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Toxins in Botanical Drugs and Plant-derived Food and Feed – from Science to Regulation: A Workshop Review

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Key words
Alkenylbenzenes, carcinogenicity, genotoxicity, pyrrolizidine alkaloids, relative toxic potency, risk assessment

received September 17, 2023
accepted after revision November 23, 2023
published online January 10, 2024

Bibliography
Planta Med 2024; 90: 219–242
DOI 10.1055/a-2218-5667
ISSN 0032-0943

Georg Thieme Verlag KG, Rüdigerstraße 14, 70469 Stuttgart, Germany

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ABSTRACT

In September 2022, the 3rd International Workshop on pyrrolizidine alkaloids (PAs) and related phytotoxins was held on-line, entitled 'Toxins in botanical drugs and plant-derived food and feed – from science to regulation'. The workshop focused on new findings about the occurrence, exposure, toxicity, and risk assessment of PAs. In addition, new scientific results related to the risk assessment of alkenylbenzenes, a distinct class of herbal constituents, were presented. The presence of PAs and alkenylbenzenes in plant-derived food, feed, and herbal medicines has raised health concerns with respect to their acute and chronic toxicity but mainly related to the genotoxic and carcinogenic properties of several congeners. The compounds are natural constituents of a variety of plant families and species widely used in medicinal, food, and feed products. Their individual occurrence, levels, and toxic properties, together with the broad range of congeners present in nature, represent a striking challenge to modern toxicology. This review tries to provide an overview of the current knowledge on these compounds and indicates needs and perspectives for future research.

Introduction

In September 2022, the 3rd International Workshop on Pyrrolizidine Alkaloids (PAs) and Related Phytotoxins was held on-line, entitled 'Toxins in botanical drugs – from science to regulation'. As with the two preceding workshops, this workshop aimed at presenting new findings on the occurrence, exposure, toxicity, and risk assessment of PAs. In addition, it was intended to cover new scientific results related to the risk assessment of alkenylbenzenes, a class of natural plant constituents present in food, feed, and herbal medicines. This widening of the scope of the conference was in recognition of the increasing concerns and subsequent numerous scientific investigations related to natural alkenylbenzenes. The conclusions presented here attempt not only to summarize the reported findings across these two topics but also intend to highlight the presumptive consequences for future

ABBREVIATIONS

ALARA	as low as reasonably achievable
API	active pharmaceutical ingredient
AUC	area under the curve
BMC	benchmark concentration
BMD	benchmark dose
BMD	lower confidence limit
CI	confidence interval
CYP	cytochrome P450
DHP	dehydropyrrolizdine
DHPA	dehydro-PA
DSS	dextran sulphate sodium
EFSA	European Food Safety Authority
EMA	European Medicines Agency
GLP	good laboratory practice
γH2AX	histone 2AX, phosphorylated form
HMP	herbal medicinal product
HMPC	Herbal Medicinal Products Committee
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
iREP	interim relative potency as defined in 11
LC-MS	liquid chromatography–mass spectrometry
LC-MS/MS	liquid chromatography–tandem mass spectrometry
LogD	log distribution coefficient
NAM	new and alternative approach methodology
NOAEL	no-observed-adverse-effect level
NOEL	no-observed-effect level
OECD	Organisation for Economic Co-operation and Development
PANO	pyrrolizidine alkaloid *N*-oxide
PA	pyrrolizidine alkaloid
PBK	physiologically based kinetic
Ph. Eur.	European Pharmacopoeia
PHH	primary human hepatocytes
PoD	point of departure
REP	relative potency
rSCH	rat sandwich culture hepatocytes
SCCS	Scientific Committee on Consumer Safety
SULT	sulfotransferase

steps in risk management and regulation in the field of public health for both classes of compounds.

PAs are found in food of plant origin, food supplements, feed, and herbal medicines [1–3]. The reported contents may reach levels that raise a health concern and warrant a refinement of the current risk assessment. To this end, a better understanding not only of the mechanism of action of PAs but also of their structure-dependent toxicokinetic and toxicodynamic properties is key to such efforts. A refined species comparison (animal species vs. human) may help to explore the quantitative meaning of animal experiments for human risk assessment and improve the basis for a risk analysis for animal species including farm animals.

