant pyrrolizidine alkaloids

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vorgelegt von

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für Mama und Papa

für Laura und Leo

Der experimentelle Teil dieser Arbeit wurde in der Zeit von April 2017 bis Mai 2020 im Fachbereich Chemie, Fachrichtung Lebensmittelchemie und Toxikologie der Technischen Universität Kaiserslautern, in der Arbeitsgruppe von Prof. Dr. med. Dr. rer. nat. Dieter Schrenk durchgeführt. Die letzten praktischen Versuche durften in den Laboren von Prof. Dr. Elke Richling und Prof. Dr. Jörg Fahrer durchgeführt werden. Dafür möchte ich mich hiermit nochmals recht herzlich bedanken.

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The experimental methods used, as well as all generated scientific results of this work are included in the publications listed below. They represent the main part of this thesis.

Publication I:

Structure-dependent hepato-cytotoxic potencies of selected pyrrolizidine alkaloids in primary rat hepatocyte culture

Lan Gao, Lukas Rutz, Dieter Schrenk

This publication appeared in peer-reviewed journal *Food and Chemical Toxicology* (Volume **135**) in January 2020.

Doi: https://doi.org/10.1016/j.fct.2019.110923

Publication 2:

Structure-dependent genotoxic potencies of selected pyrrolizidine alkaloids in metabolically competent HepG2 cells

Lukas Rutz, Lan Gao, Jan-Heiner Küpper, Dieter Schrenk

This publication appeared in peer-reviewed journal *Archives of Toxicology*, (Volume **94**) in September 2020.

Doi: https://doi.org/10.1007/s00204-020-02895-z

Publication 3:

Metabolic pattern of hepatotoxic pyrrolizidine alkaloids in liver cells

Ina Geburek*, Lukas Rutz*, Lan Gao; Jan-Heiner Küpper, Anja These, Dieter Schrenk

This publication appeared in peer-reviewed journal *Chemical Research in Toxicology* (Volume **34**) in March 2021.

*contributed equally to this work

Doi: https://dx.doi.org/10.1021/acs.chemrestox.0c00507

In this short preface, I would like to explain that these three publications are part of a joint research project between the University of Kaiserslautern and the Federal Institute for Risk Assessment Berlin. The experiments were conducted by Dr. Ina Geburek, Dr. Lan Gao and myself. In order to avoid ambiguities, I have listed below which data were generated by me.

In publication I, I conducted the experiments for the following substances: echimidine, europine, heliotrine, monocrotaline and seneciphylline. This includes the cytotoxicity studies in primary rat hepatocytes with 3 h as well as 24 h pre-incubation time and subsequent 24 or 48 h post-incubation time, as well as the cytotoxicity studies with ketoconazole as CYP inhibitor. Of Course, this also includes the corresponding data analysis. All other data were generated by Dr. Lan Gao and are therefore attributed to her thesis.

In publication II, I conducted the experiments for the following substances: echimidine, europine, heliotrine, monocrotaline, riddelliine and seneciphylline. This includes the cytotoxicity studies in naïve HepG2 cells and HepG2-C9 (CYP3A4) cells, as well as the micronucleus assay in HepG2-C9 (CYP3A4) cells. Furthermore, the substances listed above were also tested by me in the Ames fluctuation test in the two *Salmonella* strains TA 98 and TA 100 with and without metabolic activation with S9-mix. Of course, this also includes again the corresponding data analysis. All other data were generated by Dr. Lan Gao and are therefore attributed to her thesis.

In publication III, I conducted the cell culture part of the experiments for all six tested substances: echimidine, europine, lasiocarpine, lycopsamine, retrorsine and senecionine. This includes the incubations and cell harvesting for the primary rat hepatocytes as well as for the HepG2-C9 (CYP3A4) cells. The following analytic and data evaluation was performed by Dr. Ina Geburek. The generated data therefore belong equally to the work of Dr. Ina Geburek as well as to mine.

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ABBREVIATIONS

ABC	ATP binding cassette
ABCB1	ATP binding cassette subfamiliy B number 1
ADME	Absorption, Distribution, Metabolism, Excretion
ALARA	As low as reasonably achievable
ATP	Adenosine triphosphate
BCRP	Breast cancer resistance protein
BfArM	The Federal Institute for Drugs and Medical Devises
BfR	German Federal Institute for Risk Assessment
BMC	Benchmark concentration
BMCL	Benchmark concentration lower bound
BMCU	Benchmark concentration upper bound
BMD	Benchmark dose
$BMDL_{10}$	Benchmark dose for a 10% response
BROD	7-Benzoxyresorufin-*O*-Dealkylase
BSO	Buthionine sulfoximine
bw	Body weight
CBMN	Cytokinesis-block micronucleus assay
CED	Critical effect dose
CEDL	Critical effect dose lower confidence limit
CEDU	Critical effect dose upper confidence limit
COT	Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment
CONTAM	EFSA Panel on Contaminants in the Food Chain
CYP	Cytochrome P450
DHP	6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
GSH	Glutathione
GST	Glutathione-*S*-transferase
HMPC	Committee for Herbal Medicinal Products
HSC	Hepatic stellate cell
IARC	International Agency for Research on Cancer
iREP	Interim relative potency factors

JECFA	FAO/WHO Joint Expert Committee on Food Additives
LB	Lower Bound
LC-MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
LOD	Limit of detection
LSEC	Liver sinusoidal endothelial cell
MDR 1	Multi-drug resistance protein 1
MoE	Margin of Exposure
Mono-GSH-DHP	Mono-glutathione-dehydropyrrolizidine
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAT	*N*-acetyltransferase
NOAEL	No Observed Adverse Effect Level
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
OCT1	Organic cation transporter 1
PA	Pyrrolizidine alkaloids
PAPS	3′-Phosphoadenosin-5′-phosphosulfat
PBPK	Physiologically based pharmacokinetic
RPF	Relative potency factor
S9	9000 supernatant fraction
SAM	*S*-adenosylmethionine
SCE	Sister chromatid exchange
SULT	Sulfotransferase
UB	Upper bound
UGT	Uridine 5'-diphospho-glucuronosyltransferase
VOD	Veno-occlusive disease

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ABSTRACT

Pyrrolizidine alkaloids (PA) are secondary plant metabolites occurring in a great many plant species worldwide, known to exhibit hepatotoxic, genotoxic and carcinogenic properties after metabolic activation. In recent years, contamination of food, feed and herbal medicines with PA has become an increasing problem. The concept of interim relative potency factors (iREP) proposed by Merz and Schrenk in 2016 was a new approach for risk assessment of PA. While existing approaches of risk assessment assumed equivalent toxic potency for all PA congeners, the approach of Merz and Schrenk considered the structural features of individual PA congeners based on existing data from the literature. In order to generate further data on the structure-specific toxicity of PA, congeners of different structural classes were investigated in different *in vitro* test systems.

In vitro cytotoxicity was investigated in primary rat hepatocytes, HepG2 C9 cells (overespressing human CYP3A4) and naïve HepG2 cells. Overall, it could be observed that lasiocarpine and the cyclic di-esters (except monocrotaline) showed much stronger cytotoxic effects in comparison to the tested mono-esters in both, primary rat hepatocytes and in HepG2 C9 cells. Primary hepatocytes were the most sensitive cells investigating cytotoxicity of different PA congeners, followed by the HepG2 C9 cells. This is confirmed by markedly higher metabolism rates for all investigated PA in primary rat hepatocytes determined in the metabolism experiments. In naïve HepG2 cells no cytotoxic effects could be observed. The influence of cytochrome P450 (CYP) on the formation of toxic metabolites seem to play a crucial role. This assumption could be beared using ketoconazole as CYP inhibitor and testing various pre-incubation times in primary rat hepatocytes. CYP activity was measured using 7-Benzoxyresorufin-*O*-Dealkylase (BROD) assay in primary rat hepatocytes and in HepG2 C9 cells. Glutahione (GSH) depletion using buthionine sulfoximine (BSO) showed slight stronger cytotoxic effects for several PA, but not for all tested.

In contrast to the negative results of mutagenicity in ames fluctuation assay using *Salmonella* strains TA98 and TA100 with and without metabolic activation by S9 mix, all tested PA congeners showed micronuclei induction in the HepG2 C9 cell line. Again, laisocarpine and the cyclic di-esters (except monocrotaline) were the most potent ones. In conclusion, the data from cytotoxicity and genototoxicity experiments from the tested PA congeners confirm published iREP factors with a few exceptions, in particular for monocrotaline or echimidine.

Additionally, metabolism of six selected PA was studied in primary rat hepatocytes and HepG2 C9 cells. Genrally, it was found that almost all tested cyclic and open-chained di-esters (except retrorsine) showed much higher metabolism rates in both cell types, in comparison to the mono-esters, for which only low metabolism rates could be measured. The same was observed for the quantified amounts of reactive metabolites in the supernatants of both cell types. In

general, also these data bear the results from cytotoxicity and genotoxicity experiments and help to better understand the complex metabolism and the structure-specific toxicity of different PA congeners.

KURZZUSAMMENFASSUNG

Pyrrolizidinalkaloide (PA) sind sekundäre Pflanzeninhaltsstoffe, die weltweit in sehr vielen Pflanzenarten vorkommen und dafür bekannt sind, dass sie nach metabolischer Aktivierung hepatotoxische, genotoxische und karzinogene Eigenschaften aufweisen. In den letzten Jahren ist die Kontamination von Lebensmitteln, Futtermitteln und pflanzlichen Arzneimitteln mit PA zu einem zunehmenden Problem geworden. Das von Merz und Schrenk 2016 vorgeschlagene Konzept der vorläufigen relativen Wirksamkeitsfaktoren (iREP) war ein neuer Ansatz für die Risikobewertung von PA. Während bisherige Ansätze der Risikobewertung von einer gleichwertigen toxischen Potenz aller PA-Kongenere ausgingen, berücksichtigte der Ansatz von Merz und Schrenk die strukturellen Merkmale einzelner PA-Kongenere auf der Grundlage vorhandener Daten aus der Literatur. Um weitere Daten zur strukturabhängigen Toxizität von PA zu generieren, wurden Kongenere verschiedener Strukturklassen in verschiedenen *in vitro* Testsystemen untersucht.

In vitro wurde die Zytotoxizität in primären Rattenhepatozyten, HepG2-C9-Zellen (mit Überexpression von humanem CYP3A4) und naiven HepG2-Zellen untersucht. Insgesamt wurde festgestellt, dass Lasiocarpin und die zyklischen Diester (mit Ausnahme von Monocrotalin) im Vergleich zu den getesteten Monoestern sowohl in primären Rattenhepatozyten als auch in HepG2-C9-Zellen wesentlich stärkere zytotoxische Wirkungen zeigten. Die primären Hepatozyten waren die sensitivsten Zellen bei der Untersuchung der Zytotoxizität verschiedener PA-Kongenere, gefolgt von den HepG2-C9-Zellen. Dies steht im Einklang mit den deutlich höheren Metabolismusrate für alle untersuchten PA in primären Rattenhepatozyten, die in den Metabolismusexperimenten ermittelt wurden. In naiven HepG2-Zellen konnten keine zytotoxischen Wirkungen beobachtet werden. Der Einfluss von Cytochrom P450 (CYP) auf die Bildung von toxischen Metaboliten scheint eine entscheidende Rolle zu spielen. Diese Vermutung konnte durch die Verwendung von Ketoconazol als CYP-Inhibitor und die Testung verschiedener Vorinkubationszeiten in primären Rattenhepatozyten bestätigt werden. Die CYP-Aktivität wurde mit dem 7-Benzoxyresorufin-*O*-Dealkylase (BROD) Assay in primären Rattenhepatozyten und in HepG2 C9-Zellen gemessen. Die Depletion von Glutahion (GSH) mit Buthioninsulfoximin (BSO) zeigte bei mehreren PA leicht stärkere zytotoxische Wirkungen, jedoch nicht bei allen getesteten.

Im Gegensatz zu den negativen Ergebnissen hinsichtlich Mutagenität im Ames-Fluktuationstest in den *Salmonella*-Stämmen TA98 und TA100 mit und ohne metabolischer Aktivierung durch S9-Mix zeigten alle getesteten PA-Kongenere eine Induktion von Mikrokernen in der HepG2-C9-Zelllinie. Auch hier waren Laisocarpin und die zyklischen Diester die potentesten Substanzen (außer Monocrotalin). Zusammenfassend lässt sich sagen, dass die Daten aus Zytotoxizitäts- und Genotoxizitätsexperimenten mit den getesteten

PA-Kongeneren die veröffentlichten iREP-Faktoren mit einigen Ausnahmen, insbesondere für Monocrotalin und Echimidin, bestätigen.

Des Weiteren, wurde der Metabolismus von sechs ausgewählten PA in primären Rattenhepatozyten und HepG2-C9-Zellen untersucht. Im Allgemeinen wurde festgestellt, dass fast alle getesteten zyklischen und offenkettigen Diester (außer Retrorsin) in beiden Zelltypen wesentlich höhere Metabolismusraten aufwiesen als die Monoester, für die nur geringe Metabolismusraten gemessen werden konnten. Dasselbe gilt für die quantifizierten Mengen reaktiver Metaboliten in den Überständen beider Zelltypen. Im Allgemeinen stimmen auch diese Daten mit den Ergebnissen aus Zytotoxizitäts- und Genotoxizitätsexperimenten überein und tragen zu einem besseren Verständnis des komplexen Metabolismus und der strukturabhängigen Toxizität verschiedener PA-Kongenere bei.

1. INTRODUCTION

The occurrence of genotoxic carcinogens as contaminants in food has been part of scientific research for many years. A particularly large group is represented by PA, which occur as secondary plant metabolites in more than 6000 plant species worldwide (Smith and Culvenor, 1981). They are known to exhibit hepatotoxic, genotoxic and carcinogenic properties (Fu et al., 2004). Further, the so called veno-occlusive disease (VOD) is a characteristic disease of PA-intoxication and could be observed in humans and ruminants (Wiedenfeld and Edgar, 2011). Humans can be exposed to them in daily life through the consumption of contaminated foods. Products from crop-plants, i.e., tea, salad or spices contain PA because of cross-contamination with PA-containing plants during harvest. Further main sources of exposure are honey that originated from PA-producing plants or herbal food supplements (Bodi et al., 2014; Schulz et al., 2015; Mulder et al., 2018; Kaltner et al., 2020).

In the past, several cases of PA intoxication in humans due to the consumption of highly contaminated cereals have been reported from Afghanistan and India. (Kakar et al., 1976; Tandon et al., 1976; Mohabbat et al., 2010). In Jamaica and South Africa, the consumption of traditional tea infusions, known as "bush-teas" also led to severe poisonings (Bras et al., 1961; Freiman et al., 1968; Brooks et al., 1970).

A few years ago, unexpected findings of high levels of PA in tea samples (Bodi et al., 2014) and medicinal tea samples (Schulz et al., 2015) have caused a renewed focus on research on the toxic potential of PA. As early as 2013, German Federal Institute for Risk Assessment (BfR) concluded, based on available data, that there may be an increased risk of adverse health effects from cancer for adults and children who often consume certain herbal teas that are highly contaminated with PA over a pro-longed period of time (BfR, 2013). Based on new data BfR published a further opinion in 2016, which still indicated a possible risk regarding carcinogenic effects from high consumption of foods with high PA levels (BfR, 2016).

Chemically, PA can be distinguished in accordance with the type of the necine base and their esterification, which leads to the formation of mono-esters, open-chained di-esters and cyclic di-esters (Merz and Schrenk, 2016). At the moment, usually the sum of individual PA, which are assigned as equally potent, are considered for risk assessment. This is due to the fact that only a few data on the relative toxicity of individual PA are available. Therefore, it is of great scientific interest to generate more data, which will allow to compare the structure-dependent toxic effects of individual PA in order to provide a better scientific basis for risk assessment.

2. THEORETICAL BACKGROUND

In the next chapters, the theoretical background for the chemical group of PA is presented. This is followed by a chapter on the liver, the main target organ of PA toxicity.

2.1 Pyrrolizidine alkaloids

2.1.1 Occurrence

PA form a large group of secondary plant metabolites produced by more than 6000 plant species (Smith and Culvenor, 1981). PA-containing plants are probably the most common poisonous plants with effects on livestock and humans (Prakash et al., 1999; Stegelmeier et al., 1999; Wiedenfeld and Edgar, 2011) and belong mainly to the plant families *Asteraceae*, *Boraginaceae* and *Leguminosae*. (Smith and Culvenor, 1981). They are synthesized as a defense mechanism against herbivores (Liu et al., 2017). Today, more than 660 structurally different PA have already been identified either as ternary PA or as their *N*-oxide derivatives (Stegelmeier et al., 1999; Roeder, 2000).

2.1.2 Human exposure

Contaminated food is the main source from which humans are exposed to PA: Food products from crop plants, i.e., tea, salad or spices contain PA because of cross-contamination with very minor amounts of PA-containing weeds during harvest. Honey products are also contaminated because bees collect pollen and nectar from PA-containing plants. Furthermore, a carryover from feed into animal products was observed. Further sources of exposure are contaminated (herbal) food supplements or herbal medicines from non-PA-plants (Bodi et al., 2014; Schulz et al., 2015, Mulder et al., 2018; BfR, 2020; Kaltner et al., 2020).
In Germany and Europe, tea and honey are the main sources of exposure. In a german study from Bodi et al. in 2014 they analyzed 87 honey samples as well as 274 tea samples. They quantified total PA content in tea samples ranged from below the limit of detection (LOD) to 5647 µg/kg. In only a few fruit tea samples trace amounts of PA could be quantified. In honey samples a mean value of about 10 µg/kg was found (Bodi et al., 2014). In a further study from Schulz et al. in 2015 they analyzed 169 different commercially available medicinal teas, i.e., from camomile, fennel, peppermint as well as tea mixtures. Total PA concentrations ranged from below LOD to 5668 µg/kg. In eleven samples more than 300 µg/kg total PA were determined. Three tea mixtures revealed extremely high contents (Schulz et al., 2015). In a

further study from Mulder et al. in 2018 a total of 1105 samples of animal- and plant-derived products, including milk and milk products, eggs, meat and meat products, (herbal) teas and (herbal) food supplements were investigated. They were analyzed for the presence of 28 or 35 PA. They collected samples in six different european countries. Trace amounts of PA were found in milk or eggs. In meat samples no measurable amounts of PA could be determined (Mulder et al., 2018). Kaltner et al. determined the occurrence of PA in spices and culinary herbs. They investigated 305 samples from 36 countries for the presence of 44 PA, while the highest concentration of 24.6 mg/kg was determined in an oregano sample (Kaltner et al., 2020). In a statement of the BfR from 2020 it could be stated that in some food groups in Germany a reduction of the PA-content has occurred. Especially remarkable is the decrease in mean content by a factor of more than ten for green tea and peppermint tea. The food groups rocket salad, herbs/spices and herbal teas had the highest average contents (BfR, 2020).
Table 1 summarizes PA-content of selected food products from different European studies (Bodi et al., 2014; Schulz et al., 2015; Mulder et al., 2018; Kaltner et al., 2020; BfR, 2020).

Table 1: PA-content data from relevant food groups in Europe. The mean and maximum amount of the investigated product are given in each case. The number of PA examined, and the number of samples measured are also given.

product	mean [µg/kg]	maximum [µg/kg]	number of PA	number of samples	reference
peppermint tea	496	4401	28	30	Mulder et al., 2018
camomile tea	274	1394	28	35	Mulder et al., 2018
tea mixtures	253	5668	23	109	Schulz et al., 2015
green tea	109	698	17	23	Bodi et al., 2014
fennel tea	6	27	23	12	Schulz et al., 2015
honey (brand products)	15	235	17	35	Bodi et al., 2014
rocket salad	9791	166384	21	17	BfR, 2020
oregano	3199	24621	44	24	Kaltner et al., 2020
basil	12	142	44	25	Kaltner et al, 2020
milk (cow)	-	0,17	28	169	Mulder et al., 2018
eggs	-	0,12	28	205	Mulder et al., 2018

2.1.3 Structural chemical characteristics

PA are structurally ester alkaloids. They comprise of a necine base, which is esterified with one or two necine acids (**Figure 1**) (Roeder et al., 1995; Wiedenfeld et al., 2008).

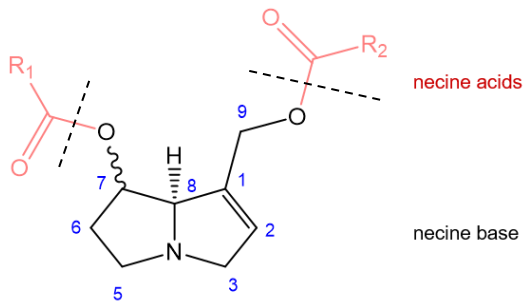

Figure 1: Chemical structure of PA. A necine base which is esterified with one or two necine acids (modifiziert nach Roeder et al., 1995).

The necine base is a bicylic ring system with a bridgehead nitrogen atom. It possesses a hydroxymethyl group at position C1 and at position C7 a second hydroxyl group commonly occurs (Wiedenfeld et al., 2008). The most abundant necine base is retronecine; other possible bases of PA are heliotridine, otonecine and platynecine (**Figure 2**). Heliotridine is *S*-configured at C7, while retronecine is *R*-configured at C7 (Fu et al., 2004). Otonecine is not a bicyclic ring system but acts as a pyrrolizidine ring because of transannular interactions between the nitrogen and the carbonyl group (Roeder et al., 1995; Wiedenfeld et al., 2008). Compared to retronecine, heliotridine, and otonecine, platynecine has no double bond in the 1,2-position. These PA are not expected to be toxic, because the 1,2-double bond is required for toxicity (Fu et al., 2004; Mattocks, 1986). The necine bases can occur as tertiary amines or as *N*-oxides. In plants PA occur mostly as water-soluble *N*-oxides. (Schramm et al., 2019).

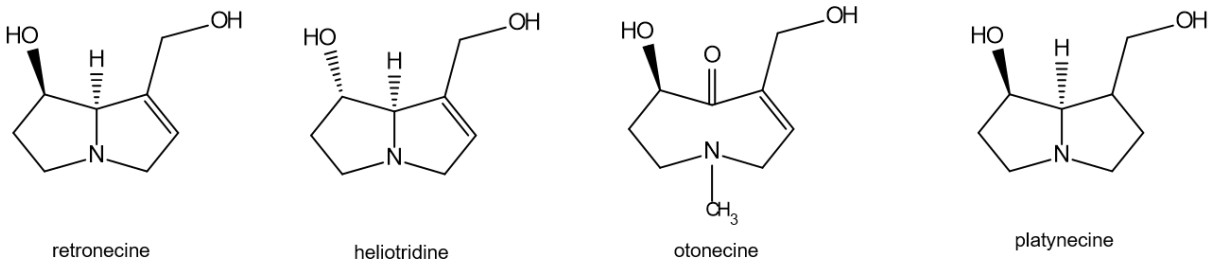

Figure 2: Different types of necine base. The most abundant is retronecine, other possible bases of PA are heliotridine, otonecine and platynecine (modifiziert nach Fu et al., 2004).

The chemical structures of PA can be divided into three groups according to the esterification mode: mono-esters, open-chained di-esters and cylic di-esters. In mono-esters, a necine base and one necine acid are linked to each other via an ester bond, usually at position C9. In the case of open-chained di esters, both hydroxyl groups of the necine base are esterified with one necine acid each. In cyclic di-esters, both hydroxyl groups are esterified with one necine acid (**Figure 3**) (Merz and Schrenk, 2016).

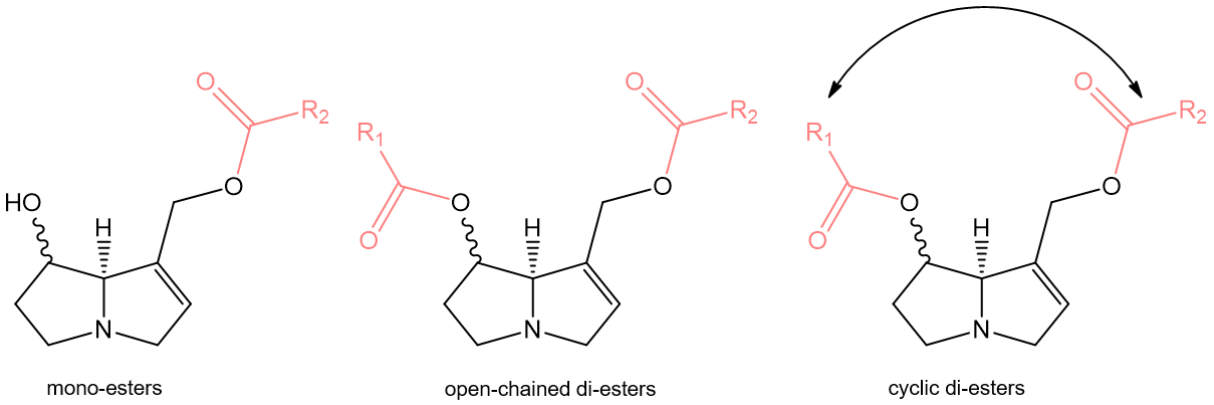

Figure 3: Chemical structure classes of PA. There are three groups according to the esterification mode: mono-esters, open-chained di-esters and cyclic di-esters (modifiziert nach Merz and Schrenk, 2016).

The necine acids are saturated or unsaturated carboxylic acids with mostly five to ten carbon atoms. They can be branched with carbon chains containing hydroxy-, alkoxy- (i.e., methoxy), epoxy- and carboxyester- groups. Branching of the necine acid side chains explains the structural diversity of PA and is also thought to influence their toxicity (Wiedenfeld et al., 2008). Furthermore, the configuration at position C7 seems to influence their toxic potency (Mattocks, 1986; Merz and Schrenk, 2016).

2.1.4 Toxicokinetics

Differences in toxicokinetics (ADME: absorption, distribution, metabolism and excretion) of different PA congeners seem to be crucial for their toxicity. Thus, potential differences in kinetics must be considered when investigating PA toxicity using *in vitro* assay systems.

2.1.4.1 Absorption

Williams et al. investigated the toxicokinetics of the PA riddelliine and two of the corresponding metabolites, riddelliine *N*-oxide and retronecine. They determined their concentrations in serum after an oral gavage dose of 10 mg/kg riddelliine in male and female rats and mice. As a result, the data showed that in all rats and mice riddelliine was fully absorbed within 30 min (Williams et al., 2002). Long et al. examined the toxicokinetics of seneciphylline and seneciphylline *N*-oxide in rat plasma after oral and intravenous administration. As a result, it was determined that seneciphylline was rapidly absorbed within 20 min into blood plasma. Further it was observed that the highest concentration was measured after oral administration. The low determined percentage of bioavailability possibly regards to the extensive metabolism in the liver (Long et al., 2021). Wang et al. compared the toxikokinetics of the PA senecionine and adonifoline and their corresponding *N*-oxides in rats after intravenous and oral

administration. Plasma concentration peaks were reached for senecionine more than twice as fast than for adonifoline. Furthermore, they determined for both, intravenous and oral administration, that the plasma concentration ratio of senecionine *N*-oxide to senecionine was significantly higher than that for adonifoline *N*-oxide and adonifoline. It was discussed that possibly the higher metabolic rate of senecionine is related to its higher toxicity (Wang et al., 2011). After oral administration, Chen et al. determined a bioavailability of monocrotaline in mice from 88.3%. The results also showed that monocrotaline is rapidly absorbed into the blood plasma (Chen et al., 2018).

Further *in vitro* studies investigated the uptake of different PA across the intestinal barrier. Yang et al. investigated the oral absorption of PA and their *N*-oxides. Differences in toxikokinetics could possibly explain the differences in their toxic potency. They found that the absorption of PA *N*-oxides was less than that of PA. Furthermore, they demonstrated in a Caco-2 cell *in vitro* model that except for senecionine *N*-oxide, retrorsine *N*-oxide and lycopsamine *N*-oxide all PA and PA *N*-Oxides examined were absorbed via passive diffusion. For this three PA *N*-Oxides an efflux transporter-mediated active transportation was found (Yang et al., 2020). Experiments from Hessel et al. in Caco-2 cells showed the highest passage rate from the apical into the basolateral compartent for senecionine (cyclic di-ester, 47%), followed by senkirkine (cyclic di-ester, 40%), heliotrine (mono-ester, 32%) and echimidine (open-chained di-ester, 13%), showing significant structure-dependent differences. This observed reduced passage rate for heliotrine and echimidine suggested an active efflux from the basolateral side to the apical side. Experiments with cells, overexpressing the transporter adenosine triphosphate (ATP) binding cassette subfamiliy B number 1 (ABCB1), confirmed an active excretion mediated by the transporter for both PA (Hessel et al., 2014). Further studies from Tu et al. showed that monocrotaline and retrorsine were substrates of the organic cation transporter 1 (OCT1). This OCT1-transporter mediates the hepatic uptake of monocrotaline and retrorsine and may play an important role in their hepatotoxicity (Tu et al., 2013; Tu et al., 2014).

2.1.4.2 Distribution and excretion

There are also some studies in the literature investigating distribution and excretion of PA. Bull et al. investigated the distribution and excretion of heliotrine in rats. They measured 6.3% of the total dose after 5 min in the liver (Bull et al., 1968). Eastman et al. investigated the distribution and excretion of senecionine and seneciphylline in mice. They showed that both PA were excreted within 16 h in a percentage value of 84% or greater via urine and feces. In the liver they found 1.5% of the PA after 16 h. A further very small amount (0.04%) of the PA was found in the milk (Eastman et al., 1982). Estep et al. conducted tissue distribution and

covalent binding studies at 4 h and 24 h after administration of ^{14}C-labeled monocrotaline. They determined distribution of monocrotaline into red blood cells, liver, kidney, lung and plasma. Furthermore, they found rapid elimination of monocrotaline (approximately 90%) in the urine and bile after 7 h (Estep et al., 1991).

2.1.4.3 Metabolism

PA metabolism mainly takes place in the liver, while it was also observed that the produced metabolites can damage other tissues (Lafranconi and Huxtable, 1984). Like many other xenobiotics, PA require metabolic activation to exert their toxic effects (Fu et al., 2004). After oral ingestion, the lipophilic PA are readily absorbed from the gut. The more hydrophilic PA *N*-oxides are absorbed into the cells in the intestine as tertiary bases only after reduction by the gut microflora (Wiedenfeld et al., 2008). In the next step then they reach the liver via the portal vein. Three major metabolic routes of the PA of the heliotridine- and retronecine-type are described (**Figure 4**): (i) hydrolysis, (ii) *N*-oxidation and (iii) oxidation (dehydrogenation). Hydrolysis and *N*-oxidation are believed to be detoxification pathways, while dehydrogenation is the major activation mechanism leading to the electrophilic dehydro-PA, which seems to be to most potent toxic metabolite (IPCS, 1988; Wiedenfeld et al., 2008).

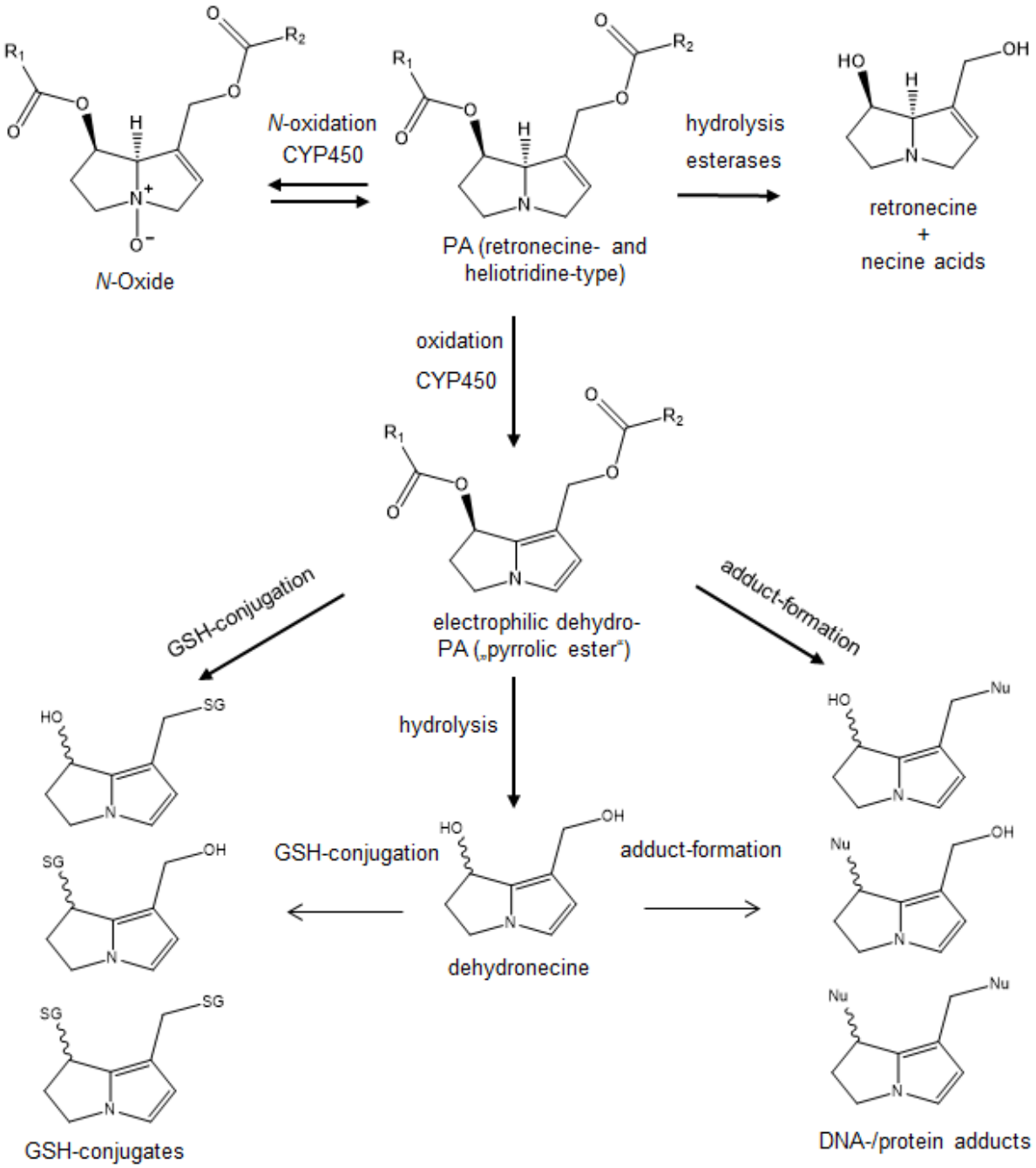

Figure 4: Major metabolic routes of PA of the retronecine- and heliotridine-type: (i) Hydrolysis of the ester groups leading to retronecine and the necine acid(s), (ii) *N*-Oxidation leading to the respective PA *N*-oxide, (iii) Oxidation resulting in the reactive electrophilic dehydro-PA, which possibly could react with DNA, proteins or GSH. Further hydrolysis of the electrophilic dehydro-PA leads to dehydronecine, which seems to be less potent (modified after IPCS, 1988; Fu et al., 2004; Ruan et al., 2014; Fu et al., 2017).

The first pathway from the retronecine- and heliotridine-type is hydrolysis of the ester linkages at positions C7 and C9 by esterases in the intestinal tract resulting in the formation of the corresponding necine base and necine acids (Fu et al., 2004). Neither the necine base nor the necin acid(s) are hepatotoxic. Necine bases are highly water-soluble and readily excreted. It is also observed that hydrolysis is reduced when PA contain highly branched necine acids

which leads to the esterases not being able to cleave the ester bond due to steric hindrance (IPCS, 1988; Culvenor et al., 1976; Mattocks, 1986).

In the second pathway the nitrogen atom of the pyrrolizidine ring can be oxidized to the corresponding PA *N*-oxide by microsomal oxidases in the liver (IPCS, 1988). The *N*-Oxides are chemically stable, water soluble and eliminated into urine without exerting a toxic effect (Mattocks, 1968; Wiedenfeld, 2011).

The third metabolic pathway is hydroxylation of the necine base at the C3 or C8 position to form the corresponding 3- or 8-hydroxynecine derivatives (Chen et al., 2010). These derivatives are very unstable and dehydrate spontaneously resulting in the corresponding dehydropyrrolizidine derivatives (electrophilic dehydro-PA) (Fu et al., 2004). In comparison to the retronecine- and heliotridine type the otonecine type contains a methylgroup at the nitrogen and a keto function at positon eight (metabolism of this type is not shown in the figure). The *N*-methyl group is hydroxylated and via a spontaneous loss of formaldehyde an unstable derivative is formed which spontaneously dehydrate to the electrophilic dehydro-PA (Culvenor et al., 1971; Wiedenfeld and Edgar, 2011). The resulting electrophilic dehydro-PA has chemically reactive centers at position C7 and C9. Since the necine acids provide a good leaving group, when esterified with the hydroxyl groups at C7 and C9, the formation of a stabilized carbonium ion is greatly facilitated. If there is no esterification at C7 and C9 the formation of the stabilized carbonium ion is not spontaneous but can be eased by protonation of the hydroxyl groups and dehydration afterwards (Culvenor et al., 1970; Wiedenfeld et al., 2008). The formed electrophilic dehydro-PA can connect with nucleophiles, i.e., proteins and nucleosides. Mercapto-, hydroxyl- and amino- groups are engaged if there was a reaction with proteins, while in DNA or RNA, the corresponding bases are alkylated. As a result, the formed adducts cannot perform their “normal” functions. DNA adduct formation possibly can result in mutations (Wiedenfeld et al., 2008). The electrophilic dehydro-PA can also react with the SH group of GSH, which possibly reduces damage to proteins and DNA or RNA (Nigra and Huxtable, 1992; Reed et al., 1992).

Electrophilic dehydro-PA also react with water *in vivo* to form dehydronecines, which are less reactive and more water soluble (Peterson and Jago, 1980; Robertson, 1982; Wiedenfeld et al., 2008). Their reduced chemical reactivity and increased hydrophilicity allows them to leave the liver cells and exert their toxicity in other organs (Peterson et al., 1972; IPCS, 1988; Prakash et al., 1999).

The sensitivity of different species and individuals to intoxication by PA regards to the relative activity of the three major metabolic routes described. Furthermore, age and gender may play a role. Males seem to be more sensitive for PA toxicity than female, while children seem to be the most sensitive (Wiedenfeld et al., 2008).

2.1.5 Toxicity

In the following section, the most important studies on the toxicity of PA will be presented. Human intoxications from the past and the symptoms of acute, sub-acute and chronic toxicity in humans are described. Furthermore, studies on cytotoxicity, genotoxicity *in vitro* as *in vivo*, carcinogenicity and teratogenicity are presented.

2.1.5.1 Human intoxications

Cases of poisoning in connection with PA-containing plants have been reported several times in the past. Toxic effects of PA were observed in humans and livestock (Stegelmeier et al., 1999; Rubbia-Brandt, 2010; Wiedenfeld and Edgar, 2011). Intoxications of humans are seen when certain plants or contaminated food are consumed in high amounts. Several cases of PA intoxication have been reported from Afghanistan and India. (Kakar et al., 1976; Tandon et al., 1976; Mohabbat et al., 2010). Highly PA-contaminated cereals seemed to be the reason for these intoxications. The consumption of traditional tea infusions, known as "bush-teas" also led to severe poisonings in Jamaica and South Africa (Bras et al., 1961; Freiman et al., 1968; Brooks et al., 1970). Furthermore, some cases of PA intoxications due to the use of traditional medicine are also reported. After consumption of a PA-contaminated herbal tea used as a folk remedy an infant diseased on a VOD (Stillman et al., 1977). In another case, a two-month-old infant with fatal PA intoxication died seven days after consumption of a PA-contaminated herbal tea (Fox et al.,1978).

2.1.5.2 Acute, sub-acute and chronic toxicity

The liver is the primary target organ of PA intoxication, which can be divided into three dose-dependent levels: acute, sub-acute, and chronic (Lafranconi and Huxtable, 1984; Wiedenfeld and Edgar, 2011). Hemorrhagic necrosis, enlarged liver and ascites are typical symptoms of acute intoxication. Furthermore, liver failure can be caused by necrosis and liver dysfunction (IPCS, 1988; Huxtable et al., 1989; Prakash et al., 1999; Wiedenfeld and Edgar, 2011). Such intoxications are very rare, far more common is the occurrence of liver changes resulting from repeated PA ingestion. The chronic toxic effects of PA are of particular interest. Over time, especially the hepatotoxic and genotoxic effects can accumulate and lead to chronic liver failure or cancer (Schrenk et al., 2022). A hallmark of PA toxicity is the damage of the liver sinusoidal endothelial cells leading to VOD which can be considered as a characteristic symptom of a PA intoxication (IPCS, 1988; Huxtable et al., 1989; Prakash et al., 1999, Fu et al., 2004; Wiedenfeld and Edgar, 2011; Schrenk et al., 2022). Consequences of chronic PA

intoxication are centrilobular congestion, necrosis, fibrosis and liver cirrhosis induced by VOD. However, other organs such as the lung, may also be damaged by PA poisoning (Wiedenfeld and Edgar, 2011). The metabolites can escape from the liver and enter the lung. There they can exert effects similar to the VOD leading to increasing pressure in the pulmonary circulation (Huxtable et al., 1989; Wiedenfeld and Edgar, 2011).

2.1.5.3 Cytotoxicity

Cytotoxicity of PA was investigated in many different *in vitro* assays using several cell culture systems. The following section summarizes the most important cytotoxic data from the literature:

Tamta et al. used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay in HepG2/C3A cells with and without metabolic activation. They observed a significant reduction in viability only in presence of the metabolic activation system for echimidine (628 µM), monocrotaline (768 µM), retrorsine (178 µM) and senkirkine (342 µM) (Tamta et al., 2012). Li et al. (2013) investigated the cytotoxicity of different types of PA in HepG2 cells in the MTT assay. They determined IC_{20}-values for clivorine (13 µM), retrorsine (270 µM) and platyphylline (850 µM) (Li et al., 2013). Field et al. determined cytotoxicity of different PA in a chicken hepatocarcinoma cell line. As a result, they estimated an order of decreasing cytotoxic potency for eleven PA: lasiocarpine, seneciphylline, senecionine, heliotrine, riddelliine, monocrotaline, riddelliine *N*-oxide, lycopsamine, intermedine, lasiocarpine *N*-oxide and senecionine *N*-oxide (Field et al., 2015). In a study from Waizenegger et al. they investigated the structure-dependent induction of apoptosis by PA in HepaRG cells. Retronecine-type PA (echimidine and senecionine) seem to act as more potent apoptosis inducers than heliotridine- (heliotrine) or otonecine-type PA (senkirkine) (Waizenegger et al., 2018). Glück et al. investigated the cytotoxic effects of 22 different PA in metabolically competent HepaRG cells using the MTT cell viability assay. They evaluated concentrations from 0.1–250 µM PA with an incubation time of 24 h. The mono-esters intermedine, indicine, lycopsamine, rinderine, echinatine and europine did not reduce cell viability or did so only to a very small extent. Open-chained or cyclic di-esters showed medium to strong cytotoxic effects (viability ≤ 70%). Platyphylline showed only a weak toxic effect (Glück et al., 2021). Green et al. tested the cyclic di-ester senecione in primary rat hepatocytes and observed significant cytotoxic effects (Green et al., 1981).

2.1.5.4 Genotoxicity and Mutagenicity

In vitro and *in vivo* genotoxicity of PA has been studied extensively. There is a large number of studies investigating the genotoxic properties of different PA congeners (Chen et al., 2010).

Formation of DNA adducts was proven *in vitro* for riddelliine (Xia et al., 2003; Zhao et al., 2012). Metabolism was conducted using rat and human liver microsomes involving the formation of 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine (DHP), which reacts with DNA to form eight different DNA adducts (Xia et al., 2003). Furthermore, the formation of DNA adducts was demonstrated for lasiocarpine and heliotrine after metabolism with rat liver homogenates (Xia et al., 2006 and 2008). Lester et al. determined the relative potencies of nine different PA by quantification of DNA adduct formation and metabolic rate in a rat sandwich culture hepatocyte cell system. The ratio DNA adduct/metabolic rate was used as a parameter to express the potency of each congener. As a result, they found that PA toxicity depends strongly on the structural features of the corresponding PA (Lester et al., 2019). DNA adduct formation was also shown *in vivo* (Yang et al., 2001; Fu et al., 2017; Wang et al., 2005a; Wang et al., 2005b). Fu et al. feed 73 cows with PA contaminated food for 42 days. They reported the quantitation of PA-derived DNA adducts from their livers and three livers of control cows feed without PA-contaminated feed. DNA adduct formation was also demonstrated with rats treated with different PA: monocrotaline, retrorsine and riddelliine (Yang et al., 2001; Wang et al., 2005a; Wang et al., 2005b).

Reactive electrophilic PA metabolites have two functional groups (at positon C7 and at position C9) able of connecting to two sites in DNA to form DNA crosslinks. PA-induced DNA-DNA cross-linking was investigated *in vivo* and *in vitro* (Chen et al., 2010). Petry et al. found DNA-DNA interstrand cross-links after intraperitoneal administration of the PA monocrotaline to adult male Sprague-Dawley rats (Petry et al., 1984). Hincks et al. characterized the ability of eight different PA to cross-link cellular DNA using bovine kidney epithelial cells. They cultured the PA for 2 h with the cells and an external metabolizing system. The cross-linking ability of senecipylline, riddelliine, retrorsine and senecionine was much higher than that of monocrotaline, heliosupine, latifoline and retronecine (Hincks et al., 1991).

In addition to the described primary DNA damage in the previous section, there are also genotoxicity studies in mammalian test systems.

Especially micronuclei induction by PA has been widely studied and the results showed that PA are strong clastogenic agents (Chen et al., 2010). Allemang et al. investigated 15 different PA (eleven PA congeners, four PA *N*-oxides) representing six different structural classes for their relative genotoxic potential, assessing micronuclei *in vitro* in HepaRG cells. They treated the cells over 24 h and determined micronuclei using flow cytometry. Lasiocarpine was the

most potent PA, while the *N*-oxides showed very weak effects (Allemang et al., 2018). In a study from Müller-Tegethoff et al. in 1997 they observed micronuclei formation in rat hepatocytes after incubation with retrorsine and monocrotaline (Müller-Tegethoff et al., 1997). Kevekordes et al. tested monocrotaline and retrorsine in the micronucleus test *in vitro*. They incubated human lymphocytes in the presence and absence of rat liver S9-mix as well as HepG2 cells. As a result, a significant increase of micronuclei for monocrotaline and retrorsine could be detected in the human lymphocytes in the presence of S9-mix and in the HepG2 cells (Kevekordes et al., 2001).

Furthermore, there are some studies in the literature observing chromosomal aberations and sister chromatid exchanges *in vitro*. Takanashi et al. saw chromosomal aberrations for heliotrine, petasitenine, lasiocarpine and senkirkine in V79 cells. Heliotrine and petasitenine caused interchromosomal exchanges, while lasiocarpine and senkirkine produced chromatid gaps (Takanashi et al., 1980). Riddelliine induced chromosomal aberrations and sister chromatid exchanges in chinese hamster ovary cells in the presence of S9-mix (Galloway et al., 1993). Müller et al. incubated V79 cells in the presence of S9-mix with monocrotaline, retrorsine *N*-oxide and retrorsine for two hours. Retrorsine showed a concentration-dependent increase in aberrations. Monocrotaline showed only weak effects, while retrorsine *N*-oxide showed no effects. In contrast, prolonged treatment (18 h) *in vitro* in the presence of primary hepatocytes resulted in significant concentration-dependent positive effects for all three PA studied (Müller et al., 1992). Sister chromatid exchanges were also observed from Bruggemann and van der Hoeven. Heliotrine, monocrotaline, seneciphylline and senkirkine were investigated in the sister chromatid exchange (SCE) assay in V79 cells with primary chick embryo hepatocytes in co-culture. The rank order for induction found was seneciphylline > senkirkine > heliotrine > monocrotaline (Bruggemann and van der Hoeven, 1985).

The mutagenicity of various PA has also been studied many times in the past, but in particular results of the Ames test are equivocal. Yamanaka et al. demonstrated the mutagenic potential of seven different PA (i.e., clivorine, heliotrine, lasiocarpine and senkirkine) in *Salmonella typhmurium* TA100 with a modified Ames´s method. Pre-incubation of the PA together with S9-mix and *Salmonella* in a liquid was crucial (Yamanaka et al., 1979). Wehner et al. observed mutagenic effects for retrorsine in bacteria strains TA1535 and TA1537 in the presence of S9-mix, indicating that retrorsine induces base pair substitution and frameshift mutations (Wehner et al., 1979). Riddelliine showed mutagenic effects in TA100 with S9-mix, no mutagenic effects were seen in the strains TA97, TA98 and TA1535 (Chan, 1993). Rubiolo et al. found that several PA showed no mutagenic effects. Retrorsine and seneciphylline showed only weak mutagenic effects (Rubiolo et al., 1992).

Furthermore, PA were evaluated for their mutagenic potential not only in bacteria but also in *Drosophila*. Frei et al. examined the mutagenic potency of 16 different PA with the help of the

“wing spot test” in *Drosophila*. All tested PA except the mono-ester supinine were clearly genotoxic. Depending on their chemical structure this test system confirmed three orders of magnitude. Macrocyclic di-ester PA were the most and 7-hydroxy C9-mono-ester PA the least genotoxic congeners, while open di-esters achieved an average value (Frei et al., 1992). Clark et al. tested nine PA in adult males of *Drosophila melanogaster*. Monocrotaline, lasiocarpine and heliotrine showed strong mutagenic effects. Echimidine, echinatine, senecionine and supinine were less potent. Furthermore, the *N*-oxides showed attenuated effects in comparison to the “parent” PA (Clark, 1960). Cook and Holt also observed that the *N*-oxides are less potent than the “parent” PA (Cook and Holt, 1966).

2.1.5.5 Carcinogenicity

The carcinogenicity of PA is attributed to their genotoxic properties. A number of PA have been shown to be carcinogenic in experimental animals, primarily in rats (Wiedenfeld et al., 2008). A *in vivo* study in Fischer 344 rats showed induction of hepatocellular tumors and angiosarcomas of the liver in both, male and female, after chronic application with lasiocarpine (NTP, 1978). In a further *in vivo* study, the PA riddelliine induced liver tumors in male and female rats and male mice. Lung neoplasms were found in female mice. Further they observed leukemia in male and female rats (NTP, 2003). In a study by Newberne and Rodgers monocrotaline induced hepatocellular carcinoma in rats, after being administered intragastrically once weekly for 55 weeks (Newberne and Rodgers, 1973). Hepatoms were induced in rats administered 0.03 g/l (drinking water study) retrorsine or retrorsine-*N*-oxide three days per week over 20 months (Schoental et al., 1954). Cook et al. observed liver tumors in rats, which were fed with alkaloids of *Senecio jacobaea* over eight months (Cook et al., 1950). Harris and Chen 1970 and Hirono et al. 1980 saw hepatic tumors in rats following chronic ingestion of *Senecio longilobus* respectively *Senecio cannabifolius* (Harris and Chen, 1970; Hirono et al., 1980).

In humans, there is no direct indication observed for carcinogenicity of PA, so the International Agency for Research on Cancer (IARC) classified lasiocarpine, monocrotaline and riddelliine as being possibly carcinogenic to humans (category 2B) (IARC, 2002; IARC 1987). Further PA were not classifiable due to lack of data (category 3) (IARC 1987; IARC 1983).

2.1.5.6 Teratogenicity

In literature there are also a few studies observing teratogenic effects of different PA. A single intraperitoneal injection of heliotrine (doses between 50 and 200 mg/kg) into female rats during the second week of pregnancy caused musculoskeletal defects and a general retardation of development (Green and Christie, 1961). Peterson and Jago administered 200 mg/kg of heliotrine intraperitoneal to rats on the 14th day of gestation and made similar observations (Peterson and Jago, 1980). Exposure of pregnant rats to PA may cause teratogenic effects, premature births and many stillbirths, but PA do not always cause liver damage in the fetuses. They appear to be more resistant to hepatotoxicity than their mothers (Wiedenfeld et al., 2008).

2.1.6 Risk assessment

The potential health risks from 1,2-unsaturated PA have been evaluated by various scientific committees. The assessment approaches have changed over the last few years (BfR, 2020). The main points of earlier assessments are briefly described below.

FAO/WHO Joint Expert Committee on Food Additives (JECFA) concluded in 2015 that rats are the most sensitive species regarding PA toxicity. Furthermore, they stated that the liver is the most sensitive target organ. They considered the long-term carcinogenicity study on riddelliine (NTP, 2003) most appropriate for dose-response-modeling. Hence, a lower limit on the benchmark dose for a 10% response ($BMDL_{10}$) of 182 µg/kg body weight (bw)/day was used as the starting point in a margin of exposure (MoE) approach, regarding to haemangiosarcoma of the liver in female rats after treatment with riddelliine. They concluded that there is a concern for adults, who consume high amounts of tea and honey, and for children, who consume average tea amounts (JECFA, 2015).

In 2011, European Food Safety Authority (EFSA) published a scientific opinion on PA in food and feed. EFSA Panel on Contaminants in the Food Chain (CONTAM) estimated acute and chronic exposure to PA based on PA levels in 14.604 honey samples. CONTAM Panel proposed the MoE approach for risk assessment because current knowledge suggests that 1,2-unsaturated PA may act as genotoxic carcinogens in humans. EFSA recommended a $BMDL_{10}$ of 70 µg/kg bw/day for the induction of liver haemangiosarcoma by the PA congener lasiocarpine in male rats as point of departure for PA risk assessment and related it to the estimated exposure to PA. The panel finally concluded that there is a potential health risk to infants and children consuming high levels of honey (EFSA, 2011).

In 2017, EFSA updated the risk characterization of PA. Compared to the scientific opinion from 2011, new data on PA levels in additional food groups and food supplements were available. Furthermore, the panel recommended a $BMDL_{10}$ of 237 µg/kg bw/day as a new reference point for PA risk assessment. The $BMDL_{10}$ used was determined in a chronic toxicity study in rats with the PA congener riddelliine (NTP, 2003; EFSA, 2017). Finally, the panel came to the conclusion that there is a possible concern for human health, especially for people who consum high amounts of tea and herbal infusions (EFSA, 2017).

In 2008, UK Committee on Toxicity of Chemicals in Food, Consumer Products, and the Environment (COT) recommended the toxicity study with riddelliine in rats to assess the non-cancer effects of PA (NTP, 2003). Finally, the committee established a no observed adverse effect level (NOAEL) of 0.1 µg/kg bw/day, including an additional uncertainty factor of 100. Furthermore, the committee also estimated the risk for cancer. A $BMDL_{10}$ of 73 µg/kg bw/day was determined in a carcionogenicity study with the PA congener lasiocarpine in rats (NTP, 1978) and in combination with an MoE of 10.000 the committee finally concluded that no risk for cancer would be expected at intakes up to 0.007 µg/kg bw/day (COT, 2008).

In agreement with COT assessment, BfR recommended not to exceed a daily intake of 0.007 µg/kg bw/day. Additionally, BfR recommended to keep the total exposure to 1,2-unsaturated PA as low as reasonably achievable (ALARA principle) (BfR, 2011). In 2016, BfR published a further opinion on the basis of new data on PA levels in additional food groups and food supplements. They determined herbal tea, green and black tea as well as honey were the main sources of exposure for humans and concluded a health concern for both children and adults in case of chronic exposure. (BfR, 2016). Two years later, BfR released a further opinion on 1,2-unsaturated PA in food. A $BMDL_{10}$ of 237 µg/kg bw/day was derived as reference point for calculating MoE values. A risk related to adverse health effects from ingestion of PA-contaminated foods according to the new calculations was still considered possible (BfR, 2018). The last update followed in 2020. The PA levels in most food groups have been significantly reduced in recent years. For normal and frequent consumers, the determined total chronic exposure across all food groups results in uptake amounts for children and adults in MoE values of more than 10.000. However, the recommendation is still to minimize the intake of these substances (BfR, 2020).

For medicinal products, Committee for Herbal Medicinal Products (HMPC) in 2014 derived an oral intake limit of 0.007 µg PA/kg bw/day, resulting in a short-time (14 days) daily intake of 0.35 µg/day for adults (50 kg bw) (EMA, 2014). Federal Institute for Drugs and Medical Devises (BfArM) released an opinion in 2016 defining a limit value of 1.0 µg PA/day for all medicinal

products (BfArM, 2016). HMPC published a statement confirming an intake limit of 1 µg PA/day for an interim period of three years (EMA, 2016). Then, in 2019, HMPC extended the interim period for a further two years (EMA, 2019) before releasing a final statement on PA in 2021, agreeing with an acceptable oral intake of 1 µg PA/day for an adult (EMA, 2021).

The concept of iREP factors for the toxicological risk assessment of PA in food and herbal medicines was published by Merz and Schrenk in 2016. After analysis of the literature regarding PA-toxicity they combined the data of genotoxicity in *Drosophila*, cytotoxicity *in vitro* and acute toxicity in adult rodents to determine the differences for different PA congeners in their toxic potency. They assigned a factor of 1.0 to the cyclic di-esters and open-chained di-esters with 7*S* configuration as the most potent ones, mono-esters with 7*S* configuration assigned a factor of 0.3, open-chained di-esters with 7*R* configuration assigned a factor of 0.1, and 7*R* mono-esters assigned a factor of 0.01. According to their concept, the amount of a single PA congener is multiplied by the corresponding potency factor. The products are then summed and a quantitative value for the toxic potential of the sample is obtained and could be compared to other samples (Merz and Schrenk, 2016).

The most recent regulation introducing limits for PA in food for the first time is the regulation of the European Union (EU) 2020/2040 of December 11, 2020 amending regulation of the European Commission (EC) No 1881/2006 as regards maximum levels for PA in certain food products from July 01, 2022. Herbal teas (dried product), for example, may contain a maximum of 200 µg/kg total PA. Food supplements with herbal ingredients are limited to a maximum content of 400 µg/kg. Further limit values for certain food products are listed in the annex to the regulation (EC, 2020).

2.2 The liver

The liver is one of the largest organs in the human body. The central functions of the liver include the synthesis and secretion of proteins, the formation of bile, homeostasis of carbohydrate and fat metabolism, and the filtration of substances entering from the intestine. Finally, the liver has a variety of metabolic and detoxifying functions. After oral uptake, xenobiotics reach the stomach and intestine and can be absorbed there. The xenobiotics then enter the liver via the portal vein before being released into the general circulation (Hengstler, 2017).

2.2.1 Structure of the liver

The human liver is morphologically divided into left, right, caudate and quadrate lobes. The smallest functional unit of the liver is called hepatic lobule, which has a hexagonal shape in cross section, with a bundle of branches in each corner (Kruepunga et al., 2019). The branches of the portal vein and hepatic artery run together with the bile ducts, in which the bile flows in the opposite direction (portal triad) (Kahl et al., 2010). The liver is supplied by two main blood vessels: the hepatic artery and the portal vein. The hepatic artery supplies the liver with oxygen while the portal vein transports blood from the stomach, gut, spleen and pancreas to the liver (Rocha, 2012). In the terminal branching area, the portal vein and hepatic artery form capillaries, called sinusoids, which flow between the hepatocytes (Kahl et al., 2010). Hepatocytes are the parenchymal cells or working cells of the liver. Finally, the blood is collected in the central vein, the central vein branches then unite as hepatic vein and leave the organ. Sinusoids are lined with specialized sinusoidal endothelial cells allowing an exchange of the blood from the sinusoids with the hepatocytes. The space between these cell types is called space of Disse. About 25% of the hepatocyte is in contact with a sinusoid and about 9% is in contact with a biliary canaliculus (Hengstler, 2017). The oxygen content in the sinusoids decreases towards the central vein and the metabolic function of the hepatocytes changes from cellular respiration to glycolysis. The enzymes of xenobiotic metabolism are enriched in the zone of lower oxygen concentrations (Kahl et al.,2010).
Next to the hepatocytes, which represent the main cell type of the liver, and the sinusoidal endothelial cells, there are two more cell types: the Ito cells and the Kupffer cells. Ito cells are located in the space of Disse. In the healthy liver, they function, among other things, as a store for vitamin A. On the blood side of the sinusoidal endothelial cells are macrophages, named Kupffer cells. Due to the close connection of the liver to the intestine, components of intestinal bacteria can enter the liver sinusoids and are phagocytosed there by the Kupffer cells (Hengstler, 2017).

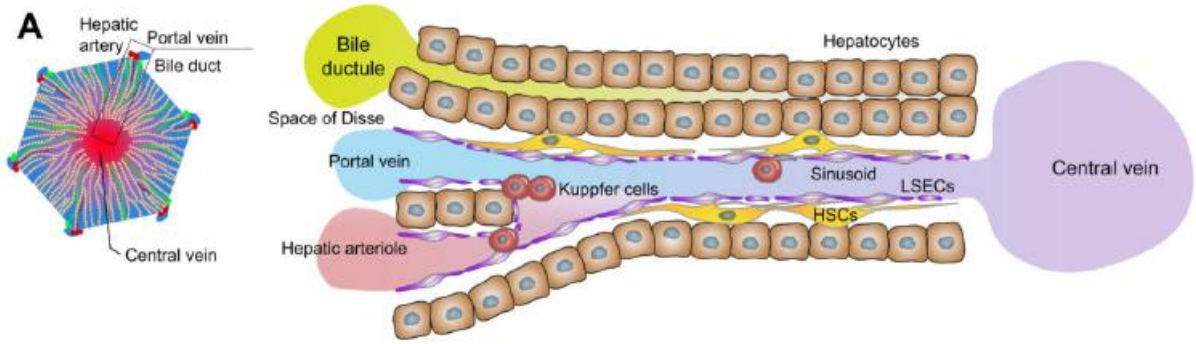

Figure 5: Structure of a hepatic lobule, the smallest functional unit of the liver (left) and detailed view of a hepatic sinusoid (right). The hepatic lobule (left) showed a hexagonal shape with a bundle of branches (portal triad: portal vein, hepatic artery and bile duct) in each corner. In the hepatic sinusoid (right) there are four important cell types: Liver sinusoidal endothelial cells (LSEC), hepatocytes, Ito cells or hepatic stellate cells (HSC) and Kupffer cells. The blood of the hepatic artery and the portal vein is pooled in the sinusoid and flows into the central vein (Iwakiri et al., 2014).

2.2.2 Xenobiotic metabolism

Humans are exposed to xenobiotics every day. These substances include i.e. environmental chemicals, plant constituents or pharmaceuticals. In order to eliminate these substances from the body again, mostly a chemical modification of lipophilic substances into hydrophilic metabolites by a xenobiotic-metabolizing-system is required (Wätjen und Fritsche, 2010).
The Toxicokinetic of xenobiotics in general can be divided into four parts, which are referred to as ADME: Absorption is the first part, it describes the uptake of substances into the blood circulation. This is followed by the distribution of substances with the blood circulation into various compartments (organs, tissues). Metabolism or biotransformation in the next step describes the metabolic transformation of lipophilic xenobiotics in the organism in the different compartments, leading to the formation of water-soluble products. This enables their excretion mainly via urine and faeces (Wätjen und Fritsche, 2010; Filser, 2017).

Metabolism or biotransformation mainly takes place in the liver and can be further divided into two different phases (Dekant, 2017):

<u>Phase I - functionalization:</u>

Enzymes of phase I reactions catalyze oxidation, reduction or hydrolysis of lipophilic xenobiotics and introduce or expose functional groups (functionalization). They include CYPs, flavin-containing monooxygenases, alcohol dehydrogenases, aldehyde dehydrogenases and esterases. The formed phase I metabolites can be classified according to their functional group

into electrophilic (i.e., epoxides) and nucleophilic compounds (i.e., hydroxyl- or carboxyl groups) (Dekant, 2017). Quantitatively, CYP reactions predominate in the organism. In the oxidation of xenobiotics by CYP enzymes, which belong to the heme proteins and thus have iron in the catalytic center, the introduction of an oxygen atom represents the key reaction (Wätjen und Fritsche, 2010). Introduced functional groups enable conjugation with endogenous polar substrates, which is described in the following (Dekant, 2017).

Phase II - conjugation:

Enzymes of phase II reactions couple the functional groups from phase I metabolism with an endogenous and very water-soluble substrate (conjugation). This further increases their hydrophilicity and allows excretion via kidney or bile by preventing reabsorption. Among the most important phase II reactions are the conjugation with glucuronic acid by uridine 5'-diphospho-glucuronosyltransferases (UGTs) or GSH by glutathione-*S*-transferases (GSTs). Further reactions are sulfonation with 3′-Phosphoadenosin-5′-phosphosulfat (PAPS) by sulfotransferases (SULT), conjugation with amino acids, acyl- or acetyl groups by *N*-acetyltransferases (NAT), as well as conjugation with methyl groups by *S*-adenosylmethionine (SAM) (Dekant, 2017).

The two-phase concept of xenobiotic metabolism is completed by the active uptake of substances into the cells, it is known as phase 0. As phase III the active excretion processes of substrates and xenobiotic conjugates from the cell and sometimes from the organism (Buters, 2008). Mainly organic anion transporting polypeptides (OATPs) and organic anion/cation transport proteins (OATs/OCTs) are responsible for the uptake of xenobiotics into the cell (Roth et al., 2011). ABC-transporters (ATP binding cassette), e.g., multi-drug resistance protein 1 (MDR 1) or breast cancer resistance protein (BCRP), are responsible for active elimination from the cell (Godoy et al., 2013).

The metabolism of xenobiotics normally ends in their detoxification and elimination from the body. However, it may also be that xenobiotics converted to intermediates that are chemically more reactive than the parent compounds and also than the metabolites to be excreted. This process is referred to as toxification, bioactivation or metabolic activation. In phase I, for example, epoxides could be formed after CYP oxygenation at a carbon double bond. The epoxides are electrophilic and can react with nucleophilic cell structures like DNA, leading to their dysfunction (Dekant, 2017). This is also the case for PA, which exert their toxic effects only after metabolic activation (Fu et al., 2004).

2.2.3 *In vitro* liver test systems

Various *in vitro* human liver test systems can be used to investigate hepatotoxicity of substances and to study their metabolism. They have been created in recent decades, including for example microsomes, 9000 supernatant fraction (S9), cell lines, transgenic cell lines, primary cells or perfused livers. A reduced complexity is a general advantage of such liver test systems (Brandon et al., 2003). The most important *in vitro* liver test systems, including those used in this work, are briefly described below.

Microsomes and S9 are liver homogenates, which are often used as *in vitro* test systems for investigation of hepatic metabolism of different substances. The S9 fractions obtained from rats treated with e.g. Aroclor 1254, which is causing a strong induction of many xenobiotic-metabolizing enzymes (Hengstler and Oesch, 2001). Therefore, a rat liver homogenate is centrifuged at 9000 x g to obtain the S9 fraction as supernatant, which contains both, microsomal and cytosolic enzymes. Further centrifugation of the S9 fraction at 100.000 x g results in separation of the endoplasmic reticulum subcellular fraction as pellet and the cytosolic enzymes as supernatant. The pellet is then resuspended in a buffer to obtain the liver microsomes. Liver microsomes mainly contain CYPs and UGTs, S9 fraction further contains the cytosolic enzymes such as aldehyde oxidase, xanthine oxidase, SULT, methyltransferases, NAT and GST (Richardson et al., 2016). The cofactor nicotinamide adenine dinucleotide phosphate (NADPH), which is necessary for the activity of the monooxygenases, is formed from NADP and isocitrate or glucose-6-phosphate by the enzymes isocitrate dehydrogenase or glucose-6-phosphate dehydrogenase. S9 fraction, NADP and isocitrate or glucose-6-phosphate form the so called S9-mix (Andrae and Martus, 2017). Microsomes are often used in co-culture with cell systems or alone, while S9-mix is often used in the Ames test for metabolic activation (Dekant, 2017). The disadvantage here is that reactive metabolites are formed outside the cells and may not be able to enter the cell interior. In most cases, S9-mix also exerts cytotoxic effects on the cells, so that experimental mammalian cells can only be incubated with S9 mix for a limited period of time (Andrae und Martus, 2017).

Another way to simulate hepatic metabolism is by using cell lines or primary cells. Primary hepatocytes, i.e., cells isolated and cultured from the liver, reflect the xenobiotic metabolism of the organ in question usually quite well, at least during the first hours after their isolation (Andrae and Martus, 2017). They can be cultured as monolayer, suspension culture or as sandwich culture and are often used for example for enzyme induction and inhibition studies. Furthermore, they are ideal for examining interspecies and interindividual differences in metabolism (Le Cluyse, 2001; Sivaraman et al., 2005; Soldatow et al., 2013). Since primary

cells normally do not proliferate, they cannot be used to study mutagenic effects (Andrae und Martus, 2017). The major disadvantage of using primary hepatocytes as *in vitro* liver test system is the fact, that their cell functionality dramatically decreases over time (Soldatow et al., 2013). Liver specific functions such as e.g., CYP expression decrease rapidly during the first 24–48 hours of culture (Nelson et al., 1982; Le Cluyse et al., 1996; Le Cluyse, 2001).

HepG2 cells or HepaRG cells are both immortalized liver-derived cell lines and are often used as alternative to primary hepatocytes to simulate hepatic metabolism (Guguen-Guillouzo and Guillouzo, 2010; Guguen-Guillouzo et al., 2010). Cell lines have gained the ability for unlimited cell division (immortalization), but therefore organ-specific xenobiotic metabolism is usually greatly reduced (Andrae and Martus, 2017). HepG2 cells express different liver-specific enzymes involved in phase I and phase II metabolism. It was found that their expression varies between different passages, which makes it difficult to compare data from different laboratories (Guguen-Guillouzo and Guillouzo, 2010). In comparison to human hepatocytes the activity of CYP450 enzymes is markedly reduced (Gerets et al., 2013). Besides HepG2 cells, HepaRG cells are also an alternative to primary hepatocytes. HepaRG cells express various CYPs and nuclear receptors in amounts similar to those found in primary human hepatocytes. They also exhibit various other capabilities such as the expression of phase II enzymes, ABC transporters and solute carrier transporters (Guillouzo et al., 2007). One of the main advantages of cell lines in general is their constant availability in unlimited amounts (Soldatow et al., 2013).

3. OBJECTIVE

In a first step, Merz and Schrenk analyzed available toxicity data of individual PA from the literature and identified a relationship between the toxic potency and the structural features of different individual PA. As a result, they proposed the concept of iREP-factors, in which they divided the structure class of PA in four different subgroups, where each subgroup was assigned a corresponding potency factor: a factor of 1.0 for cyclic di-esters and open-chained di-esters with *7S* configuration, a factor of 0.3 for mono-esters with *7S* configuration, a factor of 0.1 for open-chained di-esters with *7R* configuration and a factor of 0.01 for mono-esters with *7R* configuration (Merz and Schrenk, 2016). Due to the limited comparable data on individual PA congeners, the concept of potency factors is not yet sufficiently supported. Therefore, the project aims to further elucidate the structure-dependent PA toxicity and refine the concept of iREP factors.

Eleven different PA congeners (see **Table 2**) were selected from the subgroups to compare their toxic potency in different *in vitro* test systems. All of them are identified as important food contaminants. In the first part of this project cytotoxicity of the selected PA congeners should be examined by alamar blue assay in different human and rat liver cells. Furthermore, the role of CYP enzymes regarding to the toxic effects of PA should be investigated. In the second part, structure-dependent genotoxic effects should be determined in bacteria using the ames fluctuation assay as well as in mammalian cells using the micronucleus test. Finally, the results from the *in vitro* test system should be correlated with the quantification of known metabolic products and the identification of unknown metabolites. This correlation of *in vitro* assays and metabolism data aims to contribute to a better understanding of structure-dependent PA toxicity.