The study of cases of acute intoxication is of relevance for farm animals [4], but these cases may also reflect a risk for humans [1]. The possible risk from chronic exposure to PAs is a key question, e.g., for manufactured and/or traded food products, sometimes transported and distributed on a large scale. Here, the tracing of sources of contamination such as the origin of PAs present, e.g., in tea (*Camellia sinensis* L., *Theacea*) [5], one of the most important food items, needs special attention.

Both in humans and animals, the chronic toxicity of PAs is of particular interest since hepatotoxic and genotoxic events may accumulate over time and result in irreversible damage such as chronic liver failure or cancer (see below).

Asteraceae, Boraginaceae, and *Fabaceae* are the major plant families comprising large numbers of PA-synthesizing species [3]. Overall, more than 600 different PAs (congeners) have been found in nature, having as their major structural constituents a necine base (pyrrolizidine ring) and one or more necic acid(s) bound to the necine base via ester bridges. The PAs can be subdivided into monoesters, open-chained diesters, and cyclic diesters. All toxicologically relevant PAs bear a double bond in the 1,2-position of the necine base and require further metabolic oxidation of the pyrrolizidine ring to be activated to the reactive, toxic pyrrolic metabolites [6].

Damage to liver sinusoidal endothelial cells and hepatocytes is the most prominent adverse effect seen with PAs upon acute, sub-acute, and chronic exposure [7]. In humans and animals, necrosis of sinusoidal endothelial cells leads to veno-occlusive disease and liver failure [8], while pulmonary venous endothelial cells may also be affected. In laboratory rodents treated chronically with certain PAs, malignant tumors of the liver such as liver cell carcinoma and hemangio-endothelial sarcoma can develop [6]. Furthermore, the occurrence of extra-hepatic tumors, e.g., in the lung, the pancreas, and the intestine, has been described [1].

The reactive metabolites formed, i.e., the dehydro-pyrrolizidine (DHP) esters and/or the de-esterified DHP, the so-called pyrrolic metabolites, can undergo covalent binding to nucleophilic targets such as proteins and nucleic acids (reviewed in [7]). In fact, a number of PAs are genotoxic in bacteria, insects, and mammalian cells *in vitro*. Reaction with cellular targets is also considered responsible for non-neoplastic cell damage. The formation of reactive pyrrolic metabolites depends on an oxidative metabolism mostly catalyzed by members of the cytochrome P450 (CYP) enzyme family. Although hepatocytes are most probably the primary site of metabolic activation, secondary metabolites may be released from the hepatocytes and may affect other cell types. Furthermore, direct activation in other non-hepatic tissues may occur. The group of PA *N*-oxides (PANOs), representing products of an alternative but reversible type of oxygenation, has been the subject of recent investigations. Although not electrophilic themselves, PANO reduction can re-constitute the parent PA [9], which may then be activated following the classical ring-oxygenation pathway. Thus, the toxic potency of PANOs may be lower than that of the parent PA, although the difference is likely to depend on the actual conditions, such as the redox state of the chemical milieu, or on the reductase activities in intestinal bacteria and in tissues of the host.

Current risk assessment of PAs is focused on the formation of reactive intermediates being responsible for both cell damage and genotoxicity. The broad spectrum of PAs occurring in nature makes it difficult, however, to identify the relative toxic potencies of all individual PAs. Furthermore, a concise dose-response analysis of adverse effects including low, more relevant dose levels is challenging. The use of rodent data implies the need for a scientifically sound extrapolation to humans illustrating the requirements for an adequate use of human *in vitro* data in risk assessment. This includes the employment of toxicokinetic methodologies such as physiologically based kinetic (PBK) modeling. The application of *in vitro* data in the quantitative risk assessment of individual PA congeners, taking into account both their toxicokinetic and toxicodynamic properties, is a challenge being addressed but also a possible paradigm to apply to other groups of chemicals.

Currently, a carcinogenicity study in rats with the congener riddelliine is used as the basis for the assessment of the chronic cancer risk for all 1,2-unsaturated PAs 1, [10]. Since the marked differences in the cytotoxic and genotoxic properties between individual PAs have been widely described, this approach is scientifically problematic. Merz and Schrenk [11] have addressed this problem by suggesting interim relative potency (iREP) factors to describe the toxic and genotoxic potency of 1,2-unsaturated PAs. Briefly, this approach is based on fundamental structural considerations together with acute toxicity data from rodents, cytotoxicity data in mammalian cell culture, and genotoxicity data in *Drosophila*. The use of *in vitro* data on the toxicity and genotoxicity of PAs has been hampered, however, by a lack of data on their toxicokinetics in intact organisms. In addition, more work is needed on the detailed mode of action (MoA) of highly toxic and carcinogenic 1,2-unsaturated congeners. Furthermore, as mentioned previously, PANOs may need special considerations since the intestinal microflora are able to substantially convert *N*-oxides into the more toxic parent PAs after oral exposure. Additionally, *N*-oxides can also be reduced by the tissues and cells of the host [9].

Aspects on PA Occurrence: Stability and Transfer from Feed to Food Products

PAs are secondary metabolites produced by a wide variety of plants. They play an important role as defense compounds against attack by herbivores (insects and mammalians) and against fungal and bacterial pathogens. PAs occur in a wide structural variety, and it is estimated that approximately 2–3 % of all flowering plants are capable of producing PAs.

As PA-containing plants have a global distribution, they will also be present in areas where feed commodities are grown. When not properly removed, these weeds will end up in the fodder for livestock or in ingredients used for animal feed. Not only can this lead to adverse health effects in livestock, it is also one of the routes by which PAs can enter the food chain [12]. After ingestion by the animal, PAs are exposed to specific conditions in the gastrointestinal tract. Metabolism will take place in the liver, followed by excretion via urine and feces. Nevertheless, traces of the PAs may eventually end up in products that are used for human consumption such as milk, eggs, and meat. Although the concentrations are expected to be rather low, due to the extensive metabolism in the animals, these products constitute an important part of the dietary intake of a large part of the population [13].

In the past decade, several studies have been conducted in which the transfer of PAs from feed to the animal-derived end products was addressed in more detail. Two studies looked into the transfer of PAs to cow milk [14, 15]. Both studies indicated limited overall transfer of PAs (less than 0.1 %), but this transfer is strongly related to the chemical structure of the PA. ▶ **Table 1** presents an overview of the calculated transfer rates for individual PAs. Some PAs, such as jacoline and otosenine, show relatively high transfer rates, while for many other PAs, rates are very low, indicating extensive metabolism in the animal. PANOs were practically absent and some newly formed PA metabolites could also be tentatively identified. Very low transfer rates have been observed as well for tropane alkaloids, which are also esters [16]. In contrast, the transfer of quinolizidine alkaloids from lupin to milk is substantially higher, suggesting a higher resistance toward the metabolism of this alkaloid type [17]. PA transfer to eggs was studied by exposing laying hens to feeds mixed with small portions of five different plant species [18]. Transfer rates in the same range as observed for milk were obtained (▶ **Table 1**). Jacoline and otosenine showed relatively high transfer rates, but this was also the case for retrorsine, usaramine, sceleratine, heliosupine, and rinderine. Overall, the transfer was low (< 0.1 %), and almost no PANOs were detected in eggs.

Very recently, the uptake of PAs by insects that are raised for food or feed applications have also been studied. Black soldier fly (BSF) larvae and lesser mealworm (LMW) larvae were raised on substrate spiked with simulated extracts of the five PA plants mentioned above [19]. It was noted that upon the addition of water to the feeds, the PA composition of the substrate changed; i.e., the PANOs were effectively converted to the corresponding parent PAs. The bioaccumulation factors were therefore based on the PA composition in the feed remaining at the end of the trial (▶ **Table 1**). For LMW larvae, high transfer rates, in the order of 28–63 %, were calculated for the heliotridine-type monoesters echinatine, europine, and rinderine. For the other PAs, as well as for BSF larvae, transfer rates were 10- to 100-fold lower.