Table 2: Overview of the selected PA. The congeners are assigned to the corresponding structural classes and potency factors from Merz and Schrenk.

structure class	iREP[1]	PA
open-chained di-ester, *7S* cyclic di-ester	1.0	senecionine seneciphylline retrorsine riddelliine monocrotaline lasiocarpine
mono-ester, *7S*	0.3	heliotrine europine
open-chained di-ester, *7R*	0.1	echimidine
mono-ester, *7R*	0.01	indicine lycopsamine

[1] Merz und Schrenk, 2016

4. CUMULATIVE PART: PUBLICATIONS

The experimental methods used, as well as all generated scientific results of this work are presented and discussed in detail in the following publications. They represent the main part of this thesis.

Furthermore, it has to be mentioned that three PhD students worked on this project. Therefore, I have listed before each publication which data are assigned to whom. In this work only the already published data are cited.

4.1 Publication I: Structure-dependent hepato-cytotoxic potencies of selected pyrrolizidine alkaloids in primary rat hepatocyte culture

Reference:

Gao, L., Rutz, L., Schrenk, D., 2020. Structure-dependent hepato-cytotoxic potencies of selected pyrrolizidine alkaloids in primary rat hepatocyte culture. *Food Chem Toxicol* **135**, 110923.
Doi: https://doi.org/10.1016/j.fct.2019.110923

Contributions to this publication:

The concept of conducted experiments was developed together with my supervisor D. Schrenk and my working group colleague L. Gao. I conducted the experiments for the following substances: echimidine, europine, heliotrine, monocrotaline and seneciphylline. L. Gao conducted the experiments for further substances: indicine, lasiocarpine, lycopsamine, retrorsine and senecionine. The experiments on BROD activity and glutathione status were performed by L. Gao. Therefore, the data of L. Gao are attributed to her thesis. Following data analysis and interpretation of all results were performed by D. Schrenk, L. Gao and me. K.-H. Merz, J. Keller, K. Paulus and C. Schabelon isolated and characterized the test substance monocrotaline. J. Klingkowski isolated the primary rat hepatocytes for our experiments.

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Structure-dependent hepato-cytotoxic potencies of selected pyrrolizidine alkaloids in primary rat hepatocyte culture

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ABSTRACT

Contamination of food, feed and herbal medicines with plants containing pyrrolizidine alkaloids (PA) leads to measurable amounts of PA in many products. Since a number of PA are hepatotoxic in humans and animals and hepato-carcinogenic in animal experiments, the assessment of the relative toxic potencies of widely occurring PA contaminants warrants detailed investigation. Here, we studied the hepato-cytotoxic potencies of a number of relevant PA congeners in rat hepatocytes in primary culture. It was found that cyclic and open di-esters were much more toxic than mono-esters. Furthermore, the hepatocellular levels of cytochrome P450-catalyzed 7-benzoxyresorufin *O*-dealkylase (BROD) activity decreasing over time in culture, played an important role for activation of PA into cytotoxic metabolites. With a highly toxic PA (lasiocarpine), inhibition of BROD activity with ketoconazole markedly reduced toxicity while this was not obvious with the less toxic congener lycopsamine. Depletion of cellular glutathione with buthionine sulfoximine had no significant influence on the effects of highly toxic PA whereas it slightly increased toxicity of less potent congeners. Overall, our data partially confirm previously published structure-dependent interim Relative Potency (iREP) factors although for echimidine and monocrotaline in particular, substantial deviations were found, possibly due to specific toxicokinetic properties of these congeners.

1. Introduction

Pyrrolizidine alkaloids (PA) comprise a large group of natural compounds occurring in a wide variety of plants belonging to plant families such as *Asteraceae, Boraginaceae* or *Leguminosae* (Tasca et al., 2018). Several congeners bearing a double bond in the 1,2-position of the retronecin ring are known for theirhepatotoxic potential (Fu et al., 2004) causing damage to the hepatic endothelia and parenchymal cells (hepatocytes). Thus, exposure to certain PA can cause acute liver failure, and a special condition termed veno-occlusive disease (VOD) which is due to damage of the hepatic endothelia. These syndromes occur in laboratory animals (Chojkier, 2003) and, upon, incidental exposure in humans (Rubbia-Brandt, 2010) and livestock (Stegelmeier et al., 1999) including fatal cases. Chronic exposure to certain PA has been shown to lead to tumor formation in the liver causing the development of liver cell carcinoma and haemangiosarcoma and in some instances, tumours in extra-hepatic tissues such as lung, pancreas and intestine (Fu et al., 2004).

Hepatotoxic and carcinogenic PA were detected in black tea, green tea and herbal infusions from the food market (Bodi et al., 2014). A similar picture was obtained in medicinal teas, e.g. from camomile, fennel or peppermint (Schulz et al., 2015). Furthermore, several plants used in traditional Chinese medicine contain PA, and other plant preparations made from non-PA plants are frequently contaminated with PA. The widespread contamination of food, feed, and herbal medicines with PA is supposed to be due to cross-contamination with very minor amounts of PA-containing weeds during harvest (Bodi et al., 2014; Schulz et al., 2015).

PA require metabolic activation to exert their toxic effects (Reed et al., 1992). Mediated via an oxidation step, catalyzed by cytochromes P450 (CYP) in particular CYP3A and CYP2B isoforms (Lin et al., 2003; Fu et al., 2004), the retronecin ring of the PA molecule is converted into a highly reactive dehydroretronecine ring (Jago et al., 1970; Mattocks and White, 1971). Since this step is considered to take place mainly in the liver, hepatic damage is a hallmark of PA toxicity. The dehydropyrrolizidine derivatives (ester metabolites) thus formed can rapidly bind to nucleophilic centers in DNA, proteins, amino acids etc. (Fu et al., 2004; Chen et al., 2010). Alternatively, *N*-oxidation of the necine base leads to the *N*-oxide form of the PA which is considered to be a (reversible) step of detoxification (Mattocks and White, 1971). Furthermore, the PA can be inactivated by hydrolysis releasing the necine base and the necic acids (Chen et al., 2010).

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Due to their high reactivity, dehydropyrrolizidine ester metabolites can also react readily with water or glutathione, resulting in the formation of additional products. Alternatively, the ester bonds of PA may be saponified yielding the corresponding necic acid and necine base, e.g. retronecine. The latter may be oxidized by CYP enzymes leading to the reactive metabolite dehydroretronecine. Obviously, all types of PA require a metabolic activation in order to exert toxicity. Although the metabolic activation pathway seems to be essentially identical for all PA, the available data demonstrate marked differences in the relative toxicity of individual PA congeners (Merz and Schrenk, 2016), indicating that the cyclic di-esters have a higher potency in comparison to open-chained di-esters or mono-esters and that chirality at C7 may have an influence on toxicity.

As indicated above, contamination of food, feed, and herbal medicines with plants containing PA can be a serious problem for the safety of these products. Up to now, risk assessment is based on the carcinogenicity of highly potent PA after chronic application to rats (NTP, 1978, 2003; EFSA, 2017). Since the widely differing toxic potency of individual PA congeners is well described, interim relative potency (iREP) factors have been suggested based on literature data and emerging structure-potency relationships (Merz and Schrenk, 2016). The database for these factors is considered insufficient, however, to apply these to the current quantitative risk assessment of PA. Instead, the sum of a selected number of prominent PA congeners is used as dose metric, assuming that all PA are equally toxic to the most potent ones. Because this procedure appears inadequate, we undertake efforts to revisit and refine the concept of iREP factors in order to improve their scientific basis.

Although marked differences in hepatic metabolism of certain PAs between human and rat liver preparations have been reported (Kolrep et al., 2018). Nevertheless, studying PA toxicity in rat hepatocytes may provide important information since the rat is widely used as a model species for human risk assessment of PAs.

Here, we report on the cytotoxicity of selected PA congeners (Fig. 1) in rat hepatocytes in primary culture. Finally, we investigated the consequences of variation and inhibition of CYP activity using various incubation times and the CYP inhibitor ketoconazole on PA toxicity in hepatocytes.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin was purchased from PAA Laboratories (Pasching, Austria), Dulbecco's modified Eagle's MediumHigh Glucose (DMEM-HG), -Low Glucose (DMEM-LG), Pen/Strep solution, fetal calf serum (FCS), Percoll and the Protein Assay Dye Reagent from Life Technologies (Paisley, UK), 5,5′-dithiobis(2-nitrobenzoic acid), glucose-6-phosphate, L-glutathione, reduced and oxidized form, from PanReac AppliChem (Darmstadt, Germany), heparin from Carl Roth (Karlsruhe, Germany), glucose-6-phosphate-dehydrogenase and β-nicotinamide adenine dinucleotide phosphate hydrate (β-NADP) from Roche (Basel, Switzerland), buthionine sulfoximine (BSO), collagenase, trypan blue solution, pentobarbital, glutathione-S-reductase, Hank's Balanced Salt Solution (HBSS), 3,3′-methylene-bis(4-hydroxycoumarine), i.e., dicumarol, resorufin benzyl ether, 5-sulfosalicylic acid, and Triton X-100 from Sigma-Aldrich (Munich, Germany). All PA with the exception of monocrotaline were from Phytolab (Vestenbergsgreuth, Germany). All other chemicals were of the highest purity commercially available.

Monocrotaline was isolated from dried *Crotalaria mirabilis* seeds obtained from Germiterra Ltda. (Barreiras, Brazil). The seeds were grounded and the fine powder (3 × 30 g) was extracted with methanol (3 × 300 ml) for 2 × 24 h each in a Soxhlet apparatus. After 24 h, the solution was replaced by fresh methanol. The combined extracts were reduced by evaporation to a volume of approximately 100 ml and stored for 8 h at 4 °C. Precipitated allantoin was removed by filtration and a dark, oily residue was obtained after evaporation of the solvent. The residue was dissolved in 150 ml 5% aqueous HCl and extracted three times with 120 ml chloroform. The aqueous phase was treated with 25% aqueous NH_4OH until a pH-value of 12 was achieved. Then, the solution was extracted three times with 120 ml chloroform and the combined organic phases were evaporated to dryness. The white, yellowish residue was monocrotaline as revealed by elementary analysis, ^{1}H-NMR and ^{13}C-NMR. The latter techniques and TLC separation (visualized with *o*-chloroanil in toluene) revealed a purity of about 97%.

2.2. Animals, hepatocyte preparation and culture

Young adult male Wistar rats of a body weight of 200 ± 10 g were obtained from Janvier (Le Genest-Saint-Isle, France) and kept under standard conditions. The animals were anesthetized with pentobarbital (100 mg/kg; i.p.), and livers were perfused using a two-step procedure according to Seglen (1976) as modified by Schrenk et al. (1992). Viabilities of hepatocyte preparations were > 89% determined by trypan blue exclusion. The number of non-parenchymal cells was estimated by microscopic inspection to be less than 5%. Isolated hepatocytes (200,000 per well) were seeded in 1 ml/well medium (DMEM-LG supplemented with 10% fetal calf serum and 1% Pen/Strep solution) on rat tail collagen-coated 24-well plates at 37 °C under an atmosphere of 5% CO_2 and left for 3 h to attach. Then, medium was replaced by 1 ml/well fresh medium. Maintenance of rats and isolation of hepatocytes were carried out in compliance with Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.3. Treatment and cytotoxicity testing

In the hepatocyte cultures, medium was replaced by fresh medium after 3 or 24 h, and hepatocytes were incubated with 0.1% DMSO (solvent control) or with PA dissolved in DMSO. For testing of PA, cells were incubated with various concentrations of echimidine, europine, heliotrine, indicine, lasiocarpine, lycopsamine, monocrotaline, retrorsine, senecionine or seneciphylline, dissolved in DMSO, double distilled water or other solvents, for additional 24 or 48 h, as indicated. The concentrations used were limited by the solubility of the PAs.

Then, medium was removed and cells were analyzed for cytotoxicity using the resazurin reduction assay (Berg et al., 2015). Triton X-100 (0.1%) was used as positive control for cytotoxicity. For testing the role of GSH depletion, 1 mM buthionine sulfoximine (BSO), dissolved in double distilled water was added 3 h after seeding. For inhibition of CYP activity ketoconazole dissolved in DMSO was added to a final concentration of 5 μM. After pretreatment with BSO or ketoconazole for 2 h, medium was replaced by fresh medium and the cultures were incubated with PA.

2.4. 7-Benzoxyresorufin O-dealkylase (BROD) assay and inhibitory potency of ketoconazole

7-Benzyloxyresorufin was used as a broad model substrate for various rat CYP enzymes (Forrester et al., 1992; Kobayashi et al., 2002) including CYP2B1, 1A2 ans 3A1/2. The primary rat hepatocytes (0.2×10^6 cells/ml) were prepared and seeded in 24-well plates. After incubation, the monolayers were rinsed twice with PBS (phosphate-buffered saline, pH 7.4) to remove detached cells. The measurement was started by addition of 1 ml/well assay mixture, i.e. 1 ml PBS containing $MgCl_2$ (5 mM), dicumarol (10 μM) and 7-benzyloxyresorufin (5 μM). The amount of resorufin formed by *O*-dealkylation of 7-benzoxyresorufin was determined every 90 s up to 30 min fluorometrically (λ_{ex} = 544 nm, λ_{em} = 590 nm) with Thermo Scientific Fluoroskan Ascent FL. After the measurement, the plate was washed with PBS again and then frozen for protein determination. Resorufin standards (0.5–500 nM) were prepared by diluting a PBS solution.

The status of BROD activity in primary rat hepatocytes was

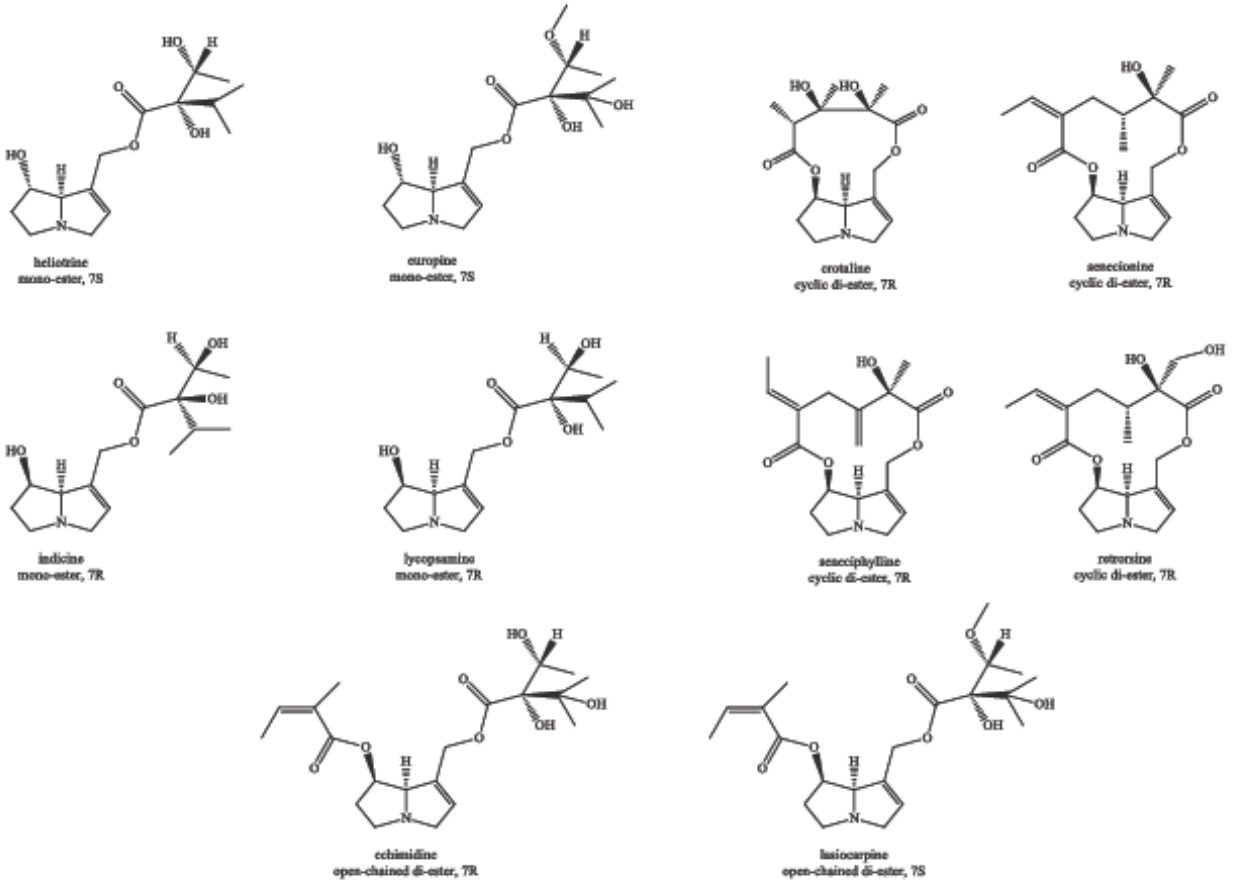

Fig. 1. Structures and structural features of tested PA congeners.

determined at different time points over up to 72 h after seeding. Ketoconazole was used as a broad inhibitor of rat CYP enzymes. For the inhibition experiment, the primary culture of rat hepatocytes was incubated in the absence and presence of ketoconazole with different concentrations (1, 5, 10 and 40 μM) for 2 h before adding the assay mixture. BROD activities were normalized to protein content that was determined with a commercially available Pierce bicinchoninic acid protein assay kit (Pierce, Rockford, USA).

2.5. Glutathione status

The determination of glutathione was based on the reduction of 5,5′-dithiobis(2-nitrobenzoic acid; DTNB; Ellman's reagent) with reduced glutathione (GSH) leading to 5-thio-2-nitrobenzoic acid (TNB) according to Anderson (1985) with the modifications used by Berg et al. (2015). The concentration of GSH was calculated as the difference between GSx and GSSG.

2.6. Data analysis

Independent experiments were carried out in triplicate. Arithmetic means and standard deviations were calculated. Statistical significance was assessed using Dunnett's test for multiple comparison with a control. Differences between two groups were analyzed using Student's t-test. A probability of error of $p \leq 0.05$, 0.01 was applied as indicative for significant ($p \leq 0.05$) or highly significant ($p \leq 0.01$) differences. EC_{50}-values were obtained after fitting concentration-response curves using OriginLab (Northampton, USA) software.

3. Results

Rat hepatocytes were initially cultured following a 24 h pre-incubation period before adding the test compounds. With two prototype PA congeners, i.e., the mono-ester lycopsamine and the open-chained di-ester lasiocarpine concentration-dependent cytotoxicity was observed in the resazurin reduction assay. It was found that lasiocarpine was several-fold more toxic than lycopsamine as estimated from the concentration-response curves. Furthermore, cytotoxicity was more pronounced after 48 h of incubation with the test compound, when compared to a 24 h incubation time. When the protocol was changed, i.e., the cells were pre-incubated for 3 h only, cytotoxicity of both compounds increased considerably, in particular after 48 incubation. Figs. 2 and 3 show the concentration-response characteristics after 3 h pre-incubation and 24 or 48 h treatment with lycopsamine (Fig. 2) or lasiocarpine (Fig. 3), respectively. The same protocol was also applied to eight other PA congeners belonging to different structural classes, i.e., to echimidine, europine, heliotrine, indicine, monocrotaline, senecionine, seneciphylline, and retrorsine. By modeling sigmoidal concentration-response curves (not shown), EC_{50}-values could be calculated for most congeners. Data are shown in Table 1, together with their standard deviations.

It was found that individual PA differ considerably in their cytotoxic potency in rat hepatocytes.

All di-esters including cyclic and open-chained congeners except monocrotaline were highly cytotoxic with EC_{50}-values between 4 and 25 μM after 3 h pre-incubation and 48 h treatment. In contrast, all mono-esters exhibited EC_{50}-values above 100 μM or even above 300 μM, the highest concentration tested. In addition, we observed that for certain PA congeners such as senecionine, the effect of a shorter pre-incubation, combined with a 48 h incubation time, was dramatic

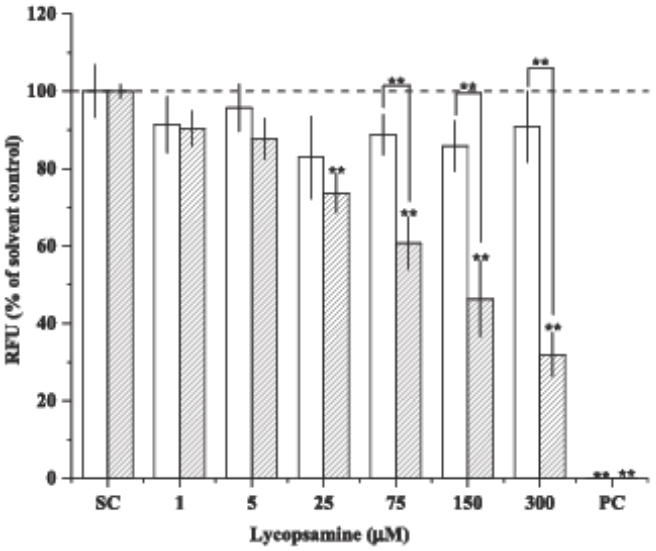

Fig. 2. Cytotoxicity (resazurin reduction assay), expressed as relative resorufin fluorescence units (RFU), of lycopsamine in rat hepatocytes in primary culture. Bars show means and S.D. from n = 3 independent series of experiments. After 3 h in culture, the cells were treated for 24 h (open bars) or 48 h (scattered bars) with the alkaloid added at the concentrations indicated, dissolved in 0.1% DMSO. Solvent controls (SC) treated with DMSO only were set as 100% RFU. Triton X-100 (0.1%) served as positive control (PC). Resorufin reduction levels were significantly different from the DMSO-treated control at *p ≤ 0.05 or **p ≤ 0.01 or from each other, as indicated, at *p ≤ 0.05 or **p ≤ 0.01.

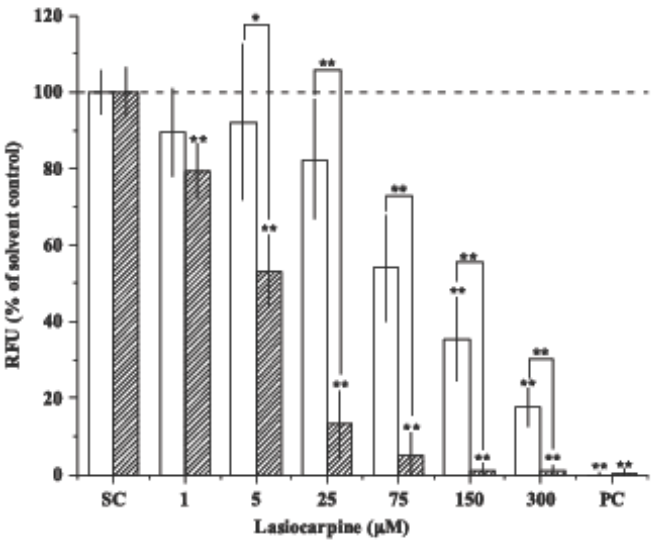

Fig. 3. Cytotoxicity (resazurin reduction assay), expressed as relative resorufin fluorescence units (RFU), of lasiocarpine in rat hepatocytes in primary culture. Bars show means and S.D. from n = 3 independent series of experiments. After 3 h in culture, the cells were treated for 24 h (open bars) or 48 h (scattered bars) with the alkaloid added at the concentrations indicated, dissolved in 0.1% DMSO. Solvent controls (SC) treated with DMSO only were set as 100% RFU. Triton X-100 (0.1%) served as positive control (PC). Resorufin reduction levels were significantly different from the DMSO-treated control at *p ≤ 0.05 or **p ≤ 0.01 or from each other, as indicated, at *p ≤ 0.05 or **p ≤ 0.01.

whereas it was rather moderate for others such as seneciphylline or heliotrine. For two congeners, retrorsine and seneciphyilline, short pre-incubation combined with a 24 h incubation time (in contrast to a 48 h incubation time) led to a reduced cytotoxicity, when compared to a 24 h pre-incubation (Table 1).

Further analysis showed that CYP-catalyzed BROD activity in rat

Table 1
Cytotoxicity of selected pyrrolizidine alkaloids (PA) in rat hepatocytes in primary culture. Data represent mean EC_{50}-values ± S.D. from n = 3 independent cell preparations. Cells were seeded 3 or 24 h after isolation (as indicated) and were treated with PA or solvent control for 24 or 48 h (as indicated). In some instances half-maximal cell death was not achieved and EC_{50} estimates could not be calculated.

PA (structural features)	EC_{50}: µM, 24 h/48 h 24 h after seeding	EC_{50}: µM, 24 h/48 h 3 h after seeding	iREP[a]
lasiocarpine (open, di, 7S)	91 ± 9/27 ± 2	86 ± 11/4 ± 1[c]	1.0
retrorsine (cyclic, di, 7R)	129 ± 9/90 ± 8	168 ± 30/19 ± 2[c]	1.0
senecionine (cyclic, di, 7R)	196 ± 31/95 ± 8	19 ± 3[c]/8 ± 1[c]	1.0
seneciphylline (cyclic, di, 7R)	109 ± 10/31 ± 4	178 ± 16[c]/19 ± 6[b]	1.0
monocrotaline (cyclic, di, 7R)	> 300/ > 300	> 300/ > 300	1.0
europine (mono, 7S)	> 300/ > 300	> 300/ > 300	0.3
heliotrine (mono, 7S)	> 300/294 ± 27	> 300/193 ± 17[c]	0.3
echimidine (open, di, 7R)	120 ± 18/48 ± 3	143 ± 7/25 ± 1[c]	0.1
indicine (mono, 7R)	> 300/ > 300	> 300/210 ± 16	0.01
lycopsamine (mono, 7R)	> 300/ > 300	> 300/114 ± 18	0.01

[a] Merz and Schrenk, (2016).
[b] Significantly different (p ≤ 0.05) from the treatment at 24 h after seeding.
[c] Highly significantly different (p ≤ 0.01) from the treatment at 24 h after seeding.

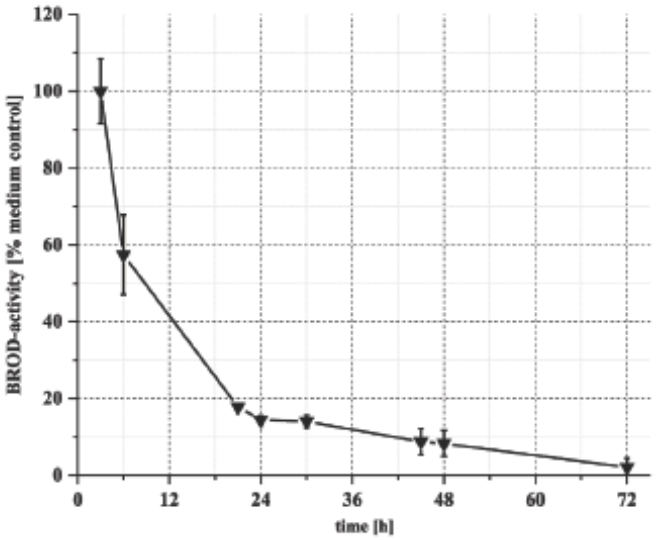

Fig. 4. 7-Benzoylresorufin O-dealkylase (BROD) activity in primary rat hepatocytes over up to 72 h after seeding. The assay was carried out in three different cell preparations. Each measurement was determined triplicately in independent culture wells, the point represents the mean ± SD of three different wells.

hepatocytes decreased by about 48 h in culture and was virtually absent after 72 h (Fig. 4). Thus, the decrease in sensitivity of the cultures towards a number of PA congeners with longer pre-incubation is likely due to a loss in relevant CYP activity. The fact that toxicity is affected by the pre-incubation time differently for different congeners, may be due a to different impact of various CYP enzymes on their metabolic activation.

Finally, we investigated the effects of inhibition of CYP and of GSH depletion on the cytotoxicity of PA congeners. We used ketoconazole as a CYP inhibitor and investigated its cytotoxicity and BROD-inhibiting potency at concentrations of 1, 5, 10 and 40 µM (data not shown). At a concentration of 5 µM ketoconazole inhibited BROD activity by about 80% without being cytotoxic. This treatment slightly reduced the cytotoxicity of lycopsamine (Fig. 5) whereas lasiocarpine was about threefold less cytotoxic under these conditions (Fig. 6). When GSH was

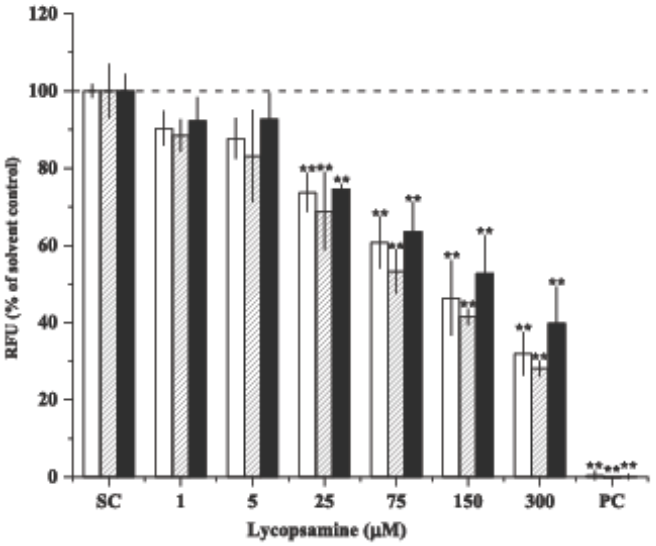

Fig. 5. Cytotoxicity (resazurin reduction assay), expressed as relative resorufin fluorescence units (RFU), of lycopsamine in rat hepatocytes in primary culture. Bars show means and S.D. from n = 3 independent series of experiments. After 3 h in culture, the cells were treated for 48 h with the alkaloid added at the concentrations indicated dissolved in DMSO only (open bars) or together with BSO (scattered bars) dissolved in double destilled water or ketoconazole (filled bars) dissolved in DMSO. Solvent controls (SC) treated with DMSO only were set as 100% RFU. Triton X-100 (0.1%) served as positive control (PC). Resorufin reduction levels were significantly different from the DMSO-treated control at *p ≤ 0.05 or **p ≤ 0.01 or from each other, as indicated, at *p ≤ 0.05 or **p ≤ 0.01.

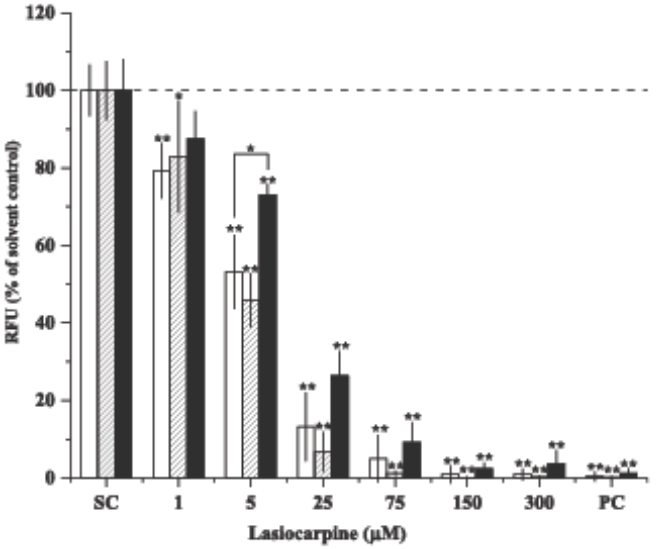

Fig. 6. Cytotoxicity (resazurin reduction assay), expressed as relative resorufin fluorescence units (RFU), of lasiocarpine in rat hepatocytes in primary culture. Bars show means and S.D. from n = 3 independent series of experiments. After 3 h in culture, the cells were treated for 48 h with the alkaloid added at the concentrations indicated dissolved in DMSO only (open bars) or together with BSO (scattered bars) dissolved in double distilled water or ketoconazole (filled bars), each dissolved in DMSO. Solvent controls (SC) treated with DMSO only were set as 100% RFU. Triton X-100 (0.1%) served as positive control (PC). Resorufin reduction levels were significantly different from the DMSO-treated control at *p ≤ 0.05 or **p ≤ 0.01 or from each other, as indicated, at *p ≤ 0.05 or **p ≤ 0.01.

depleted by about 50% (data not shown) via treatment with 1 mM BSO, a condition not causing cytotoxicity by itself (data not shown), lycopsamine and lasiocarpine both became more cytotoxic but the effect was not significant (Figs. 5 and 6). For other congeners, ketoconazole led to a slight to an about three-fold reduction in cytotoxicity (Table 2) while GSH depletion had no significant effect for the congeners senecionine and indicine, it increased the cytotoxicity of lasiocarpine after 24 h and lyopsamine after 48 h of incubation (Table 2).

4. Discussion

In a previous publication (Merz and Schrenk, 2016) we suggested interim Relative Potency (iREP) factors for a number of abundant PA suggesting a factor of 1.0 for cyclic di-esters and open-chained di-esters with 7*S* configuration, of 0.3 for mono-esters with 7*S* configuration, of 0.1 for open-chained di-esters with 7*R* configuration and of 0.01 for mono-esters with 7*R* configuration. For *N*-oxides it was suggested to apply the iREP factor of the corresponding PA. The limited database for these factors was derived from the literature. It mainly consisted of *in vivo* data on acute toxicity in rodents and genotoxic effects in Drosophila.

Since animal experiments with a large number of different PA congeners of interest are not feasible, an *in vitro*-based approach appears warranted. Such an approach has to take into account, however, the cytotoxicity (and genotoxicity) of the individual congeners at the target site as well as the target concentration resulting from oral exposure.

Both active uptake of PA (Tu et al., 2013, 2014) and metabolic activation (Fu et al., 2004) makes the hepatocyte a major target of PA toxicity. To achieve a comprehensive picture of the concentration-response characteristics of the cytotoxicity of selected congeners at the hepatocyte, we investigated selected PA in rat hepatocytes in primary culture. The first finding was that all 1,2-unsaturated congeners tested exerted a certain degree of cytotoxicity, the toxic potency being strongly dependent, however, from the congener tested. A longer incubation time of 48 h instead of 24 h led to higher toxicity in most cases suggesting that a cumulative damage such as covalent binding to proteins etc. may occur as described by others (Ma et al., 2018). Furthermore, the time of pre-incubation before adding of the test compounds had a strong influence on the general sensitivity of the cells towards PA. When studying the levels of CYP-catalyzed BROD activity, it was evident that this activity declined by more than 80% within 24 h in culture. Thus, tests after a pre-incubation time of 3 h were also included. We suggest that this procedure is more relevant since it reflects the original hepatic BROD activity much better than longer pre-incubation times. A shorter pre-incubation time of 3 h resulted in a dramatic increase in cytotoxicity for some congeners such as retrorsine (after 48 h incubation) or senecionine whereas the effects on heliotrine or echimidine were less pronounced. These differences may indicate that the toxicity of certain congeners strongly depends on certain active rat CYP enzymes whereas others can be activated by different CYP enzymes or enzymes not belonging to the CYP family (Huan et al., 1998). Evidence for the central role of CYP3A enzymes was provided for several PA congeners (Fu et al., 2004; Ruan et al., 2014) including lasiocarpine in microsomes from various species (Fashe et al., 2015), monocrotaline in rat liver (Kasahara et al., 1997; Reid et al., 1997), retrorsine in cultured rat hepatocytes Tu et al. (2014), and riddelliine and senecionine in human liver microsomes (Miranda et al., 1991; Xia et al., 2003). For monocrotaline, a study in rat astrocytes suggests that there is also role for CYP1A1 in metabolic activation (Nascimento et al., 2017). Moreover, the rat strain seems to play a role in the relative contribution of CYPs to PA metabolism as was shown by Chung and Buhler (2004). The finding that, after 3 h pre-incubation, the cytotoxicity of retrorsine and seneciphyilline after an incubation time of 24 h was significantly lower than after 24 h pre-incubation was unexpected. It may be due to a loss of detoxification pathways after 24 h pre-incubation and/or to a more important role of enzyme activities not determined in our study, in the metabolic activation of these PAs.

Table 2
Effects of ketoconazole (CYP3A inhibitor) and GSH-depletion with BSO on cytotoxicity of selected pyrrolizidine alkaloids (PA) in rat hepatocytes in primary culture. Data represent mean EC_{50}-values ± S.D. from n = 3 independent cell preparations. Cells were seeded 3 h after isolation and were treated with PA or solvent control for 24 or 48 h (as indicated). In some instances half-maximal cell death was not achieved and EC_{50} estimates could not be calculated.