In living plants, PAs are primarily present in their *N*-oxide form, as this facilitates their transport and distribution within plant tissues. However, physical, enzymatic, and microbiological factors may cause reduction or degradation of PANOs in biological matrices, e.g., during heating or storage [20]. The minimal transfer of PANOs to milk and eggs suggests a stability in the target animals that is even lower than that of PA-free bases. *In vitro* gastric rumen experiments showed that PANOs are efficiently reduced in the rumen to their parent PA form by microbial action within a few hours [15]. Subsequent metabolism of the parent PAs occurs in the liver. When excreted in milk, parent PAs are relatively stable, as thermal processing of milk and the preparation of dairy products had only a limited effect on the PA levels [21]. Microbial activity in the moist insect feeds was considered the most likely cause of why PANOs were efficiently reduced to the corresponding parent PAs.

In a general sense, these studies confirm the relatively low overall transfer of intact PAs from feed to the final products.

▶ **Table 1** Pyrrolizidine alkaloid transfer rates from feed to milk and egg and uptake rates from feed by insect larvae, in % of administered or consumed amount.

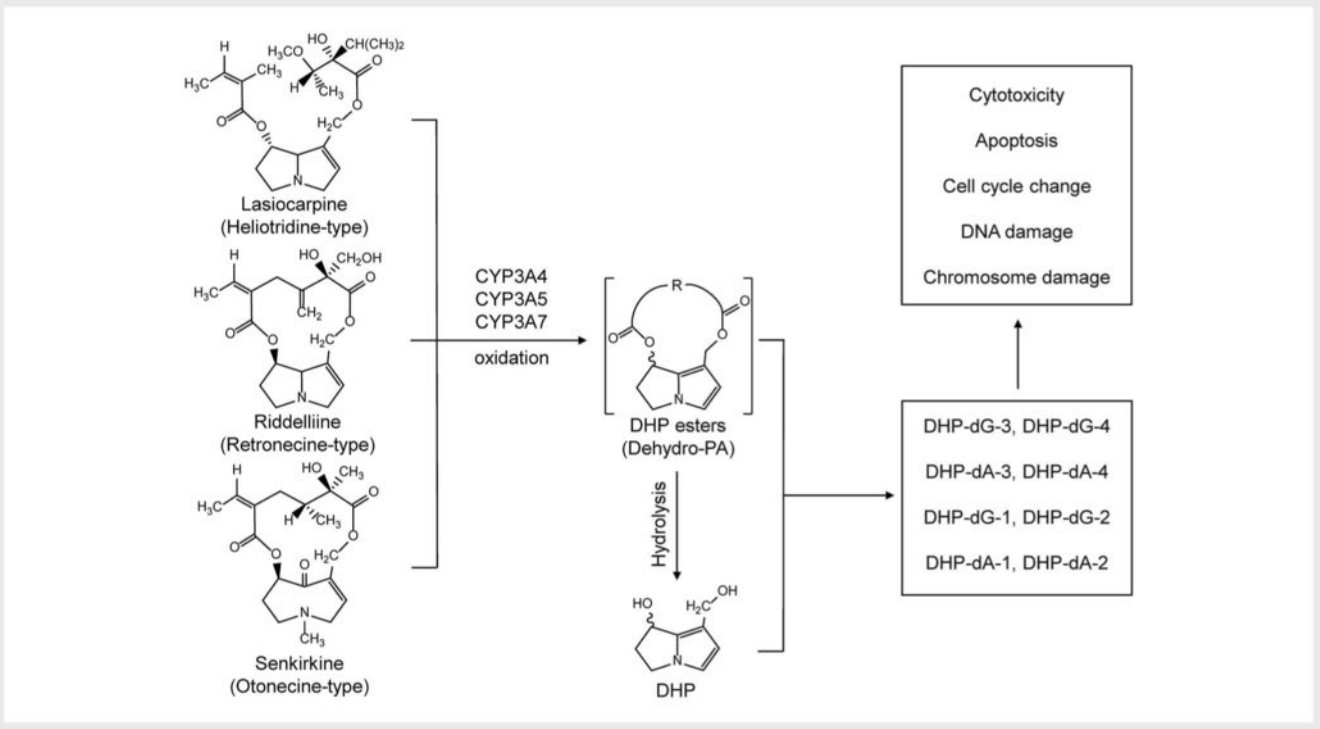
Pyrrolizidine alkaloid	Cow's milk [a] [15]	Egg [b] [18]	Black soldier fly larvae [c] [19]	Lesser meal worm larvae [c] [19]
Cyclic diesters				
Erucifoline	0.004	0.055	2.1	1.5
Florosenine	0.30			
Integerrimine	0.003–0.004	0.011–0.012	0.3–0.4	0.6–1.0
Jacobine	0.074	0.11	3.6	2.6
Jacoline	2.94	1.21	5.3	7.2–11.8
Jaconine	0.014			
Otosenine	0.73	0.40		
Riddelliine	0.017–0.083	0.074	1.2	0.6
Retrorsine	0.085–0.12	0.33–0.39	0.6–1.4	0.6–1.0
Sceleratine		0.98	1.8	3.9–5.3
Senkirkine	0.12	0.15	0.28	0.4
Senecionine	0.001	0.017–0.033	0.5–1.0	0.5–1.2
Seneciphylline	0.003–0.008	0.014–0.015	0.6–0.9	1.1–2.2
Senecivernine		0.042–0.043	0.4–0.5	0.7
Spartioidine		0.022		
Usaramine	0.038	0.23–0.49	0.9–1.5	1.2–1.4
Open-chained diesters				
Echimidine	0.29	0.013	0.7	0.5–0.8
Heliosupine		0.44		
Lasiocarpine		0.002	0.6	1.2
Mono esters				
Echinatine		0.037	3.2–4.4	28.5
Europine		0.15	4.1–4.5	50.8–63.0
Heliotrine		0.045	2.4	4.7–5.8
Lycopsamine	0.32			
Rinderine		0.35	3.8–4.1	23.9–32.2

[a] Cows given by gavage ground material of *Jacobaea vulgaris* (common ragwort), *Senecio vulgaris* (common groundsel), or *Echium vulgare* (vipers bugloss); [b] Laying hens given pelleted feed containing *J. vulgaris, S. vulgaris, S. inaequidens* (narrow-leaved ragwort), *Echium vulgare*, or *Heliotropium europaeum* (common or European heliotrope); [c] Larvae raised on substrate coated with PA mixtures simulating the composition and concentration of the feeds used in (b)

Nevertheless, the studies also show that the transfer rates of individual PAs can differ strongly, and it thus matters which PA-containing weeds are present as contaminant in the feed.

Human CYP Enzymes Account for the Metabolic Activation of PAs

It has been stated previously that PAs require metabolic activation to exert their genotoxicity [22]. For retronecine- and heliotridine-type PAs, there are three major metabolic pathways: 1) the hydrolysis of the ester functional groups to form the necine bases and the necic acids; 2) the *N*-oxidation of the necine bases to form PANOs; and 3) the hydroxylation of the necine bases by CYP enzymes followed by spontaneous dehydration to produce dehydro-PAs (DHPAs), which are then hydrolyzed into 1-hydroxymethyl-7-hydroxy-6,7-dihydropyrrolizine (DHP) in biological systems (▶ **Fig. 1**). The otonecine-type PAs requires an initial oxidative *N*-demethylation step, potentially catalyzed by CYPs, to produce DHPAs. Both DHPAs and DHP can react with DNA, generating adducts and inducing genotoxicity.



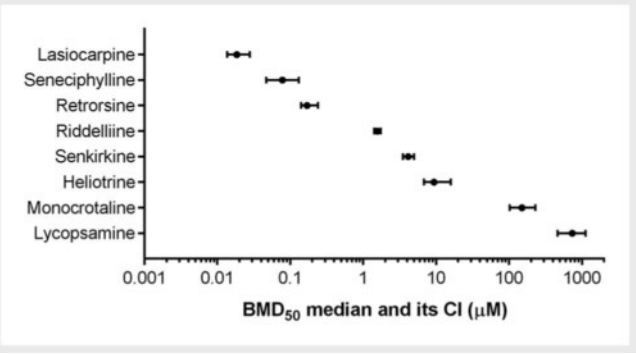
▶ **Fig. 1** Metabolic activation of lasiocarpine, riddelliine, and senkirkine by human CYP3A enzymes produces metabolites including DHP esters and DHP that react with DNA, leading to genotoxicity.