PA (structural features)	EC_{50}: μM, 24 h/48 h	EC_{50}: μM, 24 h/48 h Pre-incubation with ketoconazole (5 μM)	EC_{50}: μM, 24 h/48 h Pre-incubation with BSO (1 mM)	iREP[a]
lasiocarpine (open, di, 7*S*)	86 ± 11/4 ± 1	120 ± 10*/11 ± 1**	55 ± 14*/4 ± 1	1.0
retronsine (cyclic, di, 7*R*)	168 ± 30/19 ± 2	> 300/67 ± 6**	n. t.	1.0
senecionine (cyclic, di, 7*R*)	19 ± 3/8 ± 1	65 ± 4**/12 ± 1**	19 ± 3/8 ± 2	1.0
seneciphylline (cyclic, di, 7*R*)	178 ± 16/19 ± 6	> 300/54 ± 11**	n. t.	1.0
monocrotaline (cyclic, di, 7*R*)	> 300/ > 300	> 300/ > 300	n. t.	1.0
europine (mono, 7*S*)	> 300/ > 300	> 300/ > 300	n. t.	0.3
heliotrine (mono, 7*S*)	> 300/193 ± 17	> 300/ > 300	n. t.	0.3
echimidine (open, di, 7*R*)	143 ± 7/25 ± 1	> 300/51 ± 10*	n. t.	0.1
indicine (mono, 7*R*)	> 300/210 ± 16	> 300/248 ± 2*	> 300/191 ± 15	0.01
lycopsamine (mono, 7*R*)	> 300/114 ± 18	> 300/166 ± 26*	> 300/77 ± 11*	0.01

n.t. = not tested.
*significantly different ($p \leq 0.05$) from the respective control (PA only).
**highly significantly different ($p \leq 0.01$) from the respective control (PA only).
[a] Merz and Schrenk, (2016).

In general, the much higher cytotoxicity known for cyclic and open-chained di-esters was confirmed in our study. Furthermore, the much higher cytotoxicity for most congeners after a short pre-incubation period of 3 h in comparison with 24 h and the concomitant loss in BROD activity support the notion that certain CYP enzymes are critical, possibly rate-limiting in the toxicity of many PAs. The suppression of cytotoxicity of many PAs with ketoconazole supports this conclusion although congener-specific differences were found. For some congeners such as lasiocarpine, retrorsine or senecionine the highly significant decrease in cytotoxic potency after 24 h versus 3 h pre-incubation points to the crucial role of CYP enzymes. It has to mentioned that ketoconazole being a rather specific CYP3A inhibitor in human liver microsomes, inhibits a broad spectrum of CYP enzymes in rat liver microsomes (Eagling et al., 1998). The effect of ketoconazole was less pronounced for congeners such as seneciphylline, heliotrine or echimidine which may indicate a role of different patterns of activating enzymes.

The iREP factors previously published at least roughly reflect the relative potencies found with the exceptions of monocrotaline and echimidine. Although bearing a cyclic di-ester structure, monocrotaline was much less cytotoxic than comparable congeners, exhibiting EC_{50}-values above 300 μM. Similarly, Yang et al. (2017) found a much higher liver toxicity of retrorsine in rats in comparison to monocrotaline, which was partially explained by differences in metabolism in liver microsomes. Guinea pig carboxylesterase was able to hydrolyze monocrotaline much more effectively than senecionine (Dueker et al., 1992). A similar picture was obtained in a recent study by Allemang et al. (2018) where monocrotaline was not cytotoxic in HepaRG cells up to a concentration of 1,000 μM. The authors discuss the low levels of CYP2A6 and CYP2E1 in these cells as a possible explanation. Interestingly, monocrotaline and retrorsine are taken up by the organic cation transporter 1 (OCT1) which may add to the complexity of their toxicokinetics (Tu et al., 2013, 2014).

Another exception is the open-chained di-ester echimidine which turned out to be more cytotoxic in rat hepatocytes than expected from its iREP factor of 0.1. Likewise, Waizenegger et al. (2018) found that echimidine induced cytotoxicity in HepaRG cells with a potency only slightly lower than that of senecionine. Obviously the values suggested for iREP classification can not hold true sufficiently for *in vitro* cytotoxicity testing. Again, effects of the 7-configuration on pre-hepatic absorbance and fate of the compound may possible explain these differences. Allemang et al. (2018) recently showed a higher genotoxic potency of echimidine in HepaRG cells than expected from the iREP factor of 0.3. The authors discuss the involvement of ABCB1-driven efflux of echimidine from the gastrointestinal mucosa into the gastrointestinal lumen (Hessel et al., 2014) as a possible reason for the deviation.

Also for the mono-esters no consistent picture was obtained, i.e., the configuration at position 7 did not seem to have a strong influence on the relative cytotoxicity. The deviation of the suggested iREP factors, which were mainly based on the limited available *in vivo* data, may again be due to effects of the 7-configuration on kinetics before the target cells are reached.

In conclusion we could quantify the cytotoxic potency of ten structurally diverse PA congeners of the retronecine type in rat hepatocytes in primary culture. Our findings support the notion of a strong influence of structural features of the PA molecule on liver toxicity making many cyclic and open-chained di-esters much more toxic than mono-esters. Furthermore, we could confirm the central role of CYP enzymes for the metabolic activation of PAs although congener-dependent differences exist in the relative effects of ketoconazole. We could also confirm recently published interim REP factors with the exceptions of echimidine being more toxic and monocrotaline being less toxic *in vitro* than anticipated based on *in vivo* data. It is assumed that these deviations are due to specific differences in toxicokinetics not reflected in a simple liver cell model which prevents direct conclusions on the risk of external exposure. Furthermore, a weighted use of different endpoints of toxicity seems to be warranted when current iREP factors for PAs should be refined. Our findings illustrate, however, the usefulness of liver cells for the investigation of target toxicity but also indicate the need for additional models including human cell models, for a reliable assessment of PA toxicity and kinetics.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2019.110923.

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4.2 Publication II

Structure-dependent genotoxic potencies of selected pyrrolizidine alkaloids in metabolically competent HepG2 cells

Reference:

Rutz, L., Gao, L., Küpper, J.-H., Schrenk, D., 2020. Structure-dependent genotoxic potencies of selected pyrrolizidine alkaloids in metabolically-competent HepG2 cells. *Arch Toxicol* **94**, 4159–4172.
Doi: https://doi.org/10.1007/s00204-020-02895-z

Contributions to this publication:

The concept of conducted experiments was developed together with my supervisor D. Schrenk and my working group colleague L. Gao. I conducted the experiments for the following substances: echimidine, europine, heliotrine, monocrotaline, riddelliine and seneciphylline. L. Gao conducted the experiments for further substances: indicine, lasiocarpine, lycopsamine, retrorsine and senecionine. Therefore, the data of L. Gao are attributed to her thesis. Only the already published data are cited in this work. Following data analysis and interpretation of all results were performed by D. Schrenk, L. Gao and me. J.-H. Küpper provided HepG2 C9 cells. Analysis of PA purity was performed by I. Geburek (Federal Institute for Risk Assessment, Berlin). J. Hönes measured BROD activity. K. -H. Merz, J. Keller, K. Paulus and C. Schabelon isolated and characterized the test substance monocrotaline. J. Klingkowski and Melanie Abel-Beckmann isolated the primary rat hepatocytes for our experiments.

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GENOTOXICITY AND CARCINOGENICITY

Structure-dependent genotoxic potencies of selected pyrrolizidine alkaloids in metabolically competent HepG2 cells

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Abstract

1,2-unsaturated pyrrolizidine alkaloids (PAs) are natural plant constituents comprising more than 600 different structures. A major source of human exposure is thought to be cross-contamination of food, feed and phytomedicines with PA plants. In humans, laboratory and farm animals, certain PAs exert pronounced liver toxicity and can induce malignant liver tumors in rodents. Here, we investigated the cytotoxicity and genotoxicity of eleven PAs belonging to different structural classes. Although all PAs were negative in the fluctuation Ames test in *Salmonella*, they were cytotoxic and induced micronuclei in human HepG2 hepatoblastoma cells over-expressing human cytochrome P450 3A4. Lasiocarpine and cyclic diesters except monocrotaline were the most potent congeners both in cytotoxicity and micronucleus assays with concentrations below 3 µM inducing a doubling in micronuclei counts. Other open di-esters and all monoesters exhibited weaker or much weaker geno- and cytotoxicity. The findings were in agreement with recently suggested interim Relative Potency (iREP) factors with the exceptions of europine and monocrotaline. A more detailed micronuclei analysis at low concentrations of lasiocarpine, retrorsine or senecionine indicated that pronounced hypolinearity of the concentration–response curves was evident for retrorsine and senecionine but not for lasiocarpine. Our findings show that the genotoxic and cytotoxic potencies of PAs in a human hepatic cell line vary in a structure-dependent manner. Both the low potency of monoesters and the shape of prototype concentration–response relationships warrant a substance- and structure-specific approach in the risk assessment of PAs.

Keywords Genotoxicity · Liver cells · Micronuclei · Mutagenicity · Pyrrolizidine alkaloids · Relative potencies

Abbreviations

BMC	Benchmark concentration
BROD	7-Benzoxyresorufin-*O*-dealkylase
CYP	Cytochrome P450
DAPI	4′,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DSMZ	German collection of microorganisms and cell cultures GmbH
EC_{50}	Effective concentration 50%
EDTA	Ethylene diamine tetraacetate
EFSA	European Food Safety Authority
FAU	Formazine attenuation unit
FCS	Fetal calf serum
GSH	Glutathione
γH2AX	Gamma-histone H2AX
HG	High glucose
HVOC	Hepato-venoocclusive disease
HCl	Hydrogen chloride
iREP	Interim relative potency
KCl	Potassium chloride
LB	Lower bound
LC/MS/MS	Liquid chromatography/mass spectrometry/mass spectrometry
LDH	Lactate dehydrogenase
LOD	Limit of detection
$MgCl_2$	Magnesium chloride
MMS	Methyl methane sulfonate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NH_4OH	Ammonium hydroxide

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00204-020-02895-z) contains supplementary material, which is available to authorized users.

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NMR	Nuclear magnetic resonance
OECD	Organisation for Economic Co-operation and Development
PA	Pyrrolizidine alkaloid
PH	Primary hepatocytes
PBS	Phosphate-buffered saline
RIVM	Rijksinstituut voor Volksgezondheid en Milieu
S9 mix	Supernatant mixture obtained at 9,000 g
UB	Upper bound
UDS	Unscheduled DNA synthesis

Introduction

A large number of plants contain 1,2-unsaturated pyrrolizidine alkaloids (PAs) known for their toxic properties. Among those are plants traditionally used as food, in particular herbal teas (Bodie et al. 2014), feed or herbal medicine (Schulz et al. 2015). In addition, PA-containing plants may contaminate non-PA plants and products thereof with serious consequences for their marketing and use (Mulder et al. 2018; Steinhoff 2019). Exposure to certain PAs at critical dose levels can lead to dramatic signs of acute toxicity such as body weight loss, liver failure and death in experimental, wild and farm animals (Williams and Molyneux 1987; Stegelmeier et al. 1999; Woolford et al. 2014). Intoxications of humans have also been described after consumption of PA plants or teas made from these (Stewart and Steenkamp 2001). Furthermore, chronic exposure can result in liver failure characterized by a hepato-venous occlusive disease (HVOC) with destruction of venous endothelial cells in the liver. Severe consequences include internal bleeding, portal hypertension and cirrhosis (Stegelmeier et al. 1999). Probably on the basis of such damage, malignant tumors of the endothelia (sarcoma) or hepatocytes (carcinoma) may develop and were observed in laboratory rodents chronically treated with PAs (Fu et al. 2004).

Certain 1,2-unsaturated PA congeners exert genotoxic properties, i.e., they can form DNA adducts, elicit mutations in the Drosophila wing test, and are genotoxic in a variety of in vitro assays. Recent studies have revealed that some selected PAs are weakly or unequivocally mutagenic in the Ames test (Rubiolo et al. 1992; Wehner et al. 1979; Yamanaka et al. 1979), whereas they were able to induce micronuclei formation in mammalian cells (Allemang et al. 2018).

It is widely accepted that DNA binding and genotoxicity depend on the formation of reactive PA metabolite(s) mainly formed in the liver catalyzed by cytochrome P450 (CYP) mono-oxygenases (Li et al. 2011). Upon oxidation of the 1,2-unsaturated necine base, e.g., the retronecine ring of the PA molecule, a reactive, electrophilic dehydroretronecine, either as intact ester or after ester hydrolysis, is formed which binds covalently to DNA (Fu et al. 2004). It is also discussed that secondary metabolites formed from binding of dehydroretronecine to nucleophiles may still retain some reactivity and cause further damage (Xia et al. 2015). These findings support the notion that certain PAs act as genotoxic carcinogens and illustrate the need for an adapted toxicological risk assessment taking into account the risk from chronic exposure to low dose levels (Schrenk et al. 2020).

Since PAs are always synthesized and occur in plants as mixtures of various congeners, the risk arising from simultaneous exposure to different congeners has to be assessed. The toxic and genotoxic potencies of different congeners have been shown, however, to vary by more than two orders of magnitude (Merz and Schrenk 2016). Thus, the current approach to analyze a certain set of PAs in a sample and attribute all of them with the same potency as the most toxic congeners (lasiocarpine or riddelliine) is a clear over-estimation of the risk. Recently, we have tried to generate a suggestion for potency factors for prototypic PAs representing certain structural classes (Merz and Schrenk 2016). These data indicate that cyclic diesters and some open-chained diesters are the most toxic ones, followed by other open-chained diesters and monoesters. A classification of structures based on limited datasets from rodents, Drosophila and in vitro studies, thus, allowed the assignment of interim relative potency (iREP) factors ranging from 1.0 (for the most toxic PAs) to 0.01 (for the least toxic congeners). In was argued, however, that the basis for this classification is uncertain and needs further refinement before in can be taken into account in quantitative risk assessment (EFSA 2017).

In recent publications, good agreement was found in various in vitro test systems for cytotoxicity (Gao et al. 2019), micronucleus formation (Allemang et al. 2018), DNA adduct formation (Lester et al. 2019), and indirect markers for genotoxicity (Louisse et al. 2019) between toxic potencies in the assay and iREP factors for various PAs. For some congeners, however, especially for echimidine and monocrotaline, substantial deviations were found.

In this study, we applied systems for in vitro mutagenicity (fluctuation Ames test in *Salmonella typhimurium*) and clastogenicity/aneugenicity (micronucleus assay in mammalian cells) to a set of eleven frequently occurring PAs. We also measured cytotoxicity both in bacteria and in mammalian cells to avoid false-negative genotoxicity findings. We used two different *Salmonella* strains, TA 98 (detection of frameshift mutations) and TA 100 (detection of base pair substitutions), as well as a stably transfected human HepG2 hepatoblastoma cell line expressing human CYP3A4, one of the major enzymes catalyzing the metabolic activation of PAs (Ruan et al. 2014). The major aims of this study were the further refinement of the concept of relative toxic

potency of PA congeners as well as an investigation of the concentration–response characteristics at very low, more relevant PA concentrations.

Materials and methods

Chemicals, media and cells

Dulbecco's modified Eagle's Medium–High Glucose (DMEM-HG), Pen/Strep solution, and fetal calf serum (FCS) were from Life Technologies (Paisley, UK), blasticidin S hydrochloride from Carl Roth (Karlsruhe, Germany), dicumarol, methyl methane sulfonate (MMS), saponin, trypan blue solution and resorufin benzyl ether from Sigma-Aldrich (Merck, Darmstadt, Germany), 4′,6-diamidino-2-phenylindole (DAPI) from AppliChem (Darmstadt, Germany). All PAs with the exception of monocrotaline were from Phytolab (Vestenbergsgreuth, Germany). All other chemicals were of the highest purity commercially available.

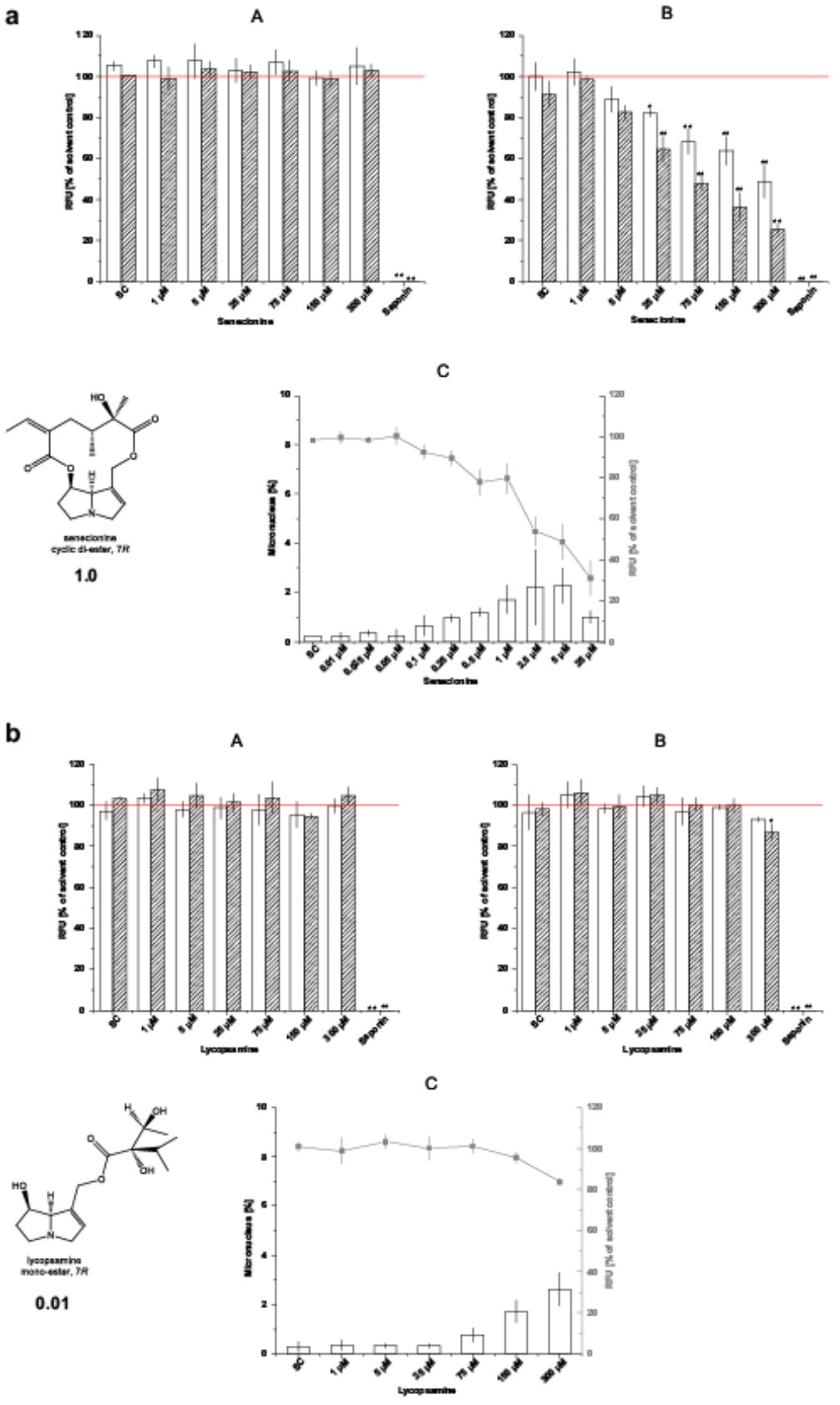
Monocrotaline was isolated from dried *Crotalaria mirabilis* seeds obtained from Germiterra Ltda. (Barreiras, Brazil). The seeds were ground and the fine powder (3 × 30 g) was extracted with methanol (3 × 300 ml) for 2 × 24 h each in a Soxhlet apparatus. After 24 h, the solution was replaced by fresh methanol. The combined extracts were reduced by evaporation to a volume of approximately 100 ml and stored for 8 h at 4 °C. Precipitated allantoin was removed by filtration and a dark, oily residue was obtained after evaporation of the solvent. The residue was dissolved in 150 ml 5% aqueous HCl and extracted three times with 120 ml chloroform. The aqueous phase was treated with 25% aqueous NH_4OH until a pH value of 12 was achieved. Then, the solution was extracted three times with 120 ml chloroform and the combined organic phases were evaporated to dryness. The white, yellowish residue was monocrotaline as revealed by elementary analysis, ^{1}H-NMR and ^{13}C-NMR. The latter techniques and TLC separation (visualized with *o*-chloroanil in toluene) revealed a purity of about 97%. In addition, all PAs were analyzed for impurities by LC/MS/MS analysis as described previously (Geburek et al. 2020). None of the PAs was contaminated by other congeners above their LODs except for heliotrine containing 0.5% heliosupine.

Salmonella typhimurium strains TA98 and TA100 were from Molecular Toxicology Inc. (Moltox, Boone, USA). HepG2 cells were from DSMZ, Heidelberg, Germany. HepG2 CYP3A4 cells (clone C9) overexpressing human CYP3A4 were genetically engineered and propagated as previously described (Herzog et al. 2015).

Fluctuation Ames test

Animal experiments were performed according to National Animal Welfare Regulations after authorization by the local authorities (Struktur- und Genehmigungsbehörde Rheinland-Pfalz, Koblenz, Germany). We used Sprague–Dawley male rats weighing approximately 200 g. To induce rat liver enzymes, we administered 500 mg/kg b.w. Aroclor 1254 via i.p. injection five days before killing from a stock solution of 200 mg/ml diluted in corn oil. The rats were given drinking water and commercial lab chow ad libitum. All steps of preparing the rat liver S9 fraction were carried out at 4 °C using cold and sterile solutions. The livers were washed in 0.15 M KCl and transferred to a beaker containing 3 ml KCl (0.15 M) per g liver. Homogenization was performed with a Potter–Elvehjem apparatus. The homogenate was centrifuged for 10 min at 9000 g and the supernatant (S9 fraction) was stored at − 80 °C.

The Ames fluctuation assay was performed as described in ISO 11350 (2012). Mutagenicity of the test compounds was analyzed in different strains, TA98 and TA100, with and without exogenous metabolic activation by rat liver S9 fraction. The overnight culture of bacteria was grown in nutrient broth medium (Oxoid Ltd., Basingstoke, UK) containing ampicillin (50 µg/ml) in an incubation shaker for 5 h at 37 °C and 125 rpm. The bacterial density was measured with a spectrophotometer at a wavelength of 595 nm. Bacteria were adjusted with exposure medium to reach an optical FAU (formazine attenuation unit) value which differs from strain to strain (TA98: 180, TA100: 45) (ISO 11350, 2012). Per test compound (dissolved in DMSO or in a DMSO/acetonitrile mixture, see below) 10 µl were placed in a 24-well plate, 490 µl bacteria were added and, in case of metabolic activation, 17 µl S9 mix (3%). The plates were incubated at 37 °C and 125 rpm for 100 min. Thereafter, 2.5 ml histidine-deficient indicator medium was added to each well and then transferred into a 384-well microplate. 48 wells were filled each with 50 µl per concentration. The 384-well plates were incubated at 37 °C for 48 h. The reversion from his- to his + in the mutagenic samples was detected by the color change of the pH indicator from purple to yellow. DMSO was used as negative control to detect spontaneous reversions (max. 10 positive wells/48 wells). Positive controls were used to examine the efficiency of the test system. The percentage of positive (yellow) wells in the positive control has to be more than 52% (25 wells/48 wells). All assays were carried out in triplicates. To indicate mutagenicity, the samples had to show a concentration-dependent statistically significant increase of positive wells above background.


a
A
B
C
RFU [% of solvent control]
Micronuclei [%]
Senecionine
senecionine
cyclic di-ester, 7R
1.0
b
A
B
C
Lycopsamine
lycopsamine
mono-ester, 7R
0.01

◂Fig. 1 **a** Cytotoxicity and micronuclei formation in HepG2 cells (upper left), HepG2-CYP3A4 cells (upper right) and micronuclei levels (lower, bars) in HepG2-CYP3A4 cells treated with senecionine. The cytotoxicity in HepG2-CYP3A4 cells under the conditions of the micronuclei assay (with recovery) is also shown (-■-). Data represent mean ± S.D. from $n = 3$ independent experiments. **b** Cytotoxicity and micronuclei formation in HepG2 cells (upper left), HepG2-CYP3A4 cells (upper right) and micronuclei levels (lower, bars) in HepG2-CYP3A4 cells treated with lycopsamine. The cytotoxicity in HepG2-CYP3A4 cells under the conditions of the micronuclei assay (with recovery) is also shown (-■-). Data represent mean ± S.D. from $n = 3$ independent experiments

Cell culture and cytotoxicity assay

For standard cytotoxicity testing, HepG2 cells and HepG2 CYP3A4 cells were seeded on 48-well plates (65,000 cells per well). Medium was replaced by fresh medium after 24 h and cultures were incubated with 0.1% DMSO (solvent control), 0.1% saponin (positive control) or with PA. For testing of PAs, cells were incubated with various concentrations of echimidine, europine, heliotrine, indicine, lasiocarpine, lycopsamine, monocrotaline or riddelliine, dissolved in DMSO, or retrorsine, senecionine or senecionphylline dissolved in a 1:1 mixture of DMSO and acetonitrile, for additional 24 or 48 h. The concentrations used were limited by the solubility of the PAs in the medium. The concentration ranges are shown in the respective figures (see below) and were selected according to previous experience with cytotoxicity testing of different PAs in mammalian cell culture (Gao et al. 2020).

Then, medium was removed and cells were analyzed for cytotoxicity using the resazurin reduction assay (Berg et al. 2015).

To obtain information on cytotoxic effects over a longer incubation time (about 1.5 cell cycles) used in the micronucleus assay, cells were also maintained over additional 72 h (recovery) without PA, medium was removed and cells were analyzed for resazurin reduction.

7-Benzoxyresorufin-O-dealkylase (BROD) assay

7-Benzyloxyresorufin is used as a model substrate for various human CYP enzymes including CYP3A4 (Alexander et al. 1999). The HepG2 or HepG2 CYP3A4 cells were cultured as described above, the monolayers were rinsed twice with PBS (phosphate-buffered saline, pH 7.4) to remove detached cells. The measurement was started by addition of 1 ml/well assay mixture, i.e., 1 ml PBS containing $MgCl_2$ (5 mM), dicumarol (10 µM) and 7-benzyloxyresorufin (5 µM). The amount of resorufin formed by *O*-dealkylation of 7-benzoxyresorufin was determined every 90 s up to 30 min fluorometrically ($\lambda_{Ex} = 544$ nm, $\lambda_{Em} = 590$ nm) with a Thermo Scientific Fluoroskan Ascent FL. After the measurement, the plate was washed with PBS again and then frozen for protein determination. Resorufin standards (0.5–500 nM) were prepared by diluting a PBS solution. BROD activities were normalized to protein content that was determined with a commercially available Pierce bicinchoninic acid protein assay kit (Pierce, Rockford, USA).

Micronucleus assay

For the micronucleus test, we followed published standard procedures (Lehmann et al. 2006) with some minor modifications. HepG2 CYP3A4 cells (7 x 10^5 cells) were seeded on 60 mm Petri dishes. After 24 h in culture (4 ml DMEM low glucose, 10% FCS, 65 µM blasticidin S hydrochloride per dish), medium was removed, test compounds were added in DMSO together with fresh DMEM high glucose, with 10% FCS and 65 µM blasticidin S hydrochlorideand incubated for 24 h. Then, the medium was replaced by fresh medium and the cells were incubated for additional 72 h. Then, the medium was removed, the cell layers were rinsed with 2 ml PBS and trypsinized with trypsin–EDTA solution (0.01%, 300 µl). Then, the cell pellets were rinsed again with PBS, 3 ml ethanol were added each and the pellets were placed in the freezer at − 20 °C for 30 min. After fixation, the liquid supernatant was removed, 750 µl DAPI staining solution was added in the dark, and the flasks were kept at − 20 °C for 10 min. Then, the cultures were inspected with a light microscope equipped with a UV lamp and a 631.25 immersion oil objective. 1000 nuclei per sample were inspected for the occurrence of micronuclei. Micronuclei showed a diameter of 1/16 to 1/3 of the main nucleus, were clearly separated from the main nucleus without overlap, and showed a strong, typical chromatin staining. The number of micronuclei was expressed as micronuclei-containing cells per 1000 cells. Controls were treated with DMSO (vehicle) only (negative control) or with methyl methane sulfonate (positive control).

Statistical analysis and modeling

Data in Fig. 1 are expressed as mean ± S.D. The data of the test compounds were analyzed with Dunnett's multiple comparison test for significant ($p \leq 0.05$) or highly significant ($p \leq 0.01$) differences from the negative control.

For modeling of concentration–response relationships (Figs. 2 and 3), we applied the benchmark calculation mode recommended by RIVM and EFSA (EFSA 2019). This software (PROAST) calculates the best fit of six different algorithms widely used in dose–response modeling. The best four out of these fits are analyzed further and weighted according to their quality of fitting. A doubling of micronuclei counts was used as a benchmark response.

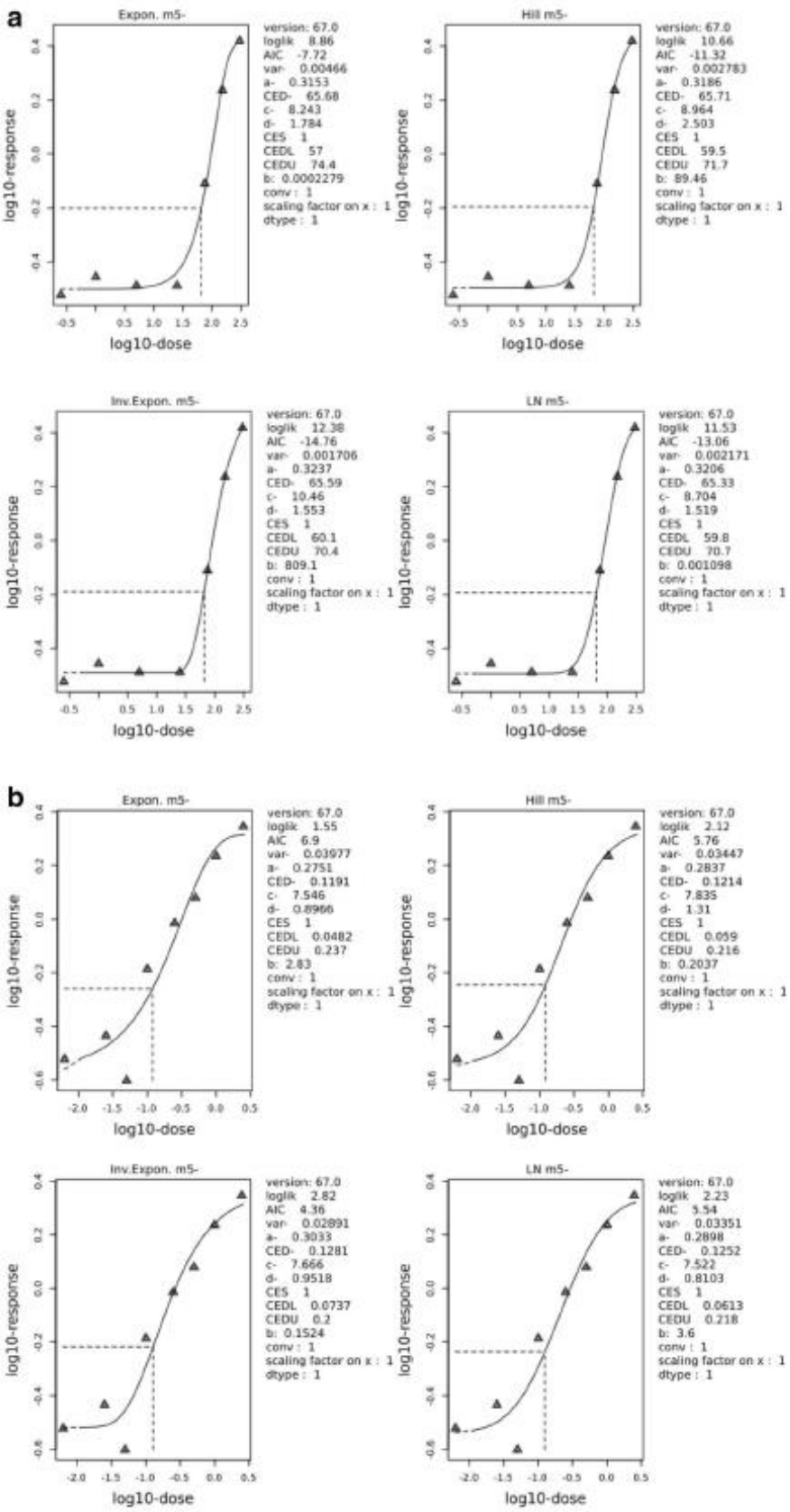


Fig. 2 **a** Benchmark-Dose modeling of micronuclei data in HepG2-CYP3A4 cells treated with various concentrations of lycospamine. **b** Benchmark-Dose modeling of micronuclei data in HepG2-CYP3A4 cells treated with various concentrations of senecionine. The panel shows the four best fits using PROAST (EFSA) software and the concentration estimate for a Benchmark effect of doubling of micronuclei counts (for details see 'Materials and Methods')

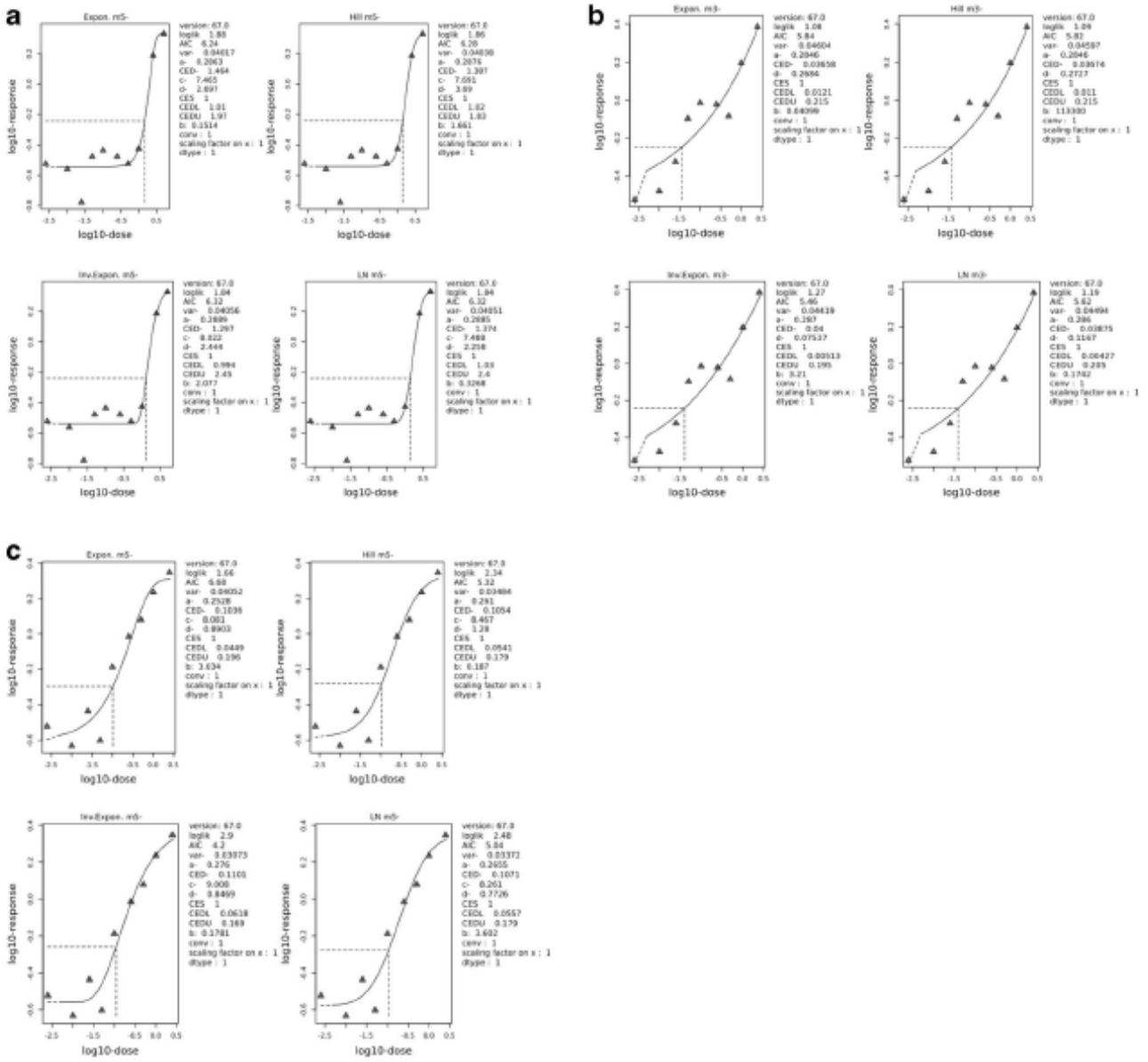


Fig. 3 **a** Benchmark-Dose modeling of micronuclei data in HepG2-CYP3A4 cells treated with various concentrations of retrorsine, with a special focus on the low concentration range below 1 µM. **b** Benchmark-Dose modeling of micronuclei data in HepG2-CYP3A4 cells treated with various concentrations of lasiocarpine, with a special focus on the low concentration range below 1 µM. **c** Benchmark-Dose modeling of micronuclei data in HepG2-CYP3A4 cells treated with various concentrations of senecionine, with a special focus on the low concentration range below 1 µM. The panels show the four best fits using PROAST (EFSA) software and the concentration estimates for a Benchmark effect of doubling of micronuclei counts (for details see 'Materials and Methods')

Results

Ames fluctuation test

In a first approach, we incubated eleven PAs belonging to different structural classes of 1,2-unsaturated congeners in the fluctuation Ames test. It was found that up to a concentration causing a more than 50% loss in bacterial viability, measured as resazurin reduction, all results were negative (see Supplementary Material). For some PAs, the maximum concentration used was 300 µM; while, others such as senecionine were more toxic to the bacteria and were,

thus, applied at lower maximum concentrations (data not shown). The addition of rat liver S9-mix had no effect on the outcome of the fluctuation assay.