As for the specific types of CYPs, early animal studies showed CYP3A and 2B subfamilies were the major CYPs accounting for the biotransformation of PAs such as clivorine, lasiocarpine, and riddelliine [22]. By using human supersomes (microsomes prepared from insect cells infected with a virus engineered to express a CYP isoform), CYP3A4 and 3A5 were identified to be critical to activate retronecine-type PAs [23]. Monocrotaline was the only exception, which was mainly bioactivated by CYP2A6. Researchers have also been using mammalian cells transfected with CYP3A4 as a tool to evaluate the genotoxicity of various PAs [24, 25].

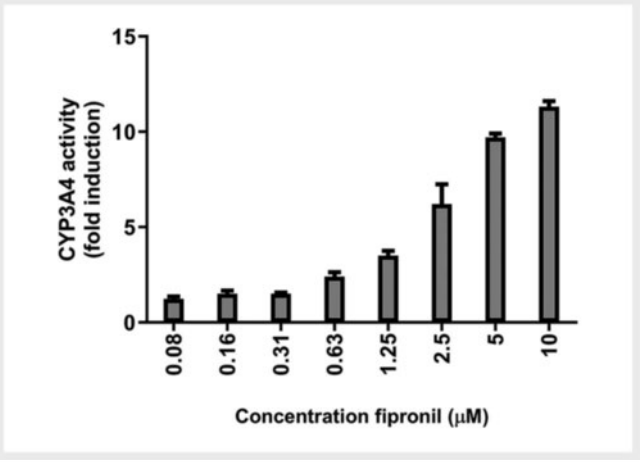
Some recent efforts have been undertaken to further identify the specific CYP enzymes that account for the bioactivation of PAs in genetically modified human lymphoblastoid TK6 cells [26]. By establishing a set of 14 TK6 cell lines, each expressing a single human CYP (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C18, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5, and 3A7) [27], the metabolism of lasiocarpine (heliotridine-type), riddelliine (retronecine-type), and senkirkine (otonecine-type) was evaluated. The LC-MS results revealed the formation of DHP, the main reactive metabolite of PAs, in CYP3A4-expressing TK6 cells exposed to all three PAs. DHP was also detected in CYP3A5- and 3A7-expressing cells after PA exposure, but to a much lesser extent. The other CYPs did not play a critical role in the biotransformation of the aforementioned PAs. The micronucleus assay, as well as the cell cycle analysis, also showed that PAs induced concentration-dependent genotoxicity in three CYP3A-variant-expressing TK6 cell lines. For relative potency analysis, a total of eight PAs were tested in CYP3A4-expressing TK6 cells (▶ **Fig. 2**). All PAs that tested positive for micronucleus induction after CYP3A4 bioactivation were ranked according to their genotoxic potency, calculated by using Bayesian benchmark dose (BMD) modeling and a critical effect size of 50%. The results showed that lasiocarpine was the most potent inducer of micronuclei, with a benchmark concentration (BMC) of 0.02 µM, while lycopsamine was the weakest among the PAs tested, with a BMC of 736 µM.

Similar to the results observed in TK6 cells, lasiocarpine was found to be the most potent PA in micronucleus induction in metabolically competent HepaRG cells, and lycopsamine was about two orders of magnitude less potent than lasiocarpine [28]. In HepG2 cells transfected with human CYP3A4, lasiocarpine and senkirkine were the most potent PAs in micronucleus induction, followed by seneciphylline, retrorsine, and riddelliine [25]. Heliotrine and lycopsamine, which are monoesters, were much weaker micronucleus inducers compared to the PAs with open or cyclic diesters in HepG2 cells. The potency ranking was almost identical (except for monocrotaline, which is metabolized by CYP2A6) to the findings in CYP3A4-expressing TK6 cells (▶ **Fig. 2**), suggesting the genotoxicity results are highly reproduceable across different types of human cells.

Overall, concordant results using different human-based systems have demonstrated that CYP3A enzymes were the main CYPs metabolizing PAs and that the genotoxicity of PAs varies by several orders of magnitude. The field of genetic toxicology is at a crossroads, transitioning from the traditional "screen-and-bin" process to include data suitable for quantitative risk assessment. Comparing the potency of PAs is becoming an increasingly impor-



▶ **Fig. 2** Bayesian benchmark dose (BMD) modeling evaluating the potency of PAs for micronucleus induction in CYP3A4-expressing TK6 cells. The BMD_{50} estimates represent a critical effect size of 0.5 (50 % increase above the controls). The bar represents the calculated lower and upper 95 % confidence interval (CI) for each BMD value.