BROD activity

For further experiments, we used the human HepG2 C9 cell line over-expressing the CYP3A4 gene. We determined a mean benzoxyresorufin-*O*-dealkylase (BROD) activity in homogenates of 0.4 pmol/mg protein per min, while the mean activity in non-transformed HepG2 cells was 0.09 pmol/mg protein per min. For comparison, the mean BROD activity in rat hepatocytes was 22.5 ± 11.9 after three h and 1.2 ± 0.3 (in pmol/mg protein per min) after 24 h in culture (unpublished data from experiments in Gao et al. 2020).

Cytotoxicity

Next, we incubated naïve HepG2 cells and the clone HepG2 C9 (CYP3A4) cells with various concentrations of eleven selected PAs (echimidine, europine, heliotrine, indicine, lasiocarpine, lycopsamine, monocrotaline, retrorsine, riddelliine, senecionine, and seneciphylline) and measured the cytotoxicity in the resazurin reduction assay after incubation over 24 and 48 h. Findings were confirmed by microscopical inspection of the cultures. In a different protocol, micronuclei counts were determined after a PA treatment over 72 h (see below). The longer treatment was required because of the special requirements for the formation of micronuclei, i.e., cytotoxicity testing over 1.5–2 cell cycles as recommended by OECD (2016). In Fig. 1, the effects of two 'prototype' PAs, i.e., the cyclic diester senecionine and the monoester lycopsamine are shown. Senecionine was non-toxic in naïve HepG2 cells up to a concentration of 300 µM, while a significant cytotoxicity was seen at 25 µM and above in HepG2 C9 cells expressing the human CYP3A4 gene (Fig. 1a). Treatment over 48 h exerted a somewhat higher cytotoxicity than the 24 h treatment. In incubations over 72 h, a continuous decline in viability being below 20% at 25 µM was found.

The monoester lycopsamine was also non-toxic in naïve HepG2 cells, and almost non-toxic in HepG2 C9 cells with a significant but minor decrease in viability after 48 h at the highest concentration tested (300 µM). Cytotoxicity was also determined over 72 h showing a very minor decline in viability which remained above 80% at all concentrations tested.

Cytotoxicity data obtained for all eleven PAs after treatment over 48 h were modeled using sigmoidal curve fitting and half-maximally cytotoxic concentrations were calculated (Table 1). With some of the PAs, a substantial cytotoxicity was measured. It was found that the cytotoxicity strongly

Table 1 Cytotoxicity and micronuclei formation in HepG2-CYP3A4 cells treated with selected PA: Half-maximally effective concentrations (EC50s) of cytotoxicity in HepG2-CYP3A4 cells treated over 48 h with selected pyrrolizidine alkaloids (PAs); Upper Bound and Lower Bound levels of concentrations causing a doubling of micronuclei counts were calculated using PROAST/EFSA software for Benchmark-Dose calculation

PA (structural features)	Cytotoxicity HepG2-CYP3A4 EC50 (µM)	Cytotoxicity PH EC50 (µM), 48 h, treated 3 h after seeding	Benchmark concentration (µM) of doubling of micronuclei counts in HepG2-CYP3A4 cells (Lower Bound)	Benchmark concentration (µM) of doubling of micronuclei counts in HepG2-CYP3A4 cells (Upper Bound)	iREP
Lasiocarpine (open, di, *7S*)	10 ± 1	4 ± 1	0.01	0.49	1.0
Monocrotaline (cyclic, di,*7R*)	> 300	> 300	23.7	153	1.0
Retrorsine (cyclic, di, *7R*)	73 ± 12	19 ± 2	1.26	1.90	1.0
Riddelliine (cyclic, di, *7R*)	97 ± 13	8 ± 1	1.29	2.29	1.0
Senecionine (cyclic, di, *7R*)	67 ± 8	8 ± 1	0.05	0.24	1.0
Seneciphylline (cyclic, di,*7R*)	73 ± 7	19 ± 6	0.66	1.34	1.0
Europine (mono, *7S*)	> 300	> 300	34.1	45.5	0.3
Heliotrine (mono, *7S*)	176 ± 31	193 ± 17	4.42	10.4	0.3
Echimidine (open, di, *7R*)	179 ± 17	25 ± 1	7.85	17.3	0.1
Indicine (mono, *7R*)	> 300	210 ± 16	34.2	76.3	0.01
Lycopsamine (mono, *7R*)	> 300	114 ± 18	59.5	73.3	0.01

Cytotoxicity data for PA (except riddelliine) in rat hepatocytes (PH) in primary culture were taken from Gao et al. (2019) and are shown for comparison; Cytotoxicity data show means ± S.D. from n=3 independent experiments; iREP factors are taken from Merz and Schrenk (2016). PAs are grouped according to iREP classes, in alphabetic order

depended on the individual PA congener. With some congeners, major cytotoxicity was found in the lower micromolar range, while others did not reach EC_{50} concentrations even when over 300 µM was added, a finding also seen in rat hepatocytes in primary culture (Table 1). In the non-transfected HepG2 cells tested, none of the PAs exhibited significant cytotoxicity up to a concentration of 300 µM (data not shown). When compared to previously published EC_{50} values of cytotoxicity in PH (Gao et al. 2020), HepG2 C9 cells turned out to be less sensitive towards most congeners tested, while heliotrine was about equally cytotoxic in both cell types. Congeners with low ($EC_{50} \geq 300$ µM) or relatively low (≥ 100 µM) cytotoxicity in PH were also weakly cytotoxic in HepG2 C9 cells. A comparison with iREP factors revealed a marked overestimation of the cytotoxicity of monocrotaline within the most toxic group assigned with an iREP factor of 1.0, and a marked overestimation of the cytotoxicity of europine (iREP factor 0.3).

Micronuclei formation

The same concentrations tested were also used for the micronuclei assay in HepG2 C9 (CYP3A4) cells. All eleven PAs induced a significant increase in micronuclei counts, with a very different potency, however. With all potent PA congeners, the amount of micronuclei detected increased with increasing concentrations; while at relatively high concentrations, the effect became smaller. This finding is in accordance with the assumption that severe cytotoxicity attenuates micronuclei formation in HepG2 cell cultures exposed to genotoxic PAs. This observation is obviously the basis for the recommendation in the OECD guideline on micronuclei testing (OECD 2016). Following this recommendation, micronuclei counts at concentrations leading to a loss of viability exceeding 50% were, thus, not considered for further modeling. With senecionine (Fig. 1a), micronuclei counts increased at concentrations in the range of 0.1 µM, reached a maximum at about 2.5–5 µM, and declined at 25 µM. In comparison, lycopsamine led to an increase in micronuclei counts at concentrations of 75 µM, rising further at 150 and 300 µM (Fig. 1b).

Modeling: relative genotoxic potencies

For a calculation of relative genotoxic potencies, we used this kind of data for all PAs tested and modeled them according to the benchmark calculation mode recommended by RIVM and EFSA. This software (PROAST) calculates the best fit of six different algorithms widely used in dose–response modeling. The best four out of these fits are analyzed further and weighted according to their quality of fitting. Figures 2a, b, show examples of the four best fittings obtained for lycopsamine and senecionine. Next, the benchmark concentrations (BMC) leading to a doubling of micronuclei counts over background and their upper and lower 10% confidence limits were calculated. These are given for each congener in Table 1 showing that all PAs tested induced micronuclei with marked differences in the BMCs, their Lower Bounds spanning over more than three, their Upper Bounds over more than two orders of magnitude. The most potent genotoxicants were lasiocarpine and senecionine with all other congeners attributed with an iREP factor of 1.0, except monocrotaline, within the range of 0.01–1.29 µM (LB) and 0.24–2.29 µM (UB). The two *7S*-monoesters europine and heliotrine differed remarkably in their potencies, heliotrine being four- (UB) to eightfold (LB) more potent than europine. Europine was much less potent than an iREP factor of 0.3 would suggest, while the potency of echimidine (iREP factor 0.1) was one order of magnitude lower than for the 'highly toxic' congeners. The *7R*-monoesters were the least genotoxic with BMCs between 34.2 and 59.5 (LB) and 73.3 and 76.3 µM (UB) indicating that an iREP factor of 0.01 is also adequate in this test system.

Finally, we wanted to obtain more information on the shape of the concentration–response relationship at the low-effect concentrations. For this purpose, we repeated the micronuclei assays for three selected PAs at these additional concentrations ranges (lasiocarpine: 0.05 and 0.25 µM, retrorsine: 0.01, 0.025 and 0.05 µM, senecionine: 0.01 µM). For retrorsine (Fig. 3a), the fine-tuning for the range of lower concentrations revealed a practical threshold in the range below 1 µM ($\log = 0$) showing no concentration-dependent increase in micronuclei counts. The picture for lasiocarpine (Fig. 3b) was different, i.e., no indication for a practical threshold at a concentration of 0.01 µM was seen. With senecionine (Fig. 3c) concentrations below 0.03 µM exhibited an apparent no-effect range, i.e., no concentration-dependent genotoxicity could be measured. The LB vs. UB intervals (retrorsine: 0.98–1.96 µM; lasiocarpine: 0.01–0.25 µM; senecionine: 0.05–0.2 µM) were quite similar to the ones determined at the higher concentration ranges (Table 1).

Discussion

Several 1,2-unsaturated PAs have been shown to exert pronounced liver toxicity in humans and animals and cyto- and genotoxicity in mammalian cell culture. It is widely accepted that the cytotoxic effects of PAs are mainly due to the formation of reactive intermediates of the dehydro-retronecine type (Mattocks and White 1971). These exert electrophilic properties and can bind covalently to cellular nucleophilic targets such as glutathione, proteins and nucleic acids (Fu et al. 2004). When structurally different congeners were tested in vitro, a structure–activity

relationship was observed with marked differences in the cytotoxic potencies between monoesters on the one hand and certain open-chained and cyclic diesters on the other hand (reviewed in Merz and Schrenk 2016). Such data were obtained, e.g., in bovine kidney epithelial cells (Kim et al. 1993) or HepG2 or HepG2/C3A hepatoblastoma cells (Li et al. 2013; Tamta et al. 2012). In primary cultures of rat hepatocytes, Green et al. (1981) found a marked cytotoxicity with senecionine. Field et al. (2015) used both the LDH and the MTT assays for measuring cytotoxicity of PAs in a chicken hepatocarcinoma cell line in the potency rank order (LDH- or MTT-assay) of lasiocarpine > seneciphylline > senecionine > heliotrine > riddelliine > monocrotaline > riddelliine-N-oxide > intermedine > lycopsamine ≈ lasiocarpine-*N*-oxide ≈ senecionine-*N*-oxide. A combined approach, suggesting iREP factors based on a limited set of date, led to the publication of interim REP factors in an attempt to quantify the relative toxic potency of PA congeners (Merz and Schrenk 2016). In general, this concept was supported by in vitro findings on PA-dependent micronuclei formation (Allemang et al. 2018), DNA adduct formation (Lester et al. 2019) and, genotoxicity-related γH2AX histone phosphorylation (Louisse et al. 2019). Recently, we could identify the cytotoxicity of ten PA congeners belonging to different structural classes in rat hepatocytes in primary culture (Gao et al. 2020). It was found that lasiocarpine and all cyclic diesters tested except monocrotaline, exerted a high cytotoxicity with EC_{50} levels between 4 and 19 µM. Furthermore, the group of monoesters showed a relatively low cytotoxicity with EC_{50} levels above 100 µM, while the open diester (*7R*) echimidine exerted a slightly lower cytotoxicity than the cyclic diesters. When these findings are compared with the data presented here on HepG2 C9 (CYP3A4) cells, it is obvious that the latter are less sensitive than rat hepatocytes towards lasiocarpine and most cyclic diesters. Again, monocrotaline was much less cytotoxic than expected from its structural classification as a cyclic diester. All other open-chained congeners were less cytotoxic with EC_{50} values above 170–300 µM. It remains to be elucidated if the lower sensitivity of human HepG2 C9 (CYP3A4) cells is due to species differences, e.g., in the pattern of metabolism including activation and detoxification pathways. Recently, no marked differences were found when human and rat liver microsomes were used to generate GSH adducts with a number of potent PAs (Geburek et al. 2020). It was shown by Ruan et al. (2014) that a variety of CYP enzymes can activate PAs in human liver microsomes to a variable extent. Thus, it cannot be excluded that the lack of certain CYP enzymes such as CYP2E1 or CYP2B subtypes not expressed in HepG2 C9 cells to a relevant degree may modify the outcome in our system. It needs to be noted, however, that permanent cell lines will always differ to a certain extent from human hepatocytes with respect to CYP expression. Furthermore, these activities are subject to considerable interindividual variability in human hepatocyte or liver microsome preparations as well (Hallifax and Houston 2009).

The possibility was also investigated that a lower level of CYP3A4 expression in HepG2 (CYP3A4) can explain the lower sensitivity in this cell line. BROD activity in HepG2 (CYP3A4) cells was in fact markedly lower than in freshly isolated rat hepatocytes but similar to rat hepatocytes after 24 h in culture. Thus, it appears unlikely that differences in CYP3A4 activity are the only explanation for the higher sensitivity in rat hepatocytes taking into account that an incubation time of 48 h was used in both cell types. Possibly, differences in transmembrane transporters playing a crucial role in the cellular disposition of at least certain PAs (Tu et al. 2013, 2014) may also be relevant.

In vitro genotoxicity of PAs was demonstrated by a number of studies to depend strongly on the congeners tested suggesting a structure–potency relationship. Xia et al. (2008) incubated PAs with rat liver microsomes in the presence of calf thymus DNA and found the following rank order of DNA adduct formation: retrorsine > retrorsine-*N*-oxide > heliotrine. Wang et al. (2005) could find identical DNA adducts formed in in vitro incubations of retrorsine with liver microsomes and in the liver of retrorsine-treated rats. Other PAs shown for their capacity to exert DNA binding in vivo were senecionine, seneciphylline (Eastman et al. 1982), retrorsine, lasiocarpine, and lycopsamine (Xia et al. 2013). Although 1,2-unsaturated PAs are widely accepted as being genotoxic in mammalian test systems, the outcome of bacterial mutagenicity tests, in particular the Ames test, is equivocal (reviewed in Merz and Schrenk 2016). A number of authors reported very minor or no mutagenic effects in bacteria treated with various PA congeners which are in agreement with our findings. Any mutagenicity of PAs in bacteria is likely to depend on external metabolic activation by S9-mix. Active metabolites then need to enter the bacterial cell to exert mutagenicity. It is unclear if the reactivity, i.e., the chemical half-life of these metabolites allows a mutagenic effect keeping in mind that active uptake mechanisms seem to be important for PA toxicity in mammalian cells (Tu et al. 2013, 2014). It remains questionable if similar or homologous transporters exist in *Salmonella*. The fact that several PAs exerted bactericidal activity in the presence of S9-mix may indicate a possible attack of reactive metabolites at the outer bacterial wall and/or membrane resulting in cell death.

With all PAs tested, a positive response was obtained in the micronucleus assay, although substantial differences in relative potencies were obtained. It was evident that micronuclei counts increased at much lower concentrations that cytotoxicity. Both outcomes although probably depending

on an electrophilic attack via the same/similar reactive intermediate/s differed in their concentrations–response characteristics. This finding is not surprising keeping in mind the different types of targets and the different assays. Similar findings were reported by Allemang et al. (2018) who found significant increases in micronuclei counts in HepaRG cells at PA concentrations which did not affect relative cell survival. Higher PA concentrations were found to lead to less pronounced increases in micronuclei. Similar results were reported in other micronuclei studies (Dorn et al. 2008, Schuler et al. 2010) and are probably due to marked cytotoxicity.

Numerous PAs turned out to be potent mutagens in *Drosophila*, bacteria and mammalian cells in culture. In Drosophila, Clark et al. (1960) identified heliotrine and lasiocarpine as 'potent', and senecionine, echimidine and echinatine as 'moderate' mutagens. *N*-oxides exerted attenuated effects in comparison to the 'parent' congener PAs. Later, Cook and Holt (1966) using the same assay, classified retrorsine as 'moderate', while retrorsine-*N*-oxide was markedly less active. Frei et al. (1992) investigated the genotoxic potency of 16 PAs using the 'wing spot test' in *Drosophila*. The relative potencies in percent were: Senkirkine 100, monocrotaline 90.0, seneciphylline 54.5, senecionine 39.1, heliotrine 13.4, retrorsine 8.3, symphytine 3.8, intermedine 0.49, indicine 0.27, and lycopsamine 0.19. With the exception of heliotrine (*7S*-configuration of the necine base), this test system confirmed a rank order of genotoxicity of cyclic diesters > open-chained diesters > monoesters.

Genotoxic effects reported for 1,2-unsaturated PAs comprise increased UDS formation in cultured rat hepatocytes with lasiocarpine and monocrotaline (Williams et al. 1980), in rat, mouse and hamster hepatocytes with monocrotaline, petasitenine, senkirkine, senecionine, and seneciphylline (Mori et al. 1985), and in rat and human hepatocytes with senkirkine (Schehrer et al. 2000). Chromosomal alterations including induction of micronuclei, chromosomal aberrations and sister chromatid exchange were also reported for a number of PAs. Retrorsine led to the formation of micronuclei in human lymphocytes incubated with rat liver S9-mix and in the human hepatoma cell line HepG2 (Kevekordes et al. 2001). In rat bone marrow (Proudlock et al. 1997) and in mouse bone marrow and fetal liver (Sanderson and Clark 1993), monocrotaline led to a significant increase in micronuclei, while a study with riddelliine in mice was negative (Mirsalis et al. 1993). In vitro studies in rat hepatocyte culture revealed enhanced micronuclei formation with retrorsine and monocrotaline with active concentrations of 1–10 µM for retrorsine and 3–30 µM for monocrotaline (Müller-Tegethoff et al. 1997). In a recent study by Allemang et al. (2018), eleven PA congeners and four PA *N*-oxides were analyzed for their potency to induce micronuclei in human HepaRG cells. Eight congeners were identical to those tested in the present study, i.e., echimidine, europine, heliotrine, indicine, lasiocarpine, lycopsamine, monocrotaline, and retrorsine. The authors treated HepaRG cells over 24 h with increasing concentrations of PAs and analyzed micronuclei levels using flow cytometry. In the present study, HepG2 C9 cells were treated over 72 h and micronuclei were counted via microscopical inspection. The latter method allows the unequivocal identification of micronuclei resulting in very low counts in untreated cells. Interestingly, the data reported by Allemang et al. are in excellent agreement with our findings for the eight congeners analyzed in both studies. For most congeners, however, the ranges (Upper bounds–Lower bounds) were somewhat lower in our study. This may be due to the longer incubation period, the lower background counts and a possible higher sensitivity of HepG2 C9 cells. In agreement with our findings, monocrotaline was much less potent than a RPF of 1.0 would indicate, i.e., in in vitro studies on DNA adduct formation (Lester et al. 2019) or gentotoxicity (Allemang et al. 2018, Louisse et al. 2019). Furthermore, europine seems to be over-estimated by the iREP factor of 0.3, at least when in vitro findings from our study and studies by others (Allemang et al. 2018, Lester et al. 2019, Louisse et al. 2019) are considered.

To obtain more detailed information on the shape of the concentration–response curves of genotoxicity at low concentrations, we fine-tuned the concentration ranges for retrorsine, lasiocarpine, and senecionine. It could be shown that the concentration–response curves for retrorsine and senecionine are hypolinear exerting a practical no-effect threshold. In this range below 0.03 (senecionine) or 1.0 µM, no increase in micronuclei counts was found. These findings indicate that a linear extrapolation of the rates of genotoxicity events from high to low dose, and probably also for suspected cancer incidences over-estimates the risk. It was interesting to find that for the most toxic congener, lasiocarpine, this assumption could not be made supporting the conclusion that considerable qualitative and quantitative differences exist between individual congeners.

Lester et al. (2019) measured PA kinetics and DNA adduct formation in rat sandwich culture hepatocytes. Linearity of the dose response of DNA adduct formation was examined for a few PAs at higher concentrations than used in the micronuclei experiments here, but comparison of the different events across dose and time seems to be pointing to the importance of cellular defence mechanisms like DNA repair at very low concentrations.

Taken together, our findings clearly demonstrate that all eleven 1,2-unsaturated PAs are genotoxic in a metabolically competent human cell model. The lack of mutagenicity in *Salmonella typhimurium* strains seems to be due to certain restrictions of the method such as the need for metabolic activation outside the bacterial cell. The potency for micronuclei induction as a hallmark of genotoxicity in

mammalian cells clearly depended on the type of PA. The open *7S*-configurated diester lasiocarpine and all cyclic diesters except monocrotaline were very potent genotoxicants, lasiocarpine being effective at very low concentrations, i.e., at 0.01–0.49 µM. Thus, a common iREP factor of 1.0 for these congeners can be confirmed. The much lower geno- and cytotoxicity of monocrotaline may be due to special properties in the uptake (Chen et al. 2019) and metabolism of this congener. The fact that monocrotaline also exerted a low cytotoxicity in rat PH suggests that the lack of CYP2B enzymes in HepG2 C9 cells is unlikely, however, to explain the low toxicity in these cells since rat PH express CYP2B. Furthermore, the very limited database for the derivation of iREP factors may explain why a factor of 1.0 for monocrotaline is not confirmed in more refined experimental models. Obviously, the relative toxicity of monocrotaline is markedly over-estimated by this factor. Another congener probably being over-estimated in the iREP concept according to our results is europine. For echimidine and heliotrine an intermediate potency seems to be warranted while the *7R*-configurated monoesters were confirmed to be of low cyto- and genotoxicity. Our findings suggest that additional studies are needed to refine the concept of REP factors but confirm the notion that the assumption of equal genotoxicity of PAs, a hallmark of the carcinogenic process, cannot be supported from a scientific perspective and is a pronounced overestimation of the risk. It is, thus, recommended to establish a more robust system of relative potencies defining, e.g., groups of congeners with high, intermediate and low genotoxic potency.

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Compliance with ethical standards

Conflict of interest The authors have no financial relationship with the organizations that sponsored the research. The authors declare that they have no conflict of interest.

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4.3 Publication III

Metabolic pattern of hepatotoxic pyrrolizidine alkaloids in liver cells

Reference:

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*contributed equally to this work

Contributions to this publication:

It has to be mentioned that this work is a cooperation between the University of Kaiserslautern and the German Federal Institute for Risk Assessment Berlin. The cell culture experiments including incubations and cell harvesting were performed at the University of Kaiserslautern. The following corresponding analytics were performed at the German Federal Institute for Risk Assessment. Following data analysis and interpretation of all results were performed by D. Schrenk, A. These, I. Geburek and me. J.-H. Küpper provided HepG2 C9 cells. K.-H. Merz, J. Keller, K. Paulus and C. Schabelon isolated and characterized the test substance monocrotaline. J. Leidner isolated the primary rat hepatocytes for our experiments.

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Article

Metabolic Pattern of Hepatotoxic Pyrrolizidine Alkaloids in Liver Cells

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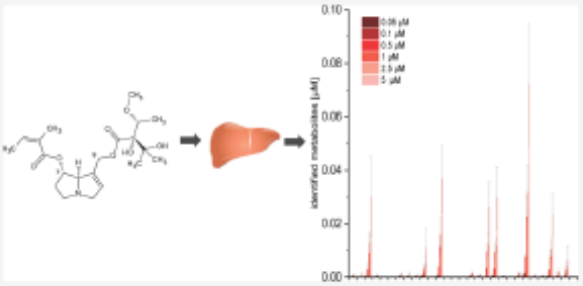
ABSTRACT: Contamination with 1,2-unsaturated pyrrolizidine alkaloids (PAs) is a serious problem for certain phytomedicines, foods, and animal feeds. Several of these PAs are genotoxic and carcinogenic, primarily in the liver, upon cytochrome P450 (CYP)-catalyzed activation into reactive (pyrrolic and pyrrole-like) metabolites. Here we investigated the metabolism of selected PAs (echimidine, europine, lasiocarpine, lycopsamine, retrorsine, and senecionine) in rat hepatocytes in primary culture and in human CYP3A4-transfected HepG2 cells. The open-chained diesters echimidine and lasiocarpine and the cyclic diester senecionine were extensively metabolized in rat hepatocytes into a broad spectrum of products released into the medium. A large portion of unidentified, possibly irreversibly bound, products remained in the cells while detectable amounts of reactive and other metabolites were found in the incubation media. In HepG2-CYP3A4 cells, lasiocarpine was more extensively metabolized than echimidine and senecionine which also gave rise to the release of pyrrolic metabolites. In human cells, no pyrrolic metabolites were detected in retrorsine or lycopsamine incubations, while no such metabolites were detected from europine in both cell types. Other types of metabolic changes comprised modifications such as side chain demethylation or oxygenation reactions like the formation of *N*-oxides. The latter, considered as a detoxification step, was a major pathway with cyclic diesters, was less distinctive for echimidine and lycopsamine and almost negligible for lasiocarpine and europine. Our data are in agreement with previously published cyto- and genotoxicity findings and suggests that the metabolic pattern may contribute substantially to the specific toxic potency of a certain congener. In addition, marked differences were found for certain congeners between rat hepatocytes and transfected human HepG2 cells, whereby a high level of bioactivation was found for lasiocarpine, whereas a very low level of bioactivation was observed for monoesters, in particular in human cells.



1. INTRODUCTION

Pyrrolizidine alkaloids (PA) are secondary plant metabolites mainly formed by the plant families *Boraginaceae*, *Asteraceae*, and *Fabaceae* and can be found worldwide in about 3% of all flowering plants.[1,2] Due to the high biodiversity, hundreds of different PAs have been documented so far while all PAs considered in this study contain a 1,2-unsaturated necine base.[3] PAs can further be grouped into monoesters, open-chained diesters, and cyclic diesters based on their type of esterification.[1,3,4]

Several PAs with a 1,2-unsaturated necine base are hepatotoxic and can cause hepatic veno-occlusive disease (HVOD) in humans and animals after acute exposure[5−7] and hemangiosarcoma and hepatocarcinoma in rodents after chronic exposure.[8−10] PA exposure in humans is primarily caused by the consumption of phytomedicine and medicinal tea[11] and foods of herbal origin or honey that are contaminated with PA-producing plants.[12−16] Because some PAs are carcinogenic in experimental animals and show genotoxic effects,[9,17] they were classified by the International Agency for Research on Cancer (IARC) as possibly carcinogenic to humans in category 2B.[18]

To exert their toxicity, PAs have to be bioactivated by xenobiotic-metabolizing enzymes like cytochrome P450s (CYPs).[19] This oxidation reaction results in the formation of reactive metabolites, which possess a further double bond in the necine base (pyrrolic metabolites) or metabolites that are carbon-oxygenated in the necine base (pyrrole-like metabolites). As a result, DNA and protein adducts and glutathione (GSH) conjugates can be formed.[20−24]

The transformation into reactive metabolites seems to be possible for all 1,2-unsaturated PAs. However, the extent of metabolic activation and the toxicokinetics of the individual compounds may differ depending on their respective structures,[25−28] probably having a marked influence on the toxic potency of different 1,2-unsaturated PAs in vivo. In

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addition to causing damage in hepatocytes reactive metabolites including GSH conjugates[29] may be released from the hepatocytes and may thus damage other typical target cells of PA toxicity, i.e., endothelial cells in liver sinusoids,[30] lung,[31] or kidney.[32]

There are several studies dealing with the different toxic potency of PAs. Merz and Schrenk[33] proposed interim relative potency factors (iREPs) for 1,2-unsaturated PAs, based on LD_{50} values in rodents and data on genotoxicity in Drosophila as well as from cytotoxicity tests in vitro. Meanwhile, several in vitro cell studies measuring cytotoxicity,[34] metabolism, DNA binding, and genotoxic effects of PAs in hepatocytes and hepatoma cell lines have further contributed to our view on the toxic potency of 1,2-unsaturated PAs.[26,35−38]

There are only a few studies analyzing the metabolic pattern of PAs in a comprehensive manner, e.g., in human liver microsomes with retrorsine,[39] in liver microsomes from various species with lasiocarpine,[40] or in rats with adonifoline[41] or senecionine.[42] Recently, we investigated the metabolism of six congeners (echimidine, Ech; europine, Eur; lasiocarpine, Las; lycopsamine, Lyc; senecionine, Sen; and retrorsine, Ret) in human and rat liver microsomes and characterized in total more than 150 different metabolites.[43] Here, we applied a similar approach to cultured rat hepatocytes and human HepG2 hepatoblastoma cells overexpressing CYP3A4, a key enzyme in PA metabolism. The studies are aimed at further elucidating the PA metabolism in the parenchymal liver cell as a major target cell type, and the role of liver cells in releasing (secondary) metabolites which may damage other target cells.

2. EXPERIMENTAL SECTION

2.1. Chemicals and Media. Bovine serum albumin was purchased from PAA Laboratories (Pasching, Austria), Dulbecco's Modified Eagle's-Medium-High Glucose (DMEM-HG), -Low Glucose (DMEM-LG), Pen/Strep solution, fetal calf serum (FCS), and Percoll were obtained from Life Technologies (Paisley, U.K.); blasticidin S hydrochloride and heparin from Carl Roth (Karlsruhe, Germany); collagenase, Hank's Balanced Salt Solution (HBSS), insulin-transferrin-selenium (ITS), pentobarbital, and trypan blue solution from Sigma-Aldrich (Merck, Darmstadt, Germany); and TriFast from VWR (Radnor, U.S.A.). All chemicals were obtained of the highest purity commercially available. All PAs were purchased from Phytolab (Vestenbergreuth, Germany). In addition, all PA solutions were tested for impurities, i.e., for the presence of other PAs by LC−MS/MS analysis. A 250 ng/mL solution was tested and the absence of any PA contamination could be proven. On the basis of a limit of detection of about 0.5 ng/mL the purity was found to be >99.8%. HepG2-CYP3A4 cells (clone C9) overexpressing human CYP3A4 were genetically engineered and propagated as previously described.[44]

2.2. Animals, Hepatocyte Culture, and Treatment. Wistar rats (male, body weight of 200 ± 10 g) were obtained from Janvier (Le Genest-Saint-Isle, France). They were anesthetized with pentobarbital (100 mg/kg, i.p.) and livers were perfused using a two-step procedure according to Seglen[45] and modified by Schrenk.[46] Viabilities of hepatocyte preparations were >89%, determined by trypan blue exclusion. The number of nonparenchymal cells was estimated by microscopic inspection to be less than 5%. Isolated hepatocytes were seeded in rat tail collagen coated 24-well-plates (200.000 cells/well; 1 mL/well). DMEM-LG supplemented with 10% FCS and 1% Pen/Strep solution was used as medium. After seeding, the plates were left at 37 °C under an atmosphere of 5% CO_2 for 3 h to ensure that the cells can attach to the surface. Then, the medium was replaced by 1 mL/well incubation medium (DMEM-LG supplemented with 1% ITS and 1% Pen/Strep solution) and hepatocytes were incubated for 24 h with PAs dissolved in DMSO. Cells were incubated with various concentrations of Lyc (1, 5, 25, 75, 150, 300 μM), Eur (1, 5, 25, 75, 150, 300 μM), Ech (0.5, 1, 2.5, 5, 12.5, 25 μM), Las (0.05, 0.1, 0.5, 1, 2.5, 5 μM), Sen (0.05, 0.1, 0.5, 1, 2.5, 5 μM), and Ret (0.1, 0.5, 1, 2.5, 5, 25 μM). The PAs and the associated concentrations were selected based on previous cytotoxicity and genotoxicity data.[34,38] The following criteria had to be fulfilled: (1) the lowest concentration level should not cause detectable genotoxic or cytotoxic effects; (2) the viability in the highest concentration level should be at least 50%; and (3) the other concentrations should be between these benchmarks. Due to the different toxic potencies observed in vitro of the selected PAs, the chosen concentrations differ. Directly after addition of the PAs, an aliquot (100 μL) of the medium was taken from each well and frozen at −20 °C. This sample represents time point t0h. After 24 h of incubation, the second aliquot representing t24h was taken and frozen at −20 °C. After washing the cells with PBS, 500 μL TriFast was added to lyse the cells. These cell samples were also frozen at −20 °C until further processing. Three different controls were analyzed for each PA. The first control consisted of cells and the medium and was considered as the blank control. The second control contained the PA at the highest tested concentration and the incubation medium, but no cells. This control was included to check whether the PA is stable in the medium. An aliquot of the incubation medium of both controls was taken immediately after adding the PA and at the end of the incubation period after 24 h. After washing with PBS, the cell sample was also taken after 24 h and frozen. The third control was composed of cells, the PA in the highest concentration, and the incubation medium. Directly after adding the PA, the incubation medium was removed, the cells were washed with PBS and the cell sample was taken. Thus, this control represents the time point t0h for the cells and was included to check whether the PAs can be completely removed by washing the cells, so that only the PA and its metabolites within the cells are detected.

2.3. Cell Culture and Treatment. HepG2-CYP3A4 cells were seeded on 48-well-plates (65 000 cells per well). Medium (DMEM-HG supplemented with 10% FCS and 1% Pen/Strep solution) was replaced after 8 h by the incubation medium (DMEM-HG supplemented with 1% ITS and 1% Pen/Strep solution). After a further 16 h, the cultures were incubated with PA dissolved in DMSO as described above. The addition of 100 μL TriFast instead of 500 μL was the only modification. All the controls described above were also performed with HepG2-CYP3A4 cells.

2.4. Sample Preparation and Analysis by Liquid Chromatography and High Resolution Mass Spectrometry. All samples of incubation medium were analyzed directly and just diluted with 5% MeOH to fit the dynamic linear range of the MS. The lysed cell samples were centrifuged at 4 °C and 10 000*g* for 10 min and the supernatants were diluted 1:10 with 5% MeOH. All analyses were performed on an UltiMate 3000 (Thermo Fisher Scientific, Waltham, U.S.A.) Ultra High Performance Liquid Chromatography (UHPLC) system. For reversed phase (RP) chromatography, a C18 Hypersil gold column (150 × 2.1 mm^2; 1.9 μm particle size) with a guard column (Thermo Fisher Scientific, Waltham, U.S.A.), with a flow rate of 0.3 mL/min and a column temperature of 40 °C was used. The injection volume was 4 μL. The mobile phase consisted of water as mobile phase A and methanol as mobile phase B, both containing 0.1% formic acid and 5 mM ammonium formate. The gradient conditions were as follows: 0−0.5 min A: 95%/B: 5%, 7.0 min A: 50%/B: 50%, 7.5 min A: 20%/B: 80%, 7.6 min A: 0%/B: 100%, 10.1−15 min A: 95%/B: 5%. Detection was carried out on a Q Exactive Focus Orbitrap Mass Spectrometer (Thermo Fisher Scientific, Waltham, U.S.A.) operating in the positive ionization mode. Full scan data were acquired in a mass range of m/z 100−1500 with a resolution of 70 000. In order to obtain MS2 data for confirmatory purposes, simultaneously a fragmentation for selected mass range windows (here m/z: 100−505; 495−1005; 995−1500) was carried out and data were recorded with a resolution of 17 500. All samples were analyzed for PAs and their microsomal metabolites recently published.[43] The amount of degraded PA was determined in proportion to the concentration used. Due to the lack of reference substances for the metabolites, their concentrations were determined

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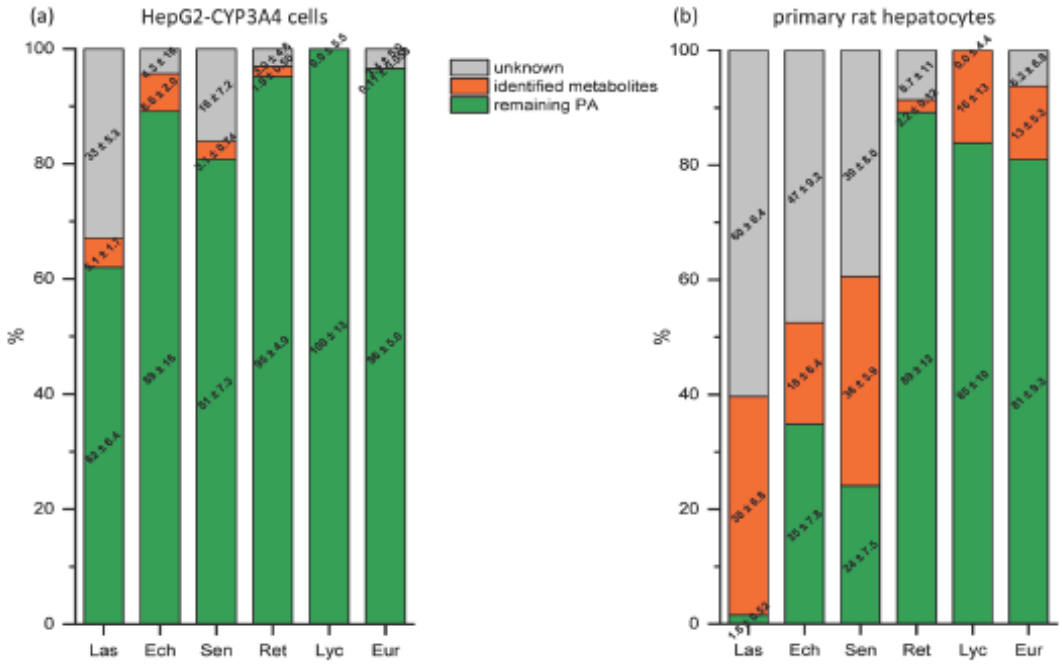

Figure 1. Percentage of nonmetabolized PA (green bar), the sum of detected known metabolites in the incubation medium (orange bar), and the "unknown" part (gray bar) after incubation of the respective PA (open chained diesters: lasiocarpine, Las; echimidine, Ech; cyclic diesters: senecionine, Sen; retrorsine, Ret; monoesters: lycopsamine, Lyc; and europine, Eur) for 24 h with (a) HepG2-CYP3A4 cells or (b) primary rat hepatocytes. The average percentages from $n = 6$ concentrations for each congener with the standard deviation are indicated.

semiquantitatively under the assumption that the metabolites show the same mass spectrometric response as the incubated PA.[43] An external 5-point matrix-matched calibration was used to determine the exact concentration at t0h for each replicate to eliminate possible deviations. The concentration of the metabolites was determined by comparing the concentration of the t0h-value and the t24h-value, which is equivalent to a 1-point calibration.

3. RESULTS

Primary rat hepatocytes (pRH) and the human HepG2 hepatoblastoma cell line overexpressing the human CYP3A4 gene were incubated with six different pyrrolizidine alkaloids (PAs) in six different concentrations for 24 h. An aliquot was taken from the incubation medium and from the cells at the start and at the end of the 24-h incubation period and analyzed by LC−MS/MS.

In a first step, we semiquantitatively determined the amounts of a variety of previously characterized metabolites[43] by assuming the same mass spectrometric response as that for the parent PA. Next, we summed up the amounts of all characterized metabolites and expressed them as a percentage of the amount of the parent compound initially added to the incubation. Since the percentage of characterized metabolites did not differ markedly between the concentrations investigated for each congener, it was possible to calculate the average yield of metabolites as a percentage of the amount of parent compound added. In all incubations a portion of the PA remained nonmetabolized and another portion could not be characterized either as the parent compound or as the known metabolite(s). Figure 1 shows these three categories, i.e., the sum of characterized metabolites, the remaining parent compound, and the "gap" between both and the initially added amount of PA. The latter portion may be considered as "unknowns".

In general, cell type-dependent differences were observed as all PAs were more extensively metabolized by pRH than by HepG2-CYP3A4 cells. In addition different metabolism rates were noticed between PAs, i.e., Las, Ech, and Sen were transformed to a higher degree than Ret and the monoesters Lyc and Eur (Figure 1). After 24 h of incubation with pRH, only about 2% of the initial Las and 35 and 24% of Ech and Sen, respectively, were recovered. In comparison, between 62% and 89% of the initial Las, Ech, and Sen and 95−100% of the other PAs were recovered unmetabolized in supernatants from HepG2-CYP3A4 cells. Proportions of detectable metabolites correlated with metabolism rates and the metabolite amounts in incubations with pRH were much higher than those in HepG2-CYP3A4 cells reaching up to 38% (Las, pRH). The gap between both, i.e., the "unknowns" was found to be the highest for Las in both cell experiments (60% pRH; 33% HepG2-CYP3A4) followed by Sen (39% pRH; 16% HepG2-CYP3A4) and Ech (47% pRH; 5% HepG2-CYP3A4), and was negligible for the monoesters Lyc and Eur (Figure 1).

In a second step, the detailed metabolite profile was investigated. A comprehensive identification of hepatic metabolites had previously been conducted for the same six PAs in both species using microsomal incubations.[43] A comparison of metabolite profiles between rat liver microsomes and primary rat hepatocytes is presented as Supporting Information (SI) for all PAs. The analyses of the cells showed either no or only traces of metabolites (data not shown). Therefore, only the results from the analysis of the medium (supernatants) were considered. The largest differences in metabolite profiles between liver cells in primary culture and microsomes were found for pyrrolic and pyrrole-like metabolites. These reactive metabolites are either formed by the hepatocytes in very low concentrations only or they are formed but their release into the medium is strongly reduced

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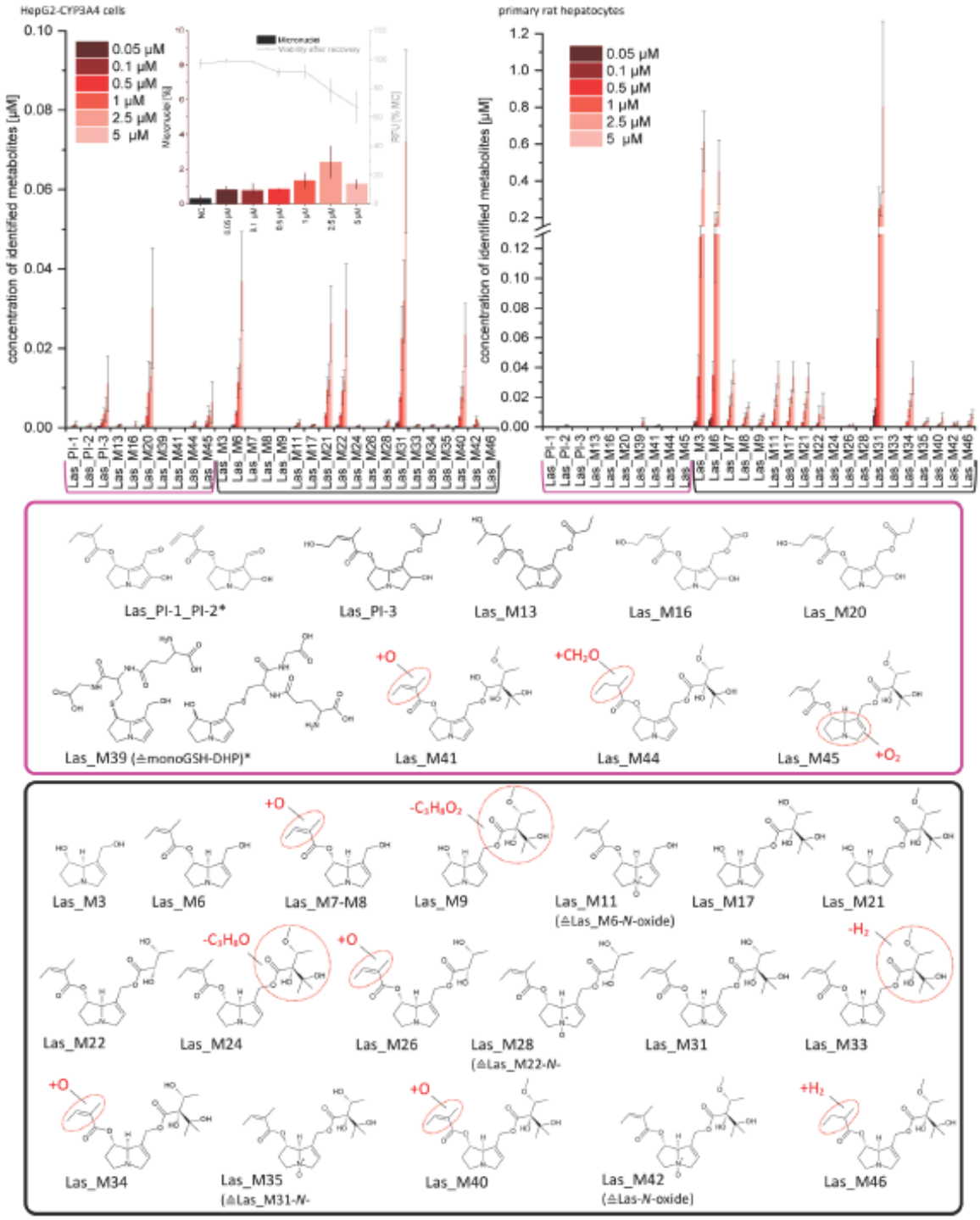

Figure 2. Amounts and structure proposals of detected metabolites in the incubation medium after incubation of various concentrations of lasiocarpine (Las) with primary rat hepatocytes or HepG2-CYP3A4 cells for 24 h. The metabolites are divided into two groups: (i) "pink framed" reactive metabolites bioactivated in the necine base moiety (dehydrogenated pyrrolic compounds or pyrrole-like metabolites carbon-oxygenated in the necine base) and (ii) "black framed" other metabolites (*N*-oxides and compounds that are modified in the necic acid moiety by, e.g., hydroxylation, epoxidation, or dealkylation). The coding of the metabolites was adopted from Geburek et al.[43] *two structure proposals possible.

(SI). Figures 2, 3, 4, 5, 6, and 7 show the amounts and structure proposals based on product ion spectra of all metabolites found in the samples of the incubation medium of HepG2-CYP3A4 cells (left) and pRH (right). The coding of the metabolites was adopted from Geburek et al.[43] Cell incubations were conducted as described by Rutz et al. and data previously obtained for micronucleus levels in HepG2-CYP3A4 cells are shown as inserts.[38]

According to the tentative structures, the metabolites were divided into two groups: (i) "pink framed" reactive metabolites

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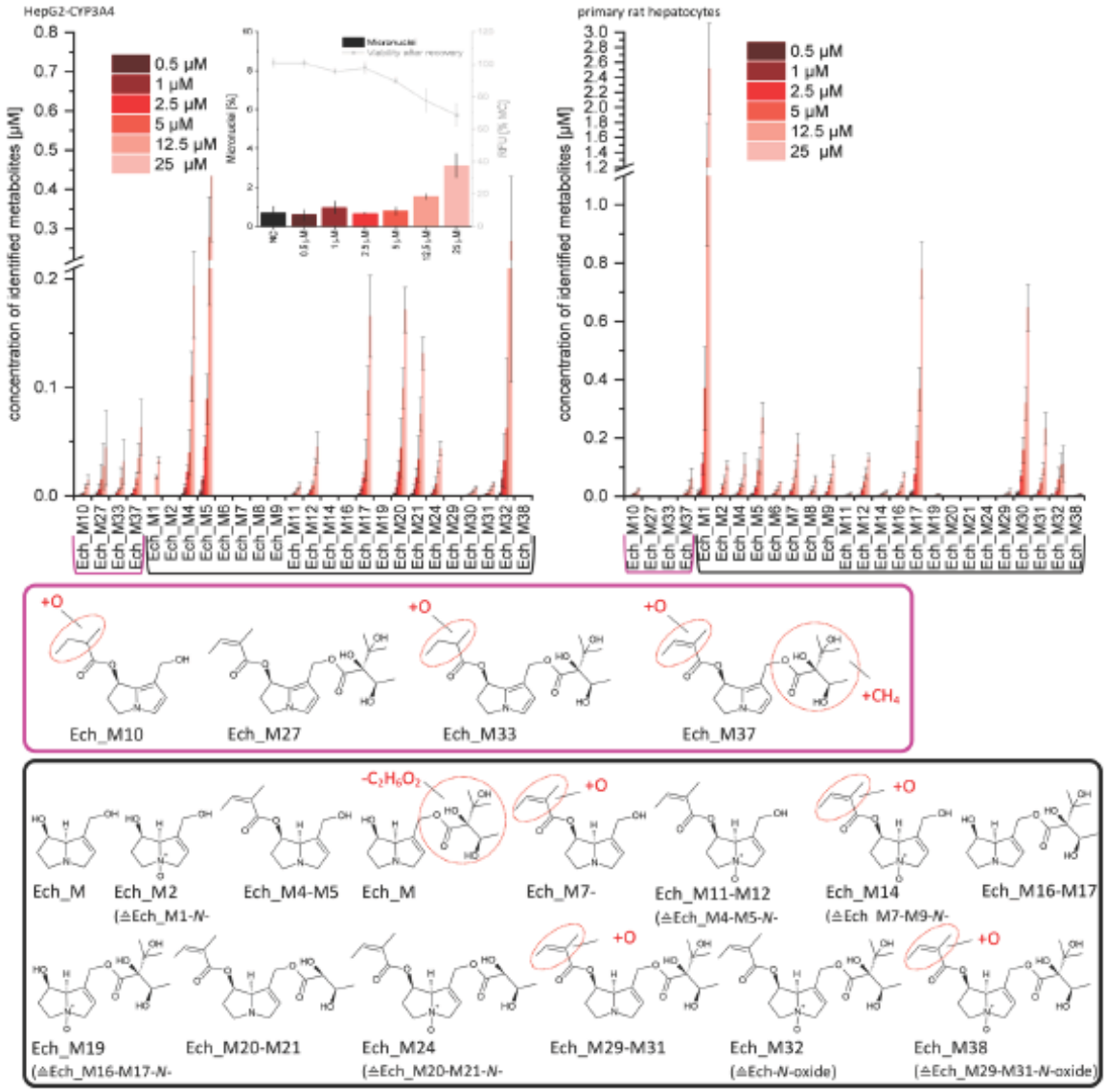

Figure 3. Amounts and structure proposals of detected metabolites in the incubation medium after incubation of various concentrations of echimidine (Ech) with primary rat hepatocytes or HepG2-CYP3A4 cells for 24 h. The metabolites are divided as described in Figure 2.

bioactivated in the necine base moiety (dehydrogenated pyrrolic compounds or pyrrole-like metabolites carbon-oxygenated in the necine base) and (ii) "black framed" other metabolites (*N*-oxides and compounds that are modified in the necic acid moiety by, e.g., hydroxylation, epoxidation, or dealkylation). While the formation of reactive metabolites is considered as toxification, the other transformation steps are considered as pathways leading to detoxification.

The amount of all detected metabolites showed a concentration-dependent increase, i.e., higher initial PA concentrations resulted in higher concentrations of metabolites. As Las resulted in the highest amount of micronuclei and was found to be the most toxic congener in several other studies, an iREP of 1.0 had been suggested (Table 1). In our study Las was the most extensively metabolized PA and the highest amount of reactive metabolites was determined (Figures 1 and 2; Table 1). On the basis of the comprehensive reference data set of metabolites characterized in liver microsomes,[43] eight reactive metabolites were detected in HepG2-CYP3A4 cells (Figure 2), of which three were present in the medium in considerable amounts (Las_PI-3; Las_M20; Las_M45). Las_PI-3 was only detectable in liver microsome incubations without added GSH[43] and is therefore considered to be an electrophilic phase I metabolite. These three pyrrole-like metabolites were formed by oxygenation of the necine base. In the mass spectrometer they were detected as the keto or enol form but it might be possible that these forms are due to gas phase reactions and the oxygen was initially bound as a hydroxyl group. In microsomal incubations these necine ring oxygenated metabolites were found with all four diesters,[43] but in the medium of HepG2-CYP3A4 cells and pRH only with Las and Sen (Sen_M16). In addition, three other metabolites bearing the same modification in the necine base were detected in much lower amounts (Las_PI-1; Las_PI-2; Las_M16). Furthermore, two metabolites that were dehydrogenated but not oxygenated at the necine base were found (Las_M13; Las_M44). In all of these necine base-oxygenated and pyrrolic metabolites, side-chain modifications had also occurred. In

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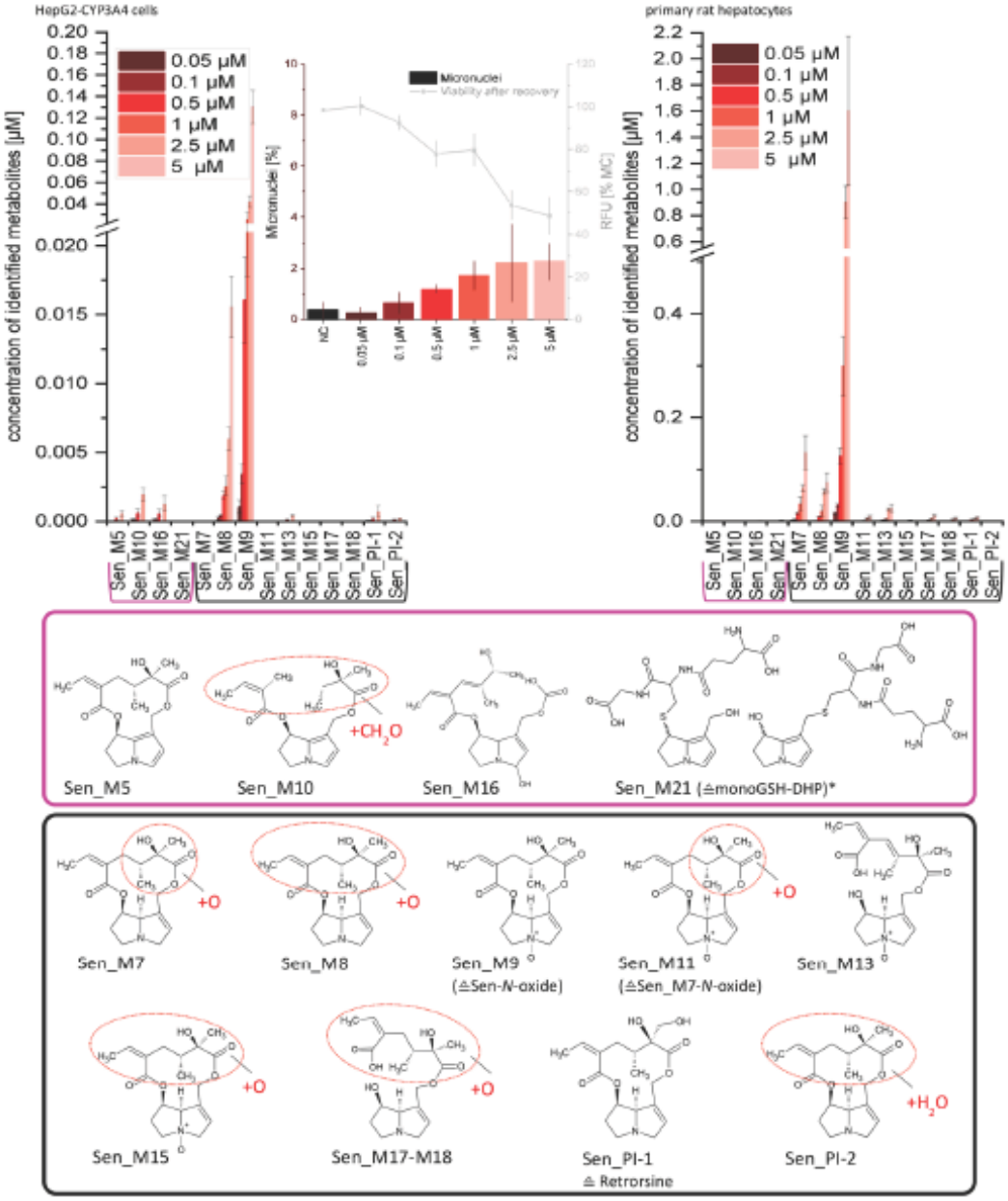

Figure 4. Amounts and structure proposals of detected metabolites in the incubation medium after incubation of various concentrations of senecionine (Sen) with primary rat hepatocytes or HepG2-CYP3A4 cells for 24 h. The metabolites are divided as described in Figure 2.

pRH incubated with Las, the mono-GSH-DHP conjugate (Las_M39) was the major metabolite detected with a dehydrogenated necine base, followed by very minor amounts of Las_M41 and Las_PI-2. GSH conjugates were not found in incubations of Las in HepG2-CYP3A4 cells.

With the other PAs (Figures 3–7), 1 necine base-oxygenated and 10 pyrrolic metabolites were detected. These comprise products with a dehydrogenated retronecine ring system or an additional oxygen atom at the necine base and are considered as having the potency to react as electrophiles with cellular constituents causing cell damage. They were either dehydrogenated at the necine base only, with intact side chain(s) (Ech_M27, Figure 3; Sen_M5, Figure 4; Lyc_M8, Figure 7), with modified side chain(s) (Ech_M10, Ech_M33, Ech_M37, Figure 3; Sen_M10, Sen_M16, Figure 4; Lyc_M21, Figure 7) or were dehydroretronecine conjugated with GSH (Sen_M21, Figure 4; Ret_M15, Figure 5). For further comparison, the total amount of reactive metabolites was calculated for each congener (Table 1), and was also expressed as a percentage of the total metabolites and as a percentage of the metabolized parent PA (sum of gray and orange bars in Figure 1). In pRH, the highest amounts of reactive metabolites from low substrate concentrations (≤25 µM) were found with Las and Ech in a concentration-dependent manner. With Lyc, reactive metabolites were also found, however, at much higher substrate concentrations. Ret and Sen led to detectable amounts of reactive metabolites at a single substrate concentration only, while Eur was completely negative in this respect. In HepG2-CYP3A4 cells, the percentage of extracellular reactive products was several-fold, in some cases over

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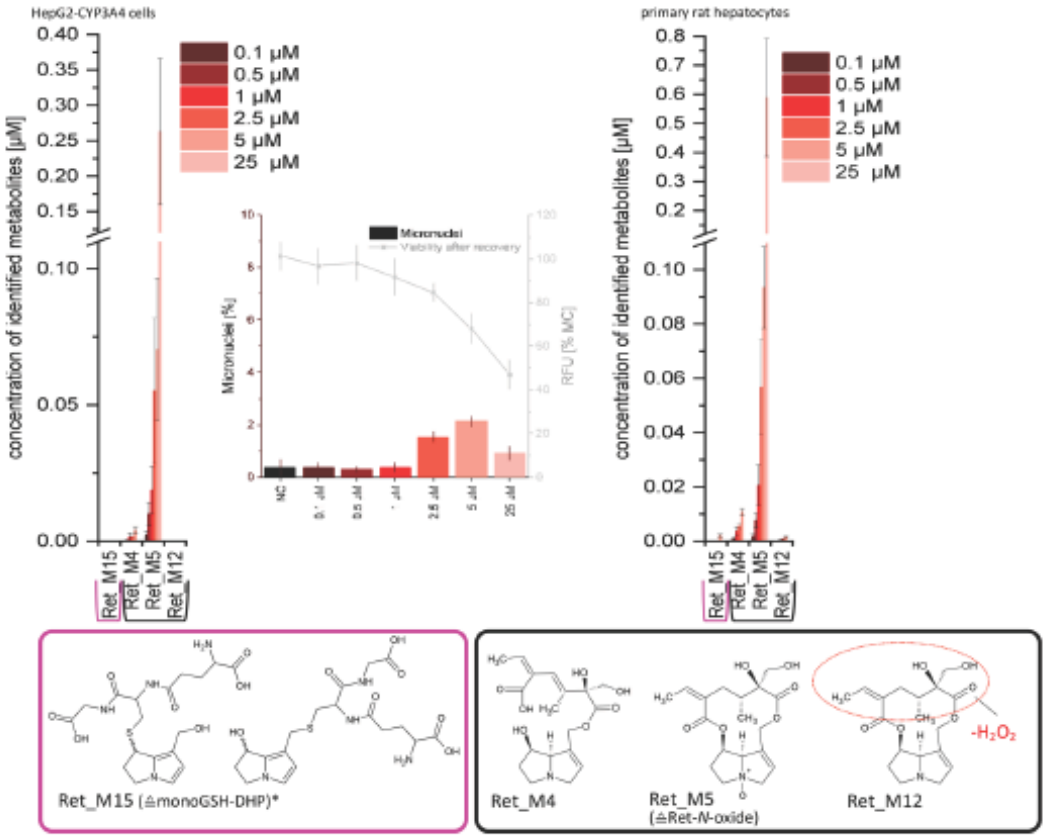

Figure 5. Amounts and structure proposals of detected metabolites in the incubation medium after incubation of various concentrations of retrorsine (Ret) with primary rat hepatocytes or HepG2-CYP3A4 cells for 24 h. The metabolites are divided as described in Figure 2.

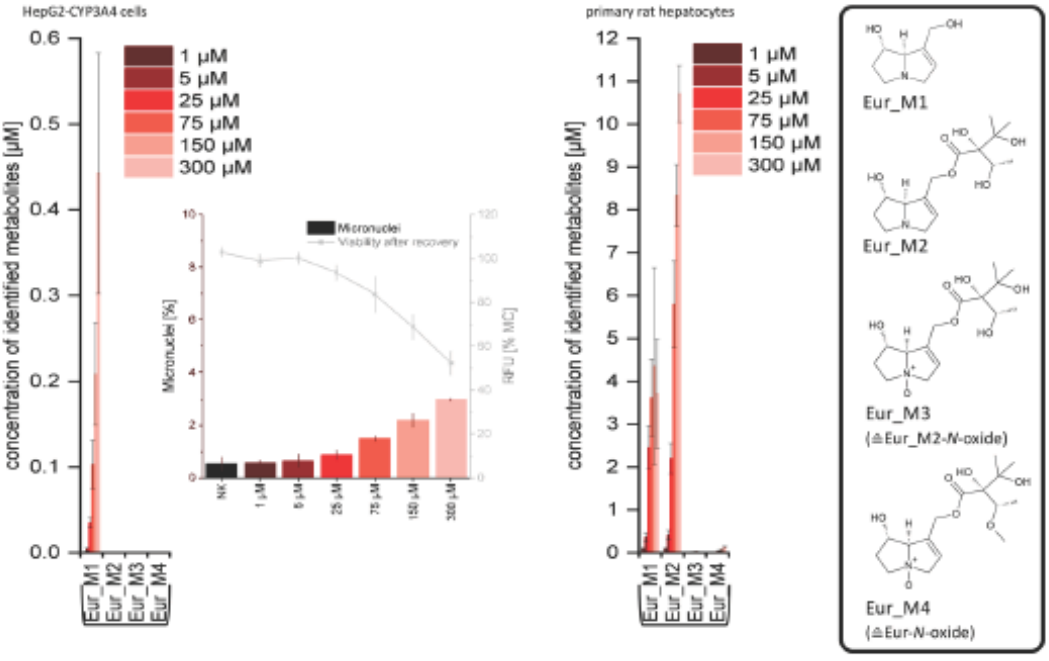

Figure 6. Amounts and structure proposals of detected metabolites in the incubation medium after incubation of various concentrations of europine (Eur) with primary rat hepatocytes or HepG2-CYP3A4 cells for 24 h. The metabolites are divided as described in Figure 2.

10-fold higher than in pRH, although the transformation rate was much lower. Within each cell model the release of reactive metabolites correlated with the overall metabolic transformation of the parent PA and consequently, there was no

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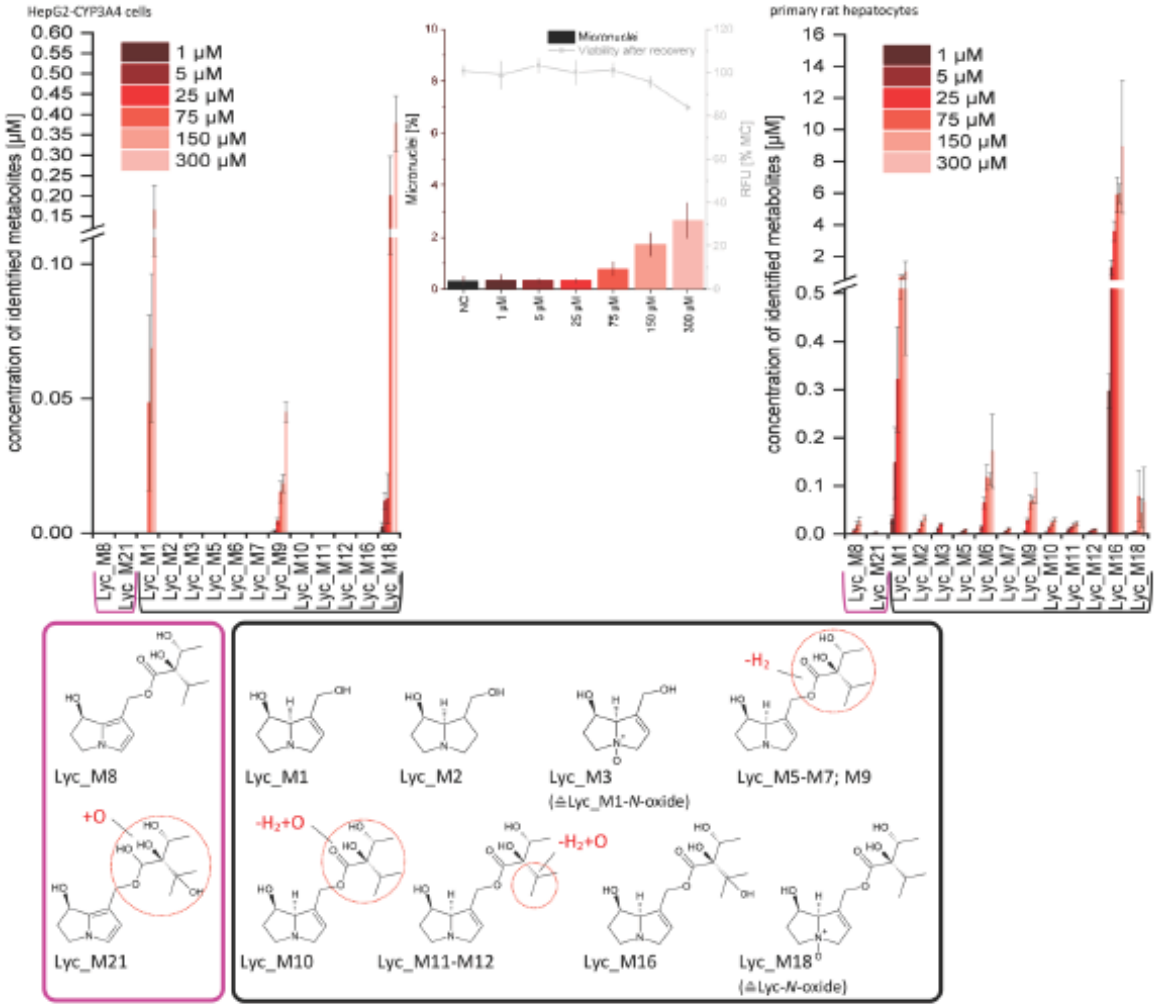

Figure 7. Amounts and structure proposals of detected metabolites in the incubation medium after incubation of various concentrations of lycopsamine (Lyc) with primary rat hepatocytes or HepG2-CYP3A4 cells for 24 h. The metabolites are divided as described in Figure 2.

indication for released reactive metabolites from Ret, Lyc, or Eur in HepG2-CYP3A4 cells. This finding coincided with a complete lack of GSH conjugates in the supernatants from this cell type. Interestingly, GSH conjugates were detected in very small amounts in incubation media with Las, Sen, or Ret with pRH only. For Ret (Figure 5), none of the dehydrogenation products was found in HepG2-CYP3A4 cells and only a single mono-GSH-DHP conjugate was detected in pRH.

The main metabolic modifications of the open-chained diesters Las and Ech lead to metabolites with shorter alkyl-chains in the necic acid moiety (about 20−25% of incubated PA). This type of metabolite was not found for the cyclic diesters, where *N*-oxidation represents the largest part of the metabolism. Five metabolites of Las and Ech were formed in both cell types by oxygenation reactions, but in rather varying amounts (Figures 2 and 3). While the total amount of oxygenation products of Las was about 0.03−0.05 μM, the pRH released about a 10-fold amount of these metabolites with the same initial concentration of Ech (compare Ech 5 μM, pRH). Micronuclei count data in HepG2-CYP3A4 treated with the PAs tested here were taken from a previous publication[38] and are shown here for comparison. As shown in the insets (Figures 2−7), the counts of micronuclei increased with increasing concentrations while the viability of the HepG2-CYP3A4 cells decreased. The number of micronuclei thus increased parallel to the amount of metabolites. According to the OECD guideline,[47] it was ensured that loss of viability did not exceed 50%. The rank order of potencies to induce micronuclei (Las ≈ Sen > Ret > Ech > Eur > Lyc)[38] was in relatively good agreement with the absolute amount of released reactive metabolites found. Although Ech led to higher levels of reactive metabolites in the medium than Ret (Table 1) the toxicities of both correlated to the percentage of "unknowns" (Figure 1).