▶ **Fig. 3** Induction of CYP3A activity upon exposure of HepaRG cells to increasing concentrations of fipronil.

tant component in risk assessment for regulatory decision-making, whereby the introduction of interim relative potency factors (iREP factors) can be a meaningful approach to effectively evaluate the risk of PAs based on their structures and the weight of genotoxicity evidence generated from different biological systems [11]. The details are described in the later sections ("Current status of the concept of relative potency factors for PAs: Perspectives and open questions").

Effects of Pesticides on the Genotoxicity of PAs

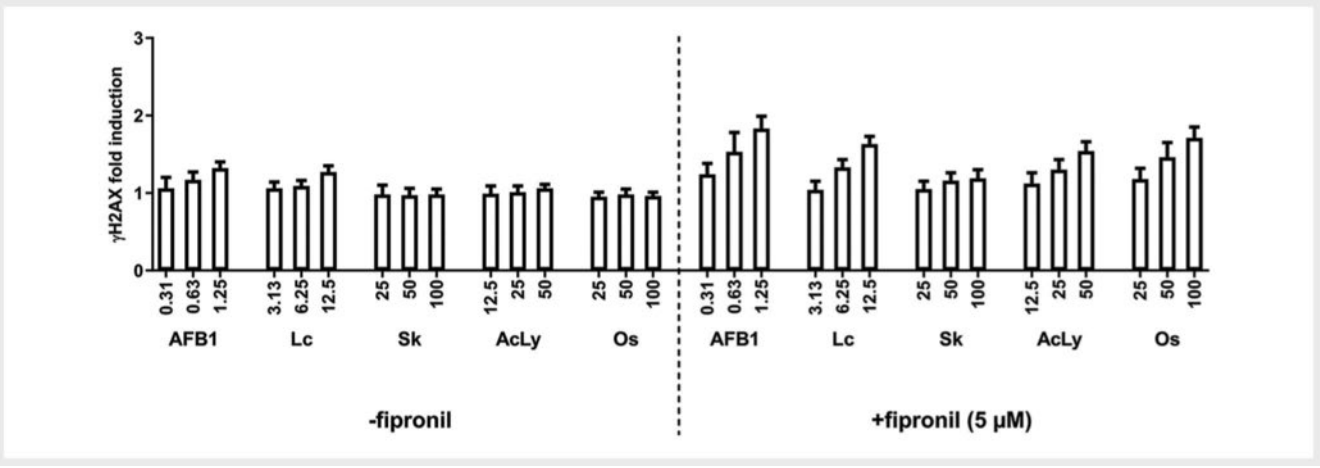
Previously, the relative genotoxic potencies of 37 PAs were determined *in vitro* in the metabolically competent human HepaRG liver cell line using the γH2AX assay, and it was found that potencies differed by several orders of magnitude [29]. The γH2AX/HepaRG assay was also applied together with analytical methods (LC-MS/MS) to extracts of European heliotrope (*Heliotropium europaeum*), and this work demonstrated the usefulness of an effect-directed analysis approach to identify less-known but potent PAs [30]. More recently, the γH2AX/HepaRG assay was used to assess whether co-exposure to pesticides could modulate the genotoxic effects of PAs (Peijnenburg et al., in preparation). The rationale of the latter research question was that some pesticides are known to induce the expression of CYP3A4, which is one of the main enzymes catalyzing the conversion of PAs into their toxic metabolites, via which the genotoxicity of PAs is exerted. Furthermore, a study on a PA intoxication incident in Tigray, Ethiopia, demonstrated that the observed hepatotoxicity was likely to be caused by co-exposure to the PA 7-acetyllycopsamine and the CYP3A4-inducing insecticide DDT [31].

In the PA–pesticide interaction study, a set of pesticides was first screened for their ability to induce CYP3A4 by exposing differentiated HepaRG cells to the pesticides for 48 h, followed by measurement of CYP3A4 activity using a CYP3A4 assay (Promega). Several pesticides, such as fipronil, o,p'-DDT