4. DISCUSSION

After having investigated the metabolism of a number of PAs in liver microsomes from humans and rats,[43] we addressed in this study the PA metabolism in intact cells in culture. We carried out the experiments using rat hepatocytes in primary culture, representing a major target with respect to metabolic bioactivation of PA. As a model for metabolism in humans we used the human HepG2 hepatoblastoma cell line transfected with human CYP3A4 cDNA thus overexpressing one of the most important CYP enzymes in PA biotransformation.[22]

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Table 1. Levels of Reactive Metabolites Detected in Supernatants of Incubations of PAs (as indicated) at Various Concentrations with Primary Rat Hepatocytes or HepG2-CYP3A4 Cells over 24 h[a]

	primary rat hepatocytes			HepG2-CYP3A4				
parent PA (μM)	reactive metabolites (μM)	% of characterized metabolites	% of metabolized parent PA	reactive metabolites (μM)	% of characterized metabolites	% of metabolized parent PA	HepG2 micronucleus (%)[b]	iREP[c]
				Lasiocarpine				
0.05	0.0003 ± 0.0001	1.9 ± 0.8	0.6 ± 0.2	0.0007 ± 0.0004	24.5 ± 5.1	4.1 ± 2.2	0.80	1
0.1	0.0002 ± 0.0001	0.9 ± 0.3	0.2 ± 0.1	0.0005 ± 0.0002	15.4 ± 4.2	1.9 ± 1.3	0.97	1
0.5	0.0004 ± 0.0001	0.3 ± 0.1	0.1 ± 0.0	0.0064 ± 0.0028	22.9 ± 9.4	3.3 ± 1.9	0.83	1
1	0.0011 ± 0.0007	0.2 ± 0.1	0.1 ± 0.1	0.0155 ± 0.0085	17.6 ± 5.9	4.6 ± 3.8	1.57	1
2.5	0.0045 ± 0.0032	0.5 ± 0.5	0.2 ± 0.1	0.0249 ± 0.0029	23.2 ± 5.14	3.1 ± 1.3	2.43	1
5	0.0049 ± 0.0018	0.3 ± 0.2	0.1 ± 0.0	0.0412 ± 0.0153	17.4 ± 1.5	2.0 ± 0.7	1.13	1
				Echimidine				
0.5	0.0005 ± 0.0002	0.8 ± 0.1	0.1 ± 0.0	0.0011 ± 0.0002	7.3 ± 1.4	1.0 ± 0.1	0.59	0.1
1	0.0006 ± 0.0002	0.7 ± 0.1	0.1 ± 0.0	0.0048 ± 0.0028	6.6 ± 1.5	1.9 ± 0.6	0.96	0.1
2.5	0.0102 ± 0.0057	2.2 ± 0.9	0.5 ± 0.3	0.0158 ± 0.0042	8.8 ± 0.7	6.8 ± 4.0	0.66	0.1
5	0.0216 ± 0.0097	1.8 ± 0.4	0.6 ± 0.3	0.0390 ± 0.0196	11.2 ± 1.5	2.3 ± 1.3	0.79	0.1
12.5	0.0465 ± 0.0254	1.5 ± 0.6	0.5 ± 0.3	0.0865 ± 0.0139	8.5 ± 1.4	3.5 ± 0.6	1.53	0.1
25	0.0856 ± 0.0384	1.5 ± 0.6	0.6 ± 0.2	0.1538 ± 0.0288	9.4 ± 1.8	6.0 ± 2.3	3.12	0.1
				Senecionine				
0.05				0.0001 ± 0.0001	4.9 ± 1.6	1.4 ± 1.0	0.25	1
0.1				0.0002 ± 0.0001	5.1 ± 1.9	1.1 ± 0.5	0.65	1
0.5				0.0013 ± 0.0007	6.5 ± 2.4	1.7 ± 0.8	1.20	1
1							1.72	1
2.5	0.0025 ± 0.0012	0.3 ± 0.1	0.1 ± 0.1	0.0037 ± 0.0012	6.9 ± 1.4	1.0 ± 0.2	2.22	1
5							2.23	1
				Retrorsine				
0.1							0.37	1
0.5							0.30	1
1							0.38	1
2.5							1.53	1
5	0.0006 ± 0.0002	0.7 ± 0.1	0.1 ± 0.1				2.13	1
25							0.85	1
				Lycopsamine				
1	0.0006 ± 0.0001	0.2 ± 0.0	0.1 ± 0.0				0.35	0.01
5	0.0024 ± 0.0008	0.2 ± 0.0	0.1 ± 0.0				0.33	0.01
25	0.0107 ± 0.0033	0.3 ± 0.0	0.2 ± 0.0				0.33	0.01
75	0.0176 ± 0.0087	0.2 ± 0.1	0.2 ± 0.1				0.78	0.01
150	0.0272 ± 0.0078	0.4 ± 0.1	0.2 ± 0.1				1.73	0.01
300							2.63	0.01
				Europine				
1							0.55	0.3
5							0.66	0.3
25							0.86	0.3
75							1.49	0.3
150							2.19	0.3
300							2.97	0.3

[a]Levels at the same (2.5 μM) or similar (5 μM) substrate concentration are printed in bold for direct comparison. Data represent means and S.D. from n = 3 independent experiments, blank cells represent levels below the respective LOD. [b]Data taken from Rutz et al.[38] [c]taken from Merz and Schrenk[3]

Cytotoxicity data from incubations of both cell types with the PAs investigated here have been published previously.[34,38]

The major aims of this study were (i) to investigate hepatic PA metabolism in intact cells, i.e., at a higher level of in vitro metabolism studies, and (ii) to obtain more information on the products released into the medium. The latter question is of particular interest since previous studies by others suggest that PA metabolites released from hepatocytes may affect other target cells, in particular endothelial cells in liver sinusoids,[30,48] lung,[31] or kidney.[32] Our results demonstrate that metabolic rates strongly differ between PAs and between cell types but all PAs investigated are metabolized in rat and human hepatic cells and various types of metabolites are released into the extracellular environment.

PRH were able to metabolize a substantially larger portion of the PA than HepG2-CYP3A4 cells. Similar results in liver microsomes may be due to differences in CYP activities toward PAs between rat and human.[43] Likewise, we have recently shown that 7-Benzoxyresorufin-O-Dealkylase (BROD) activity as a functional measure of CYP isoforms probably most relevant for PA metabolism was significantly higher in pRH than in HepG2-CYP3A4 cells, at least shortly after plating of

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the pRH although the cells tend to lose activity during culture.[38] Similarly, losses in CYP3A4 expression were observed in human hepatocytes kept under standard conditions.[49]

The metabolic differences between both cell types were most pronounced for Las, Ech, and Sen but also noted for the other PAs (Figure 1). They are likely to be due to differences in uptake, metabolism, and release on a cellular level. A good correlation between metabolism and lipophilicity-related retention times in reversed phase HPLC of the substrate PAs[50] points to possible molecular reasons for differences in cellular disposition of PAs. It remains to be elucidated if these differences also exist between rat and human hepatocytes.

Furthermore, marked differences were obtained between individual congeners with respect to overall metabolism and the amount of metabolites released. The congeners Las and Sen in HepG2-CYP3A4 cells or Las, Ech, and Sen in pRH, respectively, were metabolized most effectively as was previously also found in rat and human liver microsomes.[43]

Interestingly, those congeners being most extensively metabolized also showed the highest rate of "unknowns", i.e., the amount of substrate not recovered, neither as parent compound nor as known metabolites in the medium or in the cells. These "unknown" fractions may comprise reactive metabolites being retained within the cell as they are strongly, e.g., covalently bound to biomolecules and thus not readily assessable without additional preparation. This assumption is supported by the comparison of metabolite profiles of rat liver microsomes and primary rat hepatocytes. Markedly lower concentrations of reactive metabolites compared to other metabolites are found in the supernatants of hepatocytes. This suggests either a decreased formation or a lowered release because reactive metabolites bind to nucleophilic targets within cells (SI for all PAs). This would explain the strong correlation between the amount of "unknowns" and the relative toxicity and genotoxicity of the PA congeners (Table 1). Interestingly, the portion of unknowns being highest for Las in both cell types, was minor for Ech, Ret, and Eur in HepG2-CYP3A4 cells. Incubations with Lyc did not result in any "unknown" gap in both cell types and led to marginal levels of pyrrolic metabolites released from pRH only.

The fact that, in many cases, the yield of metabolites was more or less proportional to the initial substrate concentration suggests that the enzymatic apparatus of the cells was not saturated, except for a tendency to smaller increases at a few relatively high concentrations. It is unclear if this tendency is due to a saturation of metabolism or to first substantial cytotoxicity occurring at these PA levels.[34,38]

Reactive metabolites either bear an additional double bond in the necine ring only or are additionally oxygenated in the necine base (Figures 2−7). Generally higher concentrations of reactive metabolites were detected in HepG2-CYP3A4 media than in the media of pRH. Las metabolite Las_M20 as the major ring oxygenated metabolite was found only in HepG2-CYP3A4 incubation media in substantial amounts. Other pyrrole-like metabolites as Las_PI-1, Las_PI-3, Las_M16, and Las_M45 (or Las_M18,M19 detected in microsomes),[43] were also released from HepG2-CYP3A4 cells, although found at lower levels. As already shown for the metabolite profiles of rat liver microsomes and hepatocytes, several reactive metabolites including GSH conjugates were either not found in the supernatants or at very minor amounts. Necine base-oxygenated metabolites were found in HepG2-CYP3A4 cells with Las and Sen only. It remains to be investigated if such metabolites and/or the "simple" dehydrogenated metabolites such as Las_M44 or Las_M39 released in very small amounts may play a role in hepatocyte-mediated damage to other cell types and tissues. Interestingly, pRH only released small amounts of the mono-GSH-DHP conjugate Las_M39. In fact, such conjugates were described to be formed in rat liver microsome incubations[51] and there is good evidence that, e.g., the DHP-GSH conjugate at position 7 exerts genotoxic properties.[52] It needs to be noted that all highly toxic 1,2-unsaturated PAs should be able to be transformed enzymatically into such GSH conjugates. However, products of this type were found only in Las and Sen incubations with pRH and in Ret incubations with HepG2-CYP3A4 cells. This finding is probably due to low rates of formation or low release together with a high reactivity of the compounds. The findings seem to be in contrast with our previous findings on microsomal metabolites[43] where all PAs tested here except the monoesters Eur and Lyc released GSH conjugates, not taking into account cellular in- or uptake, however. No indication for release of cysteine conjugates described to be formed in liver microsomes[29] was obtained in our study.

A comparison of the released reactive metabolites from incubations of HepG2-CYP3A4 cells revealed similar amounts for Las and Ech and somewhat lower amounts for Sen when the same substrate concentration was considered. In pRH, the highest amounts of reactive metabolites from low substrate concentrations ($\leq$25 μM) were found with Ech and Las in a concentration-dependent manner. In HepG2-CYP3A4 cells, the percentage of extracellular reactive products was several-fold, in some cases over 10-fold higher than in pRH, at least for Las, Ech, and Sen. In contrast, there was no indication for released pyrrolic metabolites from Ret, Lyc, or Eur in HepG2-CYP3A4 cells. These findings coincide with a complete lack of GSH conjugates in the supernatants from this cell type while for Las, Sen, and Ret mono-GSH-DHP conjugates were detected in pRH. With Lyc, pyrrolic metabolites were also found, however, at much higher substrate concentrations in pRH only. It remains to be investigated if these differences reflect a lower degree of metabolic activation of certain PAs such as Ret and the monoesters Eur and Lyc or are due to the fact that HepG2-CYP3A4 cells express one of the most prominent but no other CYPs involved in PA activation. In particular, human CYP2A6 and 3A5 were found to contribute substantially to the metabolism of some of the congeners investigated here.[22] Thus, studies in human hepatocytes may be considered to show human hepatic PA metabolism in a more representative way, provided the considerable variability in CYP activities between individual hepatocyte preparations can be taken into account.

The overall picture reveals that the quantitative role of presumptive detoxification reactions such as ester clevage, side chain modifications, and *N*-oxide formation are structure-specific. In cyclic diesters such as Ret and Sen, *N*-oxide formation seems to be a predominant hepatic pathway, while it was of very minor importance in Las, Ech, and Eur incubations. The differences between Ret and Sen in released metabolites are difficult to explain as long as the overall metabolic balance has not been elucidated. However, the larger amount of "unknowns" retained in the cells together with a higher lipophilicity of Sen[50] may argue for a more extensive metabolic activation of Sen. Lyc was *N*-oxidized to a low degree in pRH but played a quantitatively dominant role in HepG2-CYP3A4

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cells. The relative contributions of the different pathways again exerted a number of similarities with results from liver microsomes. It needs to be mentioned, however, that our data can only provide information on the extracellular levels of metabolites at the time point of the measurement. These patterns do not necessarily reflect the primary or secondary events in PA metabolism within the cells in a quantitative manner.

Taken together, a number of factors seem to contribute to the pronounced cyto- and genotoxicity of Las, Ech, and Sen in comparison to the other congeners. These are a high degree of metabolism combined with a relatively high rate of formation of ring-dehydrogenated pyrrolic metabolites. For the open-chained diester congeners Las and Ech, however, *N*-oxidation played a very minor role. For the cyclic diesters Ret and Sen *N*-oxidation was prominent which may be due to a lower rate of competing oxygenation reactions at the side chain cyclus. Interestingly, Ret- and Sen-incubated HepG2-CYP3A4 cells released less (Sen) or no (Ret) detectable reactive metabolites, possibly refelecting a lower activation of these congeners than in primary rat hepatocytes. A similar picture had already been obtained in human vs rat liver microsmes.[43] Similar to human liver microsomes, no reactive metabolites were found in supernatants of incubations of HepG2-CYP3A4 cells with the monoesters Eur and Lyc. Thus, our findings confirm that Las, Ech, and Sen are effectively metabolized and activated in human and rat liver cells, while the human liver cell line may be less effective in activating the cyclic diester Ret and the monoester Lyc. No indication for a metabolic activation of the monoesters Eur and Lyc in rat or human cells was obtained.

The yield of micronuclei counts was in relatively good agreement with the percentage of "unknowns", while the correlation with reactive metabolite levels found was less convincing. A direct quantitative comparison of metabolite profiles in microsomes and hepatocytes (SI) turned out to be unfeasible since we measured the released and more or less stable and not all formed metabolites. Covalently bound electrophiles are not accessible to our LS–MS detection and thus form part of the "unknown" fraction. The amount and type of the electrophilic metabolites formed may thus play a decisive role in the release and in the possible effects on targets outside of the hepatocytes.

It remains to be elucidated why Lyc not giving rise to detectable amounts of "unknowns" and extracellular reactive metabolites in HepG2-CYP3A4 cells was able to induce micronuclei, although at relatively high concentrations. Overall, our metabolic findings contribute to our growing understanding of the complex metabolism of 1,2-unsaturated PA on the cellular level and further illustrate how metabolism contributes to the widely different toxic potencies of PAs.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.chemrestox.0c00507.

Reactive and other detected metabolites after incubation of 15 μM Las, Ech, Sen, Ret, Lyc, or Eur with rat liver microsomes in comparison to incubation of 2.5 μM Las or Sen or 5 μM Ret or 25 μM Ech, Lyc, or Eur with primary rat hepatocytes (PDF)

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Notes

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■ ABBREVIATIONS

CYP:cytochrome P450; DMEM:Dulbecco's Modified Eagle's Medium; DMSO:Dimethyl Sulfoxide; GSH:Glutathione; iREP:Interim relative potency; LC–MS/MS:Liquid chromatography–mass spectrometry/mass spectrometry; PA:Pyrrolizidine alkaloid; pRH:primary rat hepatocytes

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5. SUMMARY OF RESULTS

5.1 Publication I

Structure-dependent hepato-cytotoxic potencies of selected pyrrolizidine alkaloids in primary rat hepatocyte culture

In publication I the hepato-cytotoxic potencies of ten different food relevant PA congeners in primary rat hepatocytes were investigated. Echimidine, europine, heliotrine, indicine, lasiocarpine, lycopsamine, monocrotaline, senecionine, seneciphylline and retrorsine were selected to be examined. The selected substances are assigned to the four different potency classes published by Merz and Schrenk in 2016 (see **Table 2** in chapter 3). For all congeners the influence of CYP enzymes on their cytotoxic effects was investigated. For this purpose, two different pre-incubation times were tested. The primary hepatocytes were cultured for 3 h respectively 24 h before adding the test substances and measured the cytotoxic effect after 24 h and 48 h incubation time with the alamar blue assay. Furthermore, the primary hepatocytes were pre-incubated with the CYP inhibitor ketoconazole before adding the test substances. The effect of GSH depletion via treatment with BSO on the cytotoxicity of selected PA was only examined for indicine, lasiocarpine, lycopsamine and retrorsine. Finally, BROD activity in the primary rat hepatocytes was determined until 72 h after seeding.

In general, the observed cytotoxic effects of various PA were always concentration dependent. Furthermore, the cytotoxic effects were always stronger at 48 h incubation time compared to 24 h incubation time.

All evaluated di-esters (cyclic and open-chained congeners) except monocrotaline were cytotoxic with EC_{50}-values ranging from 91–196 µM (24 h incubation time) and from 27–95 µM (48 h incubation time) after 24 h of pre-incubation. All tested mono-esters showed only weak toxic effects with EC_{50}-values higher than 300 µM (24 h incubation time) and 294 µM– higher than 300 µM (48 h incubation time) after 24 h of pre-incubation. In addition, it was observed that the toxic effects are affected differently by the pre-incubation time for the different congeners. All PA congeners tested showed stronger cytotoxic effects with the combination of 3 h pre-incubation time and following 48 h of incubation: EC_{50}-values ranging from 4–25 µM including cyclic and open-chained di-esters (again except monocrotaline with an EC_{50} above 300 µM) and from 114– higher than 300 µM including the mono-esters. For three congeners, retrorsine, seneciphylline and echimidine, 3 h of pre-incubation and following 24 h of incubation led to a reduced toxicity, all others, especially senecionine, showed stronger cytotoxic effects. The results from the BROD-assay showed a continuously decreasing CYP activity in the primary rat hepatocytes during culture. After 24 h a decrease of more than 80% CYP activity could be observed. After pre-incubation with the CYP inhibitor ketoconazole all

tested PA showed reduced cytotoxic effects after 24 h and 48 h of incubation time. A slight to about three-fold reduction in cytotoxicity was determined. GSH depletion resulted in a significantly higher cytotoxic effect for lasiocarpine after 24 h of incubation and for lycopsamine after 48 h of incubation. For senecionine and indicine no significantly higher cytotoxic effects could be observed.

In conclusion the cytotoxic potency of ten PA congeners could be quantified in primary rat hepatocytes. This publication also showed that the structural features of individual PA strongly influence their cytotoxic potency.

5.2 Publication II

Structure-dependent genotoxic potencies of selected pyrrolizidine alkaloids in metabolically competent HepG2 cells

In publication II the *in vitro* cytotoxicity, genotoxicity and mutagenicity of eleven different food relevant PA was investigated. The same PA as in publication I were selected, only riddelliine was added. Cytotoxicity was determined in naïve HepG2 cells and the clone HepG2 C9 (CYP3A4) cells after incubation over 24 h and 48 h. HepG2 C9 is a stably transfected human HepG2 hepatoblastoma cell line overexpressing the human enzyme CYP3A4. Furthermore, the selected PA were tested for clastogenicity or aneugenicity in HepG2 C9 cells by the micronucleus assay. *In vitro* mutagenicity was determined testing the selected substances in bacteria by the ames fluctuation test, with and without exogenous metabolic activation (S9-mix). Two different *Salmonella* strains were used: TA98 for detection of frameshift mutations and TA100 for detection of base pair substitutions. Additionally, for comparison with the primary rat hepatocytes (see Publication I), BROD activity was also determined in both HepG2 cell lines.

In naïve HepG2 cells none of the tested PA showed a significant cytotoxic effect up to a concentration of 300 µM. In contrast to this, all PA showed cytotoxic effects in the transfected HepG2 C9 cell line. The EC_{50}-values for the cyclic and open-chained di-esters (except again monocrotaline with an EC_{50}-value of above 300 µM) ranging from 10–179 µM after 48 h of incubation. All tested mono-esters showed only weak toxic effects with EC_{50}-values ranging from 176– above 300 µM. In the micronucleus assay all eleven tested PA induced a significant increase in micronuclei counts. The amounts of micronuclei quantified increased with increasing PA concentrations. At higher concentrations cytotoxic effects of the PA attenuated the micronuclei formation. To compare the micronuclei data of the individual PA congeners, the benchmark concentrations (BMC) leading to a doubling of the micronuclei counts compared to background and their upper (UB) and lower (LB) 10% confidence limits were evaluated. For the tested cyclic and open-chained di-esters (again except monocrotaline (LB:

23.7 µM, UB: 153 µM)) a lower bound benchmark concentration ranging from 0.01–7.85 µM and an upper bound benchmark concentration ranging from 0.24–17.3 µM was determined. The mono-esters, except heliotrine, induced micronuclei at higher concentrations with BMCs between 34.1–59.5 µM (LB) and 45.5–76.3 µM (UB). Heliotrine was significantly more potent than the other mono-esters (LB: 4.42 µM, UB: 10.4 µM). With regard to the ames fluctuation test in TA98 and TA100 no mutagenic effects for all test PA could be observed, despite metabolic activation with S9-mix. The results of the BROD assay showed a significantly lower CYP activity in both liver cell lines in comparison to the activity in primary rat hepatocytes, described in publication I.

In conclusion the cytotoxic and genotoxic potency of eleven PA congeners in the HepG2 C9 cell line could be quantified. All tested congeners showed genotoxic effects in the micronucleus assay. Again, as in publication I, it was observed that the toxic potency clearly depended on the structural features of the individual PA congeners.

5.3 Publication III

Metabolic pattern of hepatotoxic pyrrolizidine alkaloids in liver cells

In publication III the metabolism of six selected PA was investigated: echimidine, europine, lasiocarpine, lycopsamine, retrorsine and senecionine in primary rat hepatocytes and in HepG2 C9 cells. Both cell types were incubated with each individual PA in six different concentrations for 24 h, while at the beginning and at the end of the 24 h incubation period, an aliquot was taken from the incubation supernatant and from the cells. In the following the samples were analyzed via liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS). In the first step the percentage of non-metabolized PA, the sum of detected known metabolites and the unknown part in the incubation medium were determined after 24 h of incubation for each individual PA and each tested concentration in both cell types. In the second step a metabolite profile for each PA was determined, where the metabolites were divided into two groups: (i) reactive metabolites bioactivated in the necine base moiety (dehydrogenated pyrrolic compounds or pyrrole like metabolites carbon-oxygenated in the necine base) and (ii) other metabolites (*N*-oxides and compounds that are modified in the necic acid moiety by, e.g. hydroxylation, epoxidation or dealkylation). For further comparison of the potency of the individual PA the total amount of reactive metabolites for each congener in both cell types was calculated and was expressed as a percentage of the total metabolites and as percentage of the metabolized parent PA.

Generally, it was observed that all PA showed a higher metabolism rate in the primary rat hepatocytes in comparison to the Hep G2 C9 cells. Furthermore, marked different metabolism rates between the individual PA were figured out: in both cell types echimidine, lasiocarpine

and senecionine showed a higher metabolism rate than europine, lycopsamine and retrorsine. The recovery rate for echimidine, lasiocarpine and senecionine as parent PA ranged from 1.6–35%, while the recovery rate for europine, lycopsamine and retrorsine ranged from 81–89% in the primary rat hepatocytes. In comparison, the investigations in the HepG2 C9 cells showed the following results: the recovery rate for echimidine, lasiocarpine and senecionine as parent PA ranged from 62–89%, while the recovery rate for europine, lycopsamine and retrorsine ranged from 95–100%. It was also seen that the relative amounts of known metabolites in primary rat hepatocytes were partially much higher for each individual PA than those in HepG2 C9 cells. The same was observed for the amounts of unknown metabolites.

The metabolite profiles showed a concentration-dependent increase in metabolites by incubation with increasing concentrations for all individual PA in both cell types. Figures 2–7 showed the amounts and structure proposals of the metabolites measured for each PA at each concentration tested in both cell systems. For lasiocarpine the highest amounts of reactive metabolites were determined. Six reactive metabolites were formed by oxygenation of the necine base, further two reactive metabolites were formed by dehydrogenation. GSH conjugates were only found in primary rat hepatocytes. In comparison to this, with the other PA, one oxygenated necine base and ten metabolites with a dehydrogenated retronecine ring were detected. In primary rat hepatocytes, reactive metabolites were found for lasiocarpine and echimidine in a concentration dependent manner. This was also observed for lycopsamine but at much higher tested concentrations. For retrorsine and senecionine, reactive metabolites could only be quantified at one concentration tested, while for europine no reactive metabolites were found. In the HepG2 C9 cells the percentage of quantified reactive metabolites for lasiocarpine and echimidine was much higher than in the primary rat hepatocytes, although the metabolism rate was much lower. For retrorsine, lycopsamine and europine no reactive metabolites could be quantified. Another important observation during these experiments was, that metabolites of open-chained di-esters mostly had shorter alkyl-chains in the necine acid moiety, while cyclic di-esters mostly showed *N*-oxides. When comparing with the micronucleus data from publication II, it could be observed that the number of micronuclei increased parallel to the amounts of unknown metabolites.

In conclusion, this publication shows that the metabolic profil may contribute considerably to better understand the specific toxic potency of an individual congener.

6. SUMMARIZING DISCUSSION

In this chapter the results of the three publications are linked and discussed in summary. To start the discussion, it has to be mentioned that the concept of potency factors forms the basis of this project. In 2016 Merz and Schrenk suggested the iREP-factors based on existing literature data, mainly consisting of *in vivo* data on acute toxicity in rodents, *in vitro* cytotoxicity and genotoxic effects in *Drosophila*. Four structural potency classes were proposed for the PA: a factor of 1.0 for cyclic di-esters and open-chained di-esters with 7*S* configuration, a factor of 0.3 for mono-esters with 7*S* configuration, a factor of 0.1 for open-chained di-esters with 7*R* configuration and a factor of 0.01 for mono-esters with 7*R* configuration. *N*-oxides was assigned the same potency factor as the corresponding PA (Merz and Schrenk, 2016). In general, it was the aim of these three publications to provide a better scientific basis for the iREP factors and therefore the risk assessment of PA.

The liver seems to be the primary target organ of PA toxicity, thus hepatocytes are the preferred cell system for various PA studies. Both, active uptake of PA and metabolic activation appear to play crucial roles in studying hepatotoxicity. In the first publication, primary rat hepatocytes were used as a cell system and the cytotoxicity of the selected PA was investigated. In publication II the cytotoxic effects in naïve HepG2 and HepG2 C9 cells were investigated. HepG2 C9 cells are overexpressing the human enzyme CYP3A4, which seems to play an important role in metabolic activation of PA (Ruan et al., 2014). Generally, a certain degree of cytotoxicity in primary rat hepatocytes and HepG2 C9 cells was observed, with the strength of the cytotoxic effect depending on the corresponding PA congener. Furthermore, an incubation time of 48 h instead of 24 h led to stronger cytotoxic effects in most cases. It is assumed that a cumulative damage (e.g. covalent bindings to proteins) is the reason for this (Ma et al., 2018). There are marked differences in cytotoxicity between mono-esters and certain open-chained and cyclic di-esters, except monocrotaline. In naïve HepG2 cells no cytotoxic effects were observed after 24 h and 48 h of incubation time up to a concentration of 300 µM, neither in the di-esters nor in the mono-esters. EC_{50} values in primary rat hepatocytes from lasiocarpine and the cyclic di-esters, except monocrotaline (EC_{50} above 300 µM), ranging from 4–19 µM, while the EC_{50} values of the mono-esters are all above 100 µM. Echimidine showed unexpected stronger cytotoxic effects. In HepG2 C9 cells the EC_{50} values from lasiocarpine and the cyclic di-esters, except again monocrotaline (EC_{50} above 300 µM), ranging from 10–97 µM, while the values from the mono-esters are all above 300 µM (except heliotrine with an EC_{50}-value of 176 µM). Echimidine is significantly less cytotoxic in HepG2 C9 cells than in primary rat hepatocytes.

In the literature there are many different studies investigating cytotoxicity of PA in different cell types. In HepG2/C3A cells co-incubated with S9 mix Tamta et al. observed significant toxicity for retrorsine after 6 h of incubation at concentrations above 178 µM, followed by echimidine and monocrotaline with significant cytotoxic effects only at 628 and 768 µM, respectively. Heliotrine and lycopsamine were not significantly toxic up to a concentration of 850 µM (Tamta et al., 2012). In a chicken hepatocarcinoma cell line Field et al. determined an order of decreasing cytotoxic potency for eleven PA: lasiocarpine, seneciphylline, senecionine, heliotrine, riddelliine, monocrotaline, riddelliine *N*-oxide, lycopsamine, intermedine, lasiocarpine *N*-oxide and senecionine *N*-oxide. Lasiocarpine was the most cytotoxic congener, and seneciphylline, senecionine and heliotrine were also examined as strong cytotoxic congeners (Field et al., 2015). In the here performed experiments heliotrine was also the only PA among the mono-esters that showed a stronger cytotoxic effect in HepG2 C9 cells. Both studies also show, as do the results of publication I and II, that the toxic potency of monocrotaline is possibly overestimated with an iREP of 1.0. The other cyclic di-esters showed in all three studies the strongest cytotoxic effects. In a further study from Waizenegger et al. they investigated the toxic potential of four structural different PA in HepaRG cells (Waizenegger et al., 2018). In this study, as in the study from the first publication in primary rat hepatocytes, echimidine showed a significantly higher toxic potency than would have been expected based the iREP factor. This leads to the assumption that the toxic potency of echimidine is underestimated. This was also observed in a study from Glück et al., where 22 different PA congeners were investigated regarding their cytotoxic effects in the metabolically competent human hepatoma cell line HepaRG with the MTT assay (Glück et al., 2021). The concentrations tested ranged from 0.1 to 250 µM which is similar to the cytotoxicity experiments from publication I and II. Lasiocarpine was found to be the most cytotoxic congener, followed by heliosupine, senecionine, seneciphylline and echimidine. Monocrotaline again showed similar toxic potency to the mono-esters in this study. In particular, the observation that the strength of toxicity of echimidine varies within publication I and II and between the publications in the literature, should receive special attention. The results show that HepG2 C9 cells are less sensitive than primary rat hepatocytes at almost all tested PA, while in naïve HepG2 no cytotoxic effects could be observed. This is also in agreement with the findings regarding the hepatic metabolism, where all PA showed a significantly higher metabolism rate in primary rat hepatocytes than in HepG2 C9 cells. A possible explanation may be due to species differences, in terms of metabolic activation including the spectrum of expressed CYP isoforms and their activity in the corresponding cell system. For example, Ruan et al. showed that different CYP enzymes can metabolically activate PA in human liver microsomes to varying degrees. CYP3A4 seems to play an important role in the metabolic activation of lasiocarpine (Ruan et al., 2014). The unexpected stronger cytotoxic effects of

heliotrine in HepG2 C9 cells, may also be due to a crucial role of CYP3A4 in metabolic activation. In some other studies, the influence of CYP3A enzymes was observed for different PA: retrorsine (Tu et al., 2014), senecionine (Miranda et al., 1991) and riddelliine (Xia et al., 2003). Allemang et al. discussed that CYP2A6 and CYP2E1 are partially involved in the metabolic activation of monocrotaline, while Nascimento et al. perceived a role for CYP1A1 (Nascimento et al., 2017; Allemang et al., 2018). In conclusion this means that certain CYP enzymes, which are relevant for metabolic activation of certain PA, may not be expressed or are only expressed at low levels in HepG2 C9 cells, compared with primary rat hepatocytes. In naïve HepG2 cells, the expression and activity of relevant CYP enzymes seems to be deficient. A further explanation of the attenuated cytotoxicity in HepG2 C9 cells compared with primary hepatocytes can also be derived from the results of our experiments regarding BROD activity. A reduction of BROD activity of 80% 24 h after seeding was determined in primary rat hepatocytes. Thus, we observed that the time of pre-incubation in primary rat hepatocytes had a strong influence on the sensitivity of the cells towards PA. A shorter pre-incubation time resulted in a stronger cytotoxic effect for some PA congeners, especially lasiocarpine, senecionine and retrorsine (3 h pre-incubation, 24 h incubation time). For some other PA congeners, the shorter pre-incubation has had less influence on cytotoxicity or even leads to a reduced cytotoxic effect (24 h pre-incubation, 24 h incubation time). This indicates that, first, the activity of several CYP enzymes influences cytotoxicity, second, certain CYP enzymes are responsible for the metabolic activation of certain PA and third, detoxification pathways may influence cytotoxicity. It is also possible that enzymes responsible for metabolic activation are not members of the CYP family (Huan et al., 1998). Further experiments with ketoconazole as a CYP inhibitor, inhibiting a broad spectrum of different CYP isoforms in rat liver (Eagling et al., 1998), confirm the assumption that certain CYP isoforms play a crucial role for the toxicity of certain PA. It can be seen that the attenuation of the cytotoxic effect differs among PA congeners. Another point to be considered and discussed in the toxicity of PA is the presence or absence of transporters in the corresponding cell systems. Tu et al. observed that monocrotaline and retrorsine were substrates of the OCT1. This OCT1-transporter mediates the hepatic uptake of monocrotaline and retrorsine and may play an important role in their hepatotoxicity (Tu et al., 2013; Tu et al., 2014). Furthermore, the influence of active Efflux transporters like ABCB1 *in vivo* must be taken into account and is discussed as reason for the higher as expected observed potency of echimidine *in vitro* (Allemang et al., 2018; Hessel et al., 2014). As described in the section of metabolism, there are also some known detoxification pathways (*N*-oxidation, hydrolysis and GSH-conjugation) which influence PA toxicity. Especially, the cellular amount of GSH could probably influence the toxicity of several PA congeners (Wang et al., 2000). Geburek et al. compared the GSH conjugate formation for 22 different PA in human and rat liver microsomes, to detect possible species differences, but

could not determine marked differences between the GSH conjugate levels (Geburek et al., 2020a). In the here performed experiments, GSH depletion did not significantly affect the cytotoxicity of the tested PA. This suggests, that GSH might not play an important role in PA toxicity.

Mutagenicity was determined in the ames fluctuation test for eleven selected PA with and without exogenous metabolic activation by S9-mix. We used two different *Salmonella* strains: TA98 for detection of frameshift mutations and TA100 for detection of base pair substitutions. None of the tested PA showed mutagenic effects up to a concentration of 300 µM, while at the same time not more than 50% decreased cell viability was measured. This in agreement with some studies from the literature seeing no or only weakly mutagenic effects for selected PA congeners. Rubiolo et al. observed no mutagenic effect for senecionine in TA100 despite metabolic activation with S9-mix, whereas seneciphylline and retrorsine were determined as weakly positive (Rubiolo et al., 1992). Since some positive results were also found in the literature, it cannot be excluded that the tested PA may be mutagenic despite our observed negative results. Yamanaka et al. detected mutagenic effects for heliotrine and lasiocarpine in TA100 with metabolic activation by S9-mix (Yamanaka et al., 1979). Riddelliine also showed mutagenic effects in TA100 with S9-mix (Chan, 1993). From these results, it could be concluded that external metabolic activation is very crucial for any mutagenicity in bacteria. The reactive metabolites formed are initially outside the bacteria and must be taken up by them to cause a possible mutagenic effect. Organic cation transporters seem to play an important role in PA uptake (Tu et al., 2013; Tu et al., 2014), but it is unclear whether such transporters occur in bacteria. Therefore, our negative results may be explained by a lack of uptake mechanisms. Thus, it is questionable whether the reactive metabolites can reach the cell interior. It seems to be possible that they react with nucleophiles outside the bacteria. In addition, it should also be noted that the mutagenic effects in the literature were only detected at partially high doses, confirming that the ames fluctuation test may not be sensitive enough for PA. In our experiments, we tested the PA up to its solubility limit, mostly up to a maximum of 300 µM.

In contrast to the results of the ames fluctuation assay, all PA tested showed concentration-dependent micronucleus induction in different concentration ranges. For all cyclic and open-chained di-esters, except monocrotaline, the maximum concentration determined was up to 25 µM, while the mono-esters, except heliotrine, were tested up to a concentration of 300 µM, limited by the cytotoxic effects of the different PA in HepG2 C9 cells. Lasiocarpine was the most potent congener (LB: 0.01 µM, UB: 0.49 µM) followed by senecionine (LB: 0.05 µM, UB: 0.24 µM), seneciphylline (LB: 0.66 µM, UB: 1.34 µM), retrorsine (LB: 1.26 µM, UB: 1.90 µM) and riddelliine (LB: 1.29 µM, UB: 2.29 µM). Echimidine seems to be more potent as expected from the iREPs (LB: 7.85 µM, UB: 17.3 µM), while monocrotaline seems to be overestimated

(LB: 23.7 µM, UB: 153 µM). The mono-esters, except heliotrine, induced micronuclei at higher concentrations with BMCs between 34.1–59.5 µM (LB) and 45.5–76.3 µM (UB). Heliotrine was significantly more potent than the other mono-esters (LB: 4.42 µM, UB: 10.4 µM). The induction of micronuclei in general was detected in a lower concentration range than the observed cytotoxic effects. Similar findings from the literature confirm our results: Allemang et al. detected micronuclei after incubation of PA in HepaRG cells also at concentrations where no or only weak cytotoxicity was observed. They calculated lower and upper confidence limits (CEDL, CEDU) of the critical effect dose (CED) for fifteen PA congeners. Lasiocarpine was also the most potent congener. Further significant micronuclei increase was seen for riddelliine and echimidine at concentrations five-fold higher. For heliotrine, retrorsine and europine significant increases in micronuclei were determined at concentrations 15 to 30-fold higher. For lycopsamine, indicine, intermedine, lasiocarpine *N*-oxide, riddelliine-*N*-oxide and monocrotaline concentrations between 100 to 400-fold higher than lasiocarpine were determined (Allemang et al., 2018). Louisse et al. examined the concentration-dependent genotoxicity of 37 different PA congeners using the γH2AX in cell western assay in human HepaRG cells. Based on benchmark dose (BMD) analysis of the concentration-response data, *in vitro* relative potency factors (RPFs) ranging from less than or equal to 0.01 to 1.2 were derived. On the basis of the obtained data, they suggested a higher potency for retronecine-type (7*R*) open-chained di-esters (RPF between 0.3 and 1.2 instead of an iREP of 0.1), i.e., echimidine (benchmark concentration lower bound (BMCL): 8.3 µM, benchmark concentration upper bound (BMCU): 11 µM), which is in agreement with the findings from publication II (LB: 7.85 µM, UB: 17.3 µM). Furthermore, they suggested a lower potency for heliotridine-type (7*S*) mono-esters (RPF between 0.01 and 0.1 instead of an iREP of 0.3), i.e., heliotrine (BMCL: 45 µM, BMCU: 80 µM) and europine (BMCL: 49, BMCU: 75 µM), which is in agreement with the findings for europine (LB: 34.1 µM, UB: 45.5 µM), but not for heliotrine (LB: 4.42 µM, UB: 10.4 µM) (Louisse et al.,2019). As discussed in the cytotoxicity part, possibly CYP3A4 plays a crucial role in metabolic activation of heliotrine and could explain this difference in potency. Hadi et al. showed the results of experiments from seven different PA congeners (echimidine, europine, lasiocarpine, lycopsamine, retrorsine, riddelliine and seneciphylline) tested in the cytokinesis-block micronucleus assay (CBMN) using HepG2 cells and from three different ester-types of PA (europine, lasiocarpine and seneciphylline) in the CBMN using Huh6 cells. Furthermore, they tested six different PA (echimidine, europine, lasiocarpine, lycopsamine, retrorsine and riddelliine) in a crosslink comet assay using HepG2 cells. They also determined, as do the results from publication II, lasiocarpine as the most potent PA, inducing micronuclei at a concentration of 3.2 µM. With exception of seneciphylline, they reported a similar potency rank order as we do in our results: lasiocarpine/riddelliine > retrorsine/echimidine > seneciphylline/europine/lycopsamine. Lycopsamine showed micronuclei induction at a

concentration of 100 µM and is therefore the weakest potent PA. The results from the comet assay showed significant reduced tail formation for all di-ester PA, but not for the mono-esters, confirming the influence of the structural features for their genotoxicity (Hadi et al., 2021). In another study by Lester et al., the formation of DNA adducts in a rat hepatocyte cell system in sandwich culture was measured and described as a direct expression of the formation of reactive metabolites. As a result, they determined nearly the same potency rank order as we can derived from our results: Lasiocarpine > echimidine ~riddelliine ~heliotrine > europine ~monocrotaline ~ lycopsamine ~ indicine > platyphylline (Lester et al., 2019).

In agreement with our results, the toxic potency of monocrotaline seems to be overestimated with an iREP factor of 1.0, while all other cyclic di-esters were as potent as expected from the iREPs. As described above, the much lower cyto- and genotoxicity of monocrotaline may be explained by the possibly different expression of several transporters in the different cell systems, which are responsible for the uptake of monocrotaline. Chen et al. for example determined monocrotaline to be a substrate of OCT1 and OCT2 (Chen et al., 2019). Furthermore, it is possible that the structural features from monocrotaline in comparison to the other cyclic di-esters influences its toxic potency. The cyclic side chain of monocrotaline, consisting of five carbon atoms, while the side chains of all other cyclic di-esters consisting of six carbon atoms, seems to be more unstable, since the increased ring strain facilitates hydrolysis, consequently detoxification. Dueker et al. observed that guinea pig carboxylesterase was capable of hydrolyzing monocrotaline in comparison to senecionine much more effectively (Dueker et al., 1992). Furthermore, the other cyclic di-esters have an additional allyl group connected to C7, although it is unclear how this finally affects toxicity. As described above and described in the literature, again, the influence of several CYP enzymes such as CYP1A1 (Nascimento et al., 2017), CYP2A6 and CYP2E1 discussed by Allemang et al. (Allemang et al., 2018) must be considered. Yang et al. also observed that retrorsine exerted a much higher liver toxicity in rats in comparison to monocrotaline. They determined higher amounts of pyrrole-GSH conjugates and protein covalent binding for retrorsine in comparison to monocrotaline at the same concentration. Furthermore, significant hepatic GSH depletion was determined for retrorsine, while monocrotaline did this only to a small extent (Yang et al., 2017). Europine seems also to be overestimated, while echimidine again is significantly more potent as expected. It is also interesting that heliotrine and europine differ significantly in their potency, although they are structurally very similar. For echimidine, as discussed in the cytotoxicity part, again the influence of active Efflux transporters like ABCB1 *in vivo* is discussed in the literature as reason for the unexpected higher potency *in vitro* (Allemang et al., 2018, Hessel et al., 2014). It should also be mentioned that the possible deviation of echimidine in the *in vitro* experiments performed here is due to the configuration at C7. This possibly influences the kinetics of PA, for example the interaction of PA with

corresponding uptake transporters. The observed unexpected higher potency for heliotrine in cytotoxicity and genotoxicity in HepG2 C9 cells indicated, that as also discussed above for heliotrine, CYP3A4 plays a crucial role in metabolic activation, following toxic potency. Furthermore, a structural feature in comparison to europine may could explain the differences in their potency. Europine and heliotrine differ in their chemical structure only by an additional hydroxyl group in Europine. The resulting increase in hydrophilicity certainly seems to have an influence on excretion and thus on toxicity.

Further experiments from publication III regarding hepatic metabolism of six selected PA congeners in the supernatants of incubated primary rat hepatocytes and HepG2 C9 cells contribute to our better understanding of PA metabolism and thus toxicity. Generally, in the supernatants of primary rat hepatocytes all PA showed a higher metabolism rate than in the supernatants of the human cell line HepG2 C9. This is in agreement with our results regarding BROD activity, which was significantly higher in primary rat hepatocytes than in HepG2 C9 cells. Similar results were observed in microsomes due to differences in CYP activities towards several PA between rat and human (Geburek et al., 2020b). Lasiocarpine and senecionine in HepG2 C9 cells and lasiocarpine, senecionine and echimidine in primary rat hepatocytes showed the highest metabolism rate, and also the highest amount of `unknowns`, which is the difference between the initially added amount of PA and the sum of remaining parent PA and identified metabolites. This is also confirmed of the results from experiments in rat and human liver microsomes (Geburek et al., 2020b). It is possible that this fraction of `unknowns` contains reactive metabolites covalently bound and retained within the cell. This is supported by determination of significantly lower concentrations of reactive metabolites compared to other metabolites in the supernatants of the hepatocytes then in rat liver microsomes. It also would possibly explain the correlation between the amount of `unknows` and the observed cytotoxicity and genotoxicity of the PA congeners. Lasiocarpine showed the highest amounts of `unknows` in both cell types and is also the most potent PA in our cytotoxicity and genotoxicity experiments. Lycopsamine showed no `unknown-fraction` in both cell types as well as no reactive metabolites in the supernatants of the HepG2 C9 cells, although the substance induced micronuclei. This observation remains to be elucidated. No reactive metabolites could also be determined for europine and confirms a possible overestimation of the toxic potency of europine as we observed in the micronucleus experiments. However, the potency factor for lycopsamine of 0.01 can be confirmed. Regarding reactive metabolites, we measured general higher concentrations in the supernatants of HepG2 C9 cells than in the supernatants of the primary rat hepatocytes. Several ring oxygenated metabolites were measured only in the supernatants of the HepG2 C9 cells with lasiocarpine and senecionine. It is possible that these released metabolites could play an important role in damaging other cell types. Regarding GSH conjugation, mono-glutathione-dehydropyrrolizidine (mono-GSH-

DHP) conjugates could be measured in small amounts for lasiocarpine and senecionine in the supernatants of primary rat hepatocytes. In the supernatants of the HepG2 C9 cells, such conjugates could only be determined for retrorsine. As already seen in the cytotoxicity experiments, this may also be a reason for the lower sensitivity of HepG2 C9 cells towards the most PA congeners. HepG2 C9 cells express CYP3A4 but not some other CYPs for example CYP2A6 and CYP3A5, which are also responsible for the metabolic activation of certain PA, as observed from Ruan et al. (Ruan et al., 2014). Furthermore, the fact that we investigated the supernatants of intact cells seems to be crucial, and the presence of these conjugates depends on whether they are released from the cell. In comparison, GSH conjugates could be measured in human and rat microsomes in almost all PA examined (Geburek et al., 2020b). Furthermore, Chen et al. also observed such conjugates in liver microsomes and in treated rats (Chen et al., 2016). Furthermore, we saw that *N*-oxide formation is PA-specific. For the cyclic di-esters retrorsine and senecionine *N*-oxidation seems to be a predominant pathway, while for the open-chained di esters as well as europine this pathway was only from minor importance. For lycopsamine *N*-oxidation played a quantitatively dominant role only in HepG2 C9 cells. In combination with the various side-chain modifications and necine base metabolites, clear structure-specific differences can also be identified with regard to metabolism.

7. CONCLUSION AND FUTURE PERSPECTIVES

In conclusion we were able to determine the cytotoxic potency of ten PA congeners in primary rat hepatocytes and of eleven congeners in naïve HepG2 cells and HepG2 C9 cells. Our results show that the primary rat hepatocytes seem to be the most sensitive cell system for investigating cytotoxicity of pyrrolizidine alkaloids, followed by the HepG2 C9 cells. This is in line with our results from the observed metabolism in the tested liver cell systems, whereas primary rat hepatocytes showed a significantly higher metabolism rate than HepG2 C9 cells for all investigated PA. In naïve HepG2 cells no cytotoxicity was observed. In addition, we could confirm the central role of CYP enzymes in the metabolic activation of PA, in particular by using our experiments on CYP inhibition with ketoconazole or the variation of the pre-incubation time. In the micronucleus assay we could clearly demonstrate the genotoxic potency of all eleven tested PA congeners in HepG2 C9 cells. The ames fluctuation assay in *Salmonella typhimurium* seems not to be suitable for detecting mutagenicity of PA, possibly due to certain limitations of the method. The metabolic activation of the PA outside the bacteria cell as well as the possible lack of corresponding uptake transporters seem to be crucial. Taken together the observed cytotoxicity in primary rat hepatocytes and HepG2 C9 cells as well as the genotoxicity in HepG2 C9 cells clearly dependent on the structural type of PA. Our findings support the approach of a strong influence of structural features of PA on their toxicity. It could clearly be showed that the cyclic di-esters, except monocrotaline, and the open-chained di-esters are more potent than the tested mono-esters. This is also supported by the results of the metabolism studies, in which high metabolism rates were found for lasiocarpine, echimidine and senecionine compared to the mono-esters, and high rates of ring-dehydrogenated pyrrole metabolites were quantified. Steric hindrance of the side chains, especially the length and branching, of the di-esters seems to be crucial to prevent enzymatic hydrolysis. For lasiocarpine, an open-chained di-ester with 7*S* configuration and all investigated cyclic di-esters, except monocrotaline, an iREP factor of 1.0 can be confirmed. Especially the structural feature of monocrotaline, with only five carbon atoms in the side chain, seems to play a crucial role compared to the other cyclic di-esters with six carbon atoms in the side chain. In terms of structural features, we also found that the hydrophilicity of the side chains could play an important role: the more hydroxyl groups present, the higher the renal excretion. In general, we could confirm the iREP factors proposed by Merz and Schrenk in most cases, with a few exceptions. In both the micronucleus assay and cytotoxicity experiments, echimidine was more toxic and monocrotaline was clearly less toxic than expected from the iREP factors. This deviation is probably due to PA specific differences in toxicokinetics, which cannot be simulated by a simple liver cell model, which means that, among other things, further studies on PA toxicokinetics are necessary to refine the concept

of iREP factors. Especially, physiologically based pharmacokinetic (PBPK) modeling studies could help to better understand ADME of individual structurally different PA congeners. The results for heliotrine regarding cytotoxicity and genotoxicity in HepG2 C9 cells indicate that CYP3A4 appears to play a key role in metabolic activation of heliotrine. Because of that more studies are needed to investigate which CYPs are responsible for the metabolic activation of certain PA. The primary rat hepatocytes as well as HepG2 C9 cells are well suited for studying PA hepatotoxicity. However, the results also indicate that other test systems, such as primary human hepatocytes, could be useful for hepatotoxicity testing. Furthermore, we showed that the connection of toxicological endpoints with the corresponding metabolic pattern contribute considerably to the elucidation of the structure-dependent toxicity of the individual PA congeners.

In general, our generated results have made an important contribution to the risk assessment of PA and improved the scientific basis of the iREP factors. Further, the assumption that different PA congeners are not equally toxic could be confirmed.

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9. SUPPLEMENTARY DATA

9.1 Publication I: Structure-dependent hepato-cytotoxic potencies of selected pyrrolizidine alkaloids in primary rat hepatocyte culture

No supplementary data available. Only the forms for the disclosure of potential conflicts of interest are attached as an appendix.

9.2 Publication II: Structure-dependent genotoxic potencies of selected pyrrolizidine alkaloids in metabolically competent HepG2 cells

Table S1–S2 show raw data of ames fluctuation assay from all tested PA congeners in bacteria strains TA 98 and TA 100. Means and standard deviations from three independent experiments are given.

Table S1. Fluctuation Ames test in *S. typhimurium* TA98. The data represent means and standard deviations (S.D.) from n= 3 independent experiments.

Compound	Concentration [µM]	- S9-mix positive wells [%]	S. D. [%]	+ S9-mix positive wells [%]	S. D. [%]
echimidine	1	6.94	2.60	2.08	1.70
	5	4.17	1.70	1.39	0.98
	25	5.56	3.93	1.39	1.96
	75	2.08	2.95	2.78	0.98
	150	0.69	0.98	2.78	1.96
	300	6.25	3.40	4.17	3.40
europine	1	2.78	2.60	2.78	0.98
	5	5.56	0.98	4.17	1.70
	25	7.64	0.98	0.69	0.98
	75	4.86	3.54	1.39	1.96
	150	1.39	1.96	2.78	0.98
	300	5.56	3.54	3.47	0.98
heliotrine	1	9.72	5.20	2.78	2.08
	5	4.17	2.95	3.47	2.71
	25	5.56	0.98	2.08	1.73
	75	2.08	2.95	3.47	2.71
	150	4.86	1.96	4.17	3.45
	300	2.78	0.98	4.17	2.33
indicine	1	2.08	2.77	2.77	3.18
	5	0.69	0.69	0.69	1.20
	25	2.08	2.08	2.08	2.08
	75	0.69	0.69	0.69	1.20
	150	0.69	1.38	1.38	2.40
	300	2.77	0.69	0.69	1.20
lasiocarpine	1	2.08	2.08	0.00	0.00
	5	0.69	1.20	1.38	1.20
	25	2.77	2.40	3.47	1.20
	75	1.38	1.20	2.08	0.00
	150	0.00	0.00	0.69	1.20
	300	1.38	2.40	2.08	2.08
lycopsamine	1	2.08	3.60	1.38	1.20
	5	2.08	0.00	0.00	0.00
	25	2.08	2.08	0.69	1.20
	75	1.38	1.20	1.38	1.20
	150	0.00	0.00	0.69	1.20
	300	0.00	0.00	1.38	1.20
monocrotaline	1	6.25	4.50	4.17	2.95
	5	4.17	4.50	5.56	3.54
	25	1.39	0.98	2.08	1.70
	75	1.39	0.98	2.78	2.60
	150	1.39	0.98	2.08	2.95
	300	2.08	1.70	2.78	2.60
retrorsine	1	0.00	0.00	2.08	2.08
	5	3.47	1.20	2.08	2.08
	25	1.38	2.40	0.00	0.00
	75	1.38	2.40	1.38	2.40
	150	2.08	2.08	0.69	1.20
	300	2.77	3.18	0.69	1.20
riddelliine	1	2.08	1.70	1.39	0.98
	5	3.47	0.98	2.08	1.70
	25	4.17	1.70	3.47	0.98
	75	3.47	2.60	2.08	1.70
	150	3.47	0.98	3.47	0.98
	300	7.64	2.60	2.78	0.98
senecionine	1	1.38	1.20	0.69	1.20
	5	3.47	3.18	1.38	2.40
	25	2.77	1.20	1.38	1.20
	75	0.69	1.20	2.08	0.00
	150	2.08	0.00	2.77	1.20
	300	0.00	0.00	2.77	2.40
seneciphylline	1	2.78	3.93	1.39	0.98
	5	0.00	0.00	2.08	1.70
	25	2.08	1.70	0.69	0.98
	75	0.69	0.98	0.69	0.98
	150	2.78	2.60	2.78	1.96
	300	2.78	2.60	0.69	0.98

Table S2. Fluctuation Ames test in *S. typhimurium* TA100. The data represent means and standard deviations (S.D.) from n= 3 independent experiments.

Compound	Concentration [µM]	- S9-mix positive wells [%]	S. D. [%]	+ S9-mix positive wells [%]	S. D. [%]
echimidine	1	9.03	2.60	3.47	0.98
	5	6.25	3.40	9.03	2.60
	25	9.03	0.98	2.78	0.98
	75	7.64	5.20	5.56	1.96
	150	7.64	2.60	6.25	1.70
	300	6.94	2.60	4.86	2.60
europine	1	8.33	3.40	9.03	2.60
	5	6.94	3.54	7.64	0.98
	25	10.42	1.70	4.86	2.60
	75	15.28	0.98	6.25	1.70
	150	13.19	3.54	6.25	1.70
	300	10.42	3.40	4.17	1.70
heliotrine	1	5.56	3.54	3.47	0.98
	5	9.03	3.54	5.56	1.96
	25	4.86	2.60	6.94	4.91
	75	8.33	4.50	3.47	1.96
	150	4.17	3.40	5.56	1.96
	300	11.11	1.96	7.64	3.93
indicine	1	2.08	2.08	2.77	3.18
	5	2.77	3.18	1.38	2.40
	25	3.47	2.40	1.38	2.40
	75	2.08	3.60	1.38	2.40
	150	1.38	1.20	3.47	2.40
	300	4.16	3.60	6.25	2.08
lasiocarpine	1	2.08	2.08	2.60	1.04
	5	2.77	1.20	3.12	2.68
	25	4.86	3.18	6.25	2.94
	75	2.08	2.08	6.77	3.12
	150	3.47	1.20	7.29	3.60
	300	4.16	4.16	6.77	4.61
lycopsamine	1	4.16	2.08	4.86	3.18
	5	2.77	2.40	0.69	1.20
	25	4.86	3.18	3.47	4.33
	75	4.86	4.81	1.38	1.20
	150	0.69	1.20	3.47	4.33
	300	4.16	2.08	4.16	3.60
monocrotaline	1	6.94	1.96	8.33	2.95
	5	7.64	0.98	6.94	3.54
	25	6.94	5.47	7.64	3.93
	75	10.42	1.70	3.47	0.98
	150	12.50	1.70	2.78	0.98
	300	9.03	2.60	4.17	1.70
retrorsine	1	3.47	1.20	1.56	1.99
	5	5.55	2.40	3.64	2.62
	25	5.55	4.33	5.72	3.55
	75	3.47	4.33	5.72	3.55
	150	6.25	2.08	4.16	3.80
	300	4.16	2.08	6.77	1.99
riddelliine	1	9.03	2.60	2.78	1.96
	5	6.94	0.98	2.08	1.70
	25	10.42	1.70	2.08	1.70
	75	11.81	5.47	2.78	0.98
	150	11.81	3.54	6.94	0.98
	300	11.11	2.60	6.94	0.98
senecionine	1	6.25	3.60	2.77	1.20
	5	6.25	5.51	4.16	2.08
	25	4.86	4.81	6.25	3.60
	75	5.55	1.20	6.25	7.51
	150	4.16	2.08	6.94	5.24
	300	5.55	1.20	6.94	6.01
seneciphylline	1	4.17	2.95	7.64	6.87
	5	4.86	1.96	7.64	5.20
	25	1.39	1.96	6.25	3.40
	75	10.42	4.50	7.64	6.44
	150	9.03	5.47	7.64	2.60
	300	5.56	3.54	6.25	4.50

9.3 Publication III

Figures S1–S6 show amounts of detected metabolites after incubation of all six tested PA congeners with liver microsomes (left side) and primary hepatocytes (right side). Reactive metabolites are shown in red, all other metabolites are shown in gray. Data of liver microsomes are taken from Geburek et al., 2020b.

The metabolic pattern of hepatotoxic pyrrolizidine alkaloids in liver cells

- Supporting Information -

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Keywords

Pyrrolizidine alkaloids, metabolic pattern, primary rat hepatocytes, HepG2 cells, genotoxicity

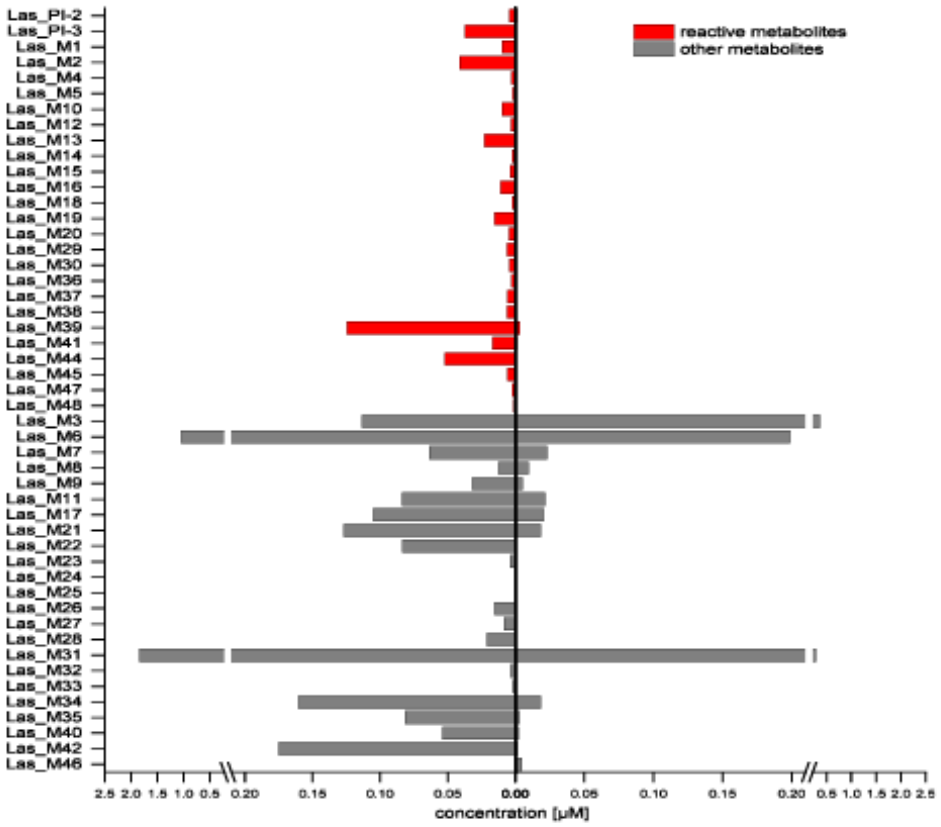

Figure S1: Amount of detected metabolites after incubation of 15 µM lasiocarpine with rat liver microsomes for 60 minutes[1] (left side) and after incubation of 2.5 µM lasiocarpine with primary rat hepatocytes for 24 hours (right side). Reactive metabolites are shown in red, all other metabolites are shown in gray. [1]Data taken from Geburek et al.[43]

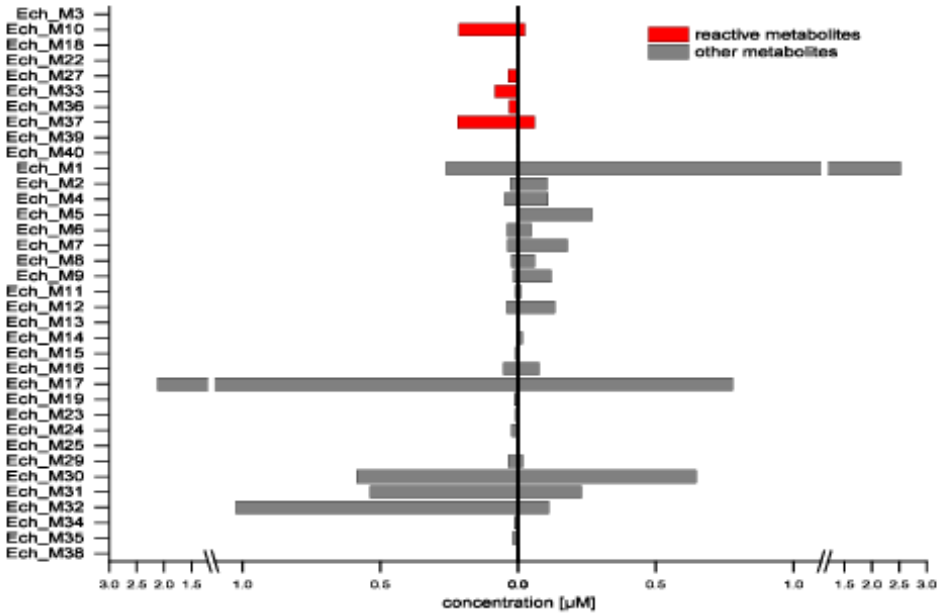

Figure S2: Amount of detected metabolites after incubation of 15 µM echimidine with rat liver microsomes for 60 minutes[1] (left side) and after incubation of 25 µM echimidine with primary rat hepatocytes for 24 hours (right side). Reactive metabolites are shown in red, all other metabolites are shown in gray. [1]Data taken from Geburek et al.[43]

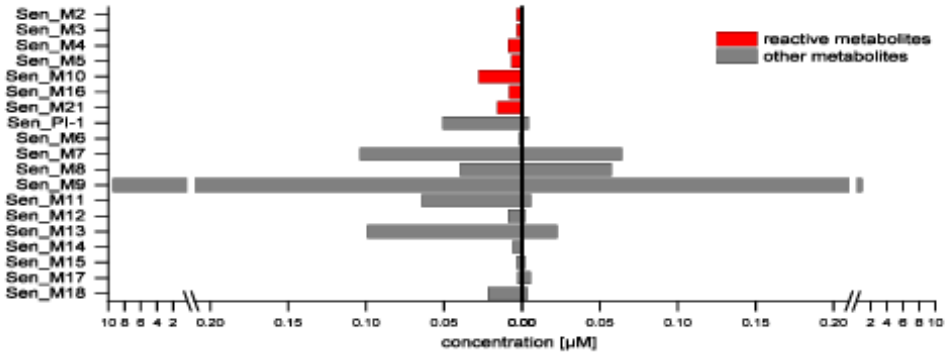

Figure S3: Amount of detected metabolites after incubation of 15 µM senecionine with rat liver microsomes for 60 minutes[1] (left side) and after incubation of 2.5 µM senecionine with primary rat hepatocytes for 24 hours (right side). Reactive metabolites are shown in red, all other metabolites are shown in gray. [1]Data taken from Geburek et al.[43]

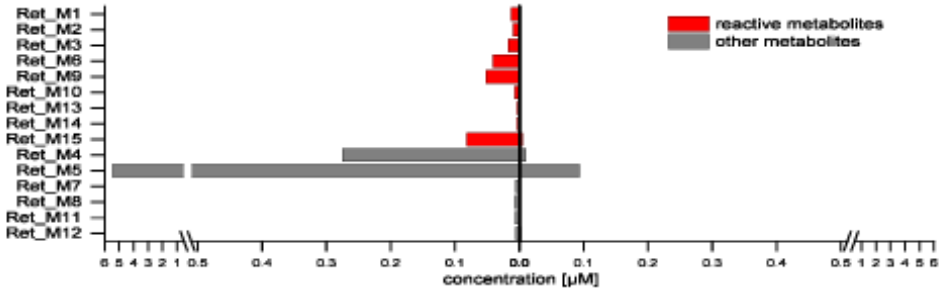

Figure S4: Amount of detected metabolites after incubation of 15 µM retrorsine with rat liver microsomes for 60 minutes[1] (left side) and after incubation of 5 µM retrorsine with primary rat hepatocytes for 24 hours (right side). Reactive metabolites are shown in red, all other metabolites are shown in gray. [1]Data taken from Geburek et al.[43]

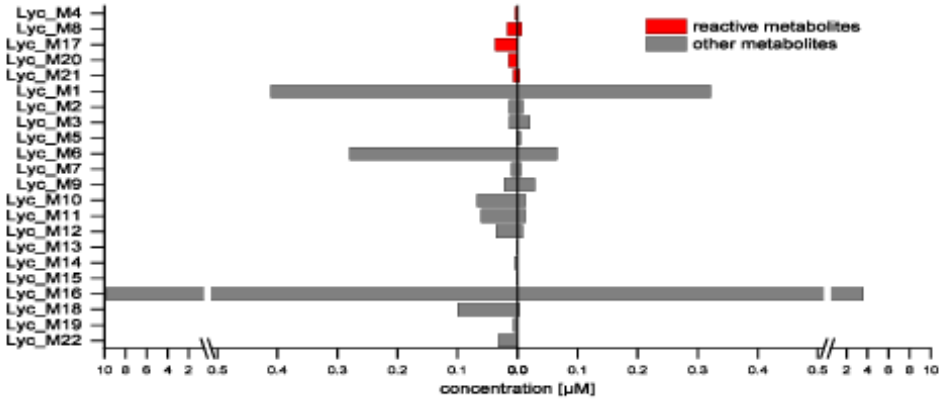

Figure S5: Amount of detected metabolites after incubation of 15 µM lycopsamine with rat liver microsomes for 60 minutes[1] (left side) and after incubation of 25 µM lycopsamine with primary rat hepatocytes for 24 hours (right side). Reactive metabolites are shown in red, all other metabolites are shown in gray. [1]Data taken from Geburek et al.[43]

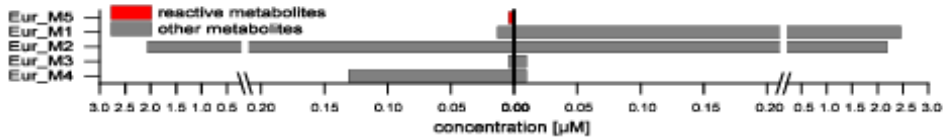

Figure S6: Amount of detected metabolites after incubation of 15 µM europine with rat liver microsomes for 60 minutes[1] (left side) and after incubation of 25 µM europine with primary rat hepatocytes for 24 hours (right side). Reactive metabolites are shown in red, all other metabolites are shown in gray. [1]Data taken from Geburek et al.[43]

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CONGRESS CONTRIBUTIONS

Parts of this work were presented oral or as poster in the following congress contributions:

Oral presentations:

PA-Workshop Toxicity and risk assessment of pyrrolizidine alkaloids current status and way forward, 12.09-13.09.2018, Kaiserslautern
Toxicological risk assessment of pyrrolizidine alkaloids - Investigation of cytotoxicity and mutagenicity of individual congeners
Lukas Rutz, Lan Gao, Karl-Heinz Merz und Dieter Schrenk

Phytotherapiekongress, 20.09.2019, Münster
Zytotoxizität ausgewählter Pyrrolizidinalkaloide (PAs) in primären Rattenhepatozyten
Lukas Rutz, Lan Gao und Dieter Schrenk

Poster presentations:

54th Congress of the European Societies of Toxicology, 08.09-11.09.2019, Helsinki
Toxicological risk assessment of pyrrolizidine alkaloids - Investigations of the hepatotoxic and genotoxic potential
Lukas Rutz, Lan Gao, Jan-Heiner Küpper und Dieter Schrenk

84th Annual Meeting of the German Society for Experimental and Clinical Pharmacology and Toxicology (DGPT), 25.02-28.02.2019, Stuttgart
Investigations of the hepatotoxic potential of selected pyrrolizidine alkaloids and synthesis of dehydropyrrolizidine alkaloid glutathione adducts
Lukas Rutz, Lan Gao, Karl-Heinz Merz und Dieter Schrenk

83rd Annual Meeting of the German Society for Experimental and Clinical Pharmacology and Toxicology (DGPT), 26.02-01.03.2018, Göttingen
Investigations of hepatotoxicity in the Alamar blue assay and test for mutagenic potential in the Ames fluctuation assay
Lukas Rutz, Lan Gao, and Dieter Schrenk

CURRICULUM VITAE

Lukas Rutz

BERUFLICHER WERDEGANG	
06/2022	Regulatorischer Toxikologe, BASF SE
03/2021 - 05/2022	Stellvertretender Laborleiter, Fa. Hochwald
04/2017 - 06/2020	Promotion im AK Prof. Dr. Dr. Schrenk (Fachbereich Lebensmittelchemie und Toxikologie, TU Kaiserslautern) „*In vitro* investigations on the hepatotoxicity and genotoxicity of food relevant pyrrolizidine alkaloids (PA)“
10/2016 - 04/2017	Diplomarbeit im AK Prof. Dr. Dr. Schrenk „Analyse von Muconsäure im *in vitro* Metabolismus von Benzen“
04/2012 - 04/2017	Studium der Lebensmittelchemie (Diplom-Studiengang), TU Kaiserslautern
03/2011	Allgemeine Hochschulreife Sickingen-Gymnasium, Landstuhl

WEITERBILDUNGEN	
	Fortbildungskurse zum Fachtoxikologen der Deutschen Gesellschaft für Pharmakologie und Toxikologie (DGPT):
09/2017	Lebensmitteltoxikologie TU Kaiserslautern
02/2018	Grundlagen der Organtoxikologie und -pathologie I Institut für Pharmakologie und Toxikologie, Erfurt
03/2018	Molekulare Zelltoxikologie Helmholtz Zentrum, München
03/2018	Klinische Toxikologie Universität des Saarlandes, Homburg
08/2019	Fremdstoffmetabolismus / Toxikokinetik Institut für Pharmakologie und Toxikologie, Zürich
10/2019	10/2019 Versuchstierkunde I /Biostatistik Institut für Pharmakologie und Toxikologie, Erfurt
10/2016 - 03/2017	Englisch-Sprachkurs „C1 Business Conversation“
07/2020	Statistik-Seminar „Introduction into statistics and statistical thinking“

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Danke Mama und danke Papa!

Danke Laura und danke Leo!

DECLARATION OF INTEREST

Hiermit erkläre ich, Lukas Rutz, dass ich die vorliegende Dissertation mit dem Titel „*In vitro* investigations on the hepatotoxicity and genotoxicity of food relevant pyrrolizidine alkaloids“ selbstständig verfasst und nur die in der Arbeit angegebenen Quellen und Hilfsmittel genutzt habe. Alle Ergebnisse anderer Personen wurden entsprechend gekennzeichnet.

Die vorliegende Dissertation habe ich weder im Ganzen noch in Teilen als Prüfungsarbeit bei einem anderen Fachbereich eingereicht und kein anderes Promotionsverfahren bei einer anderen Hochschule beantragt oder eröffnet. Die geltende Promotionsordnung des Fachbereichs Chemie der Technischen Universität Kaiserslautern vom 25. März 2014 ist mir bekannt.

Kaiserslautern, den 05.10.2022 _____

(Lukas Rutz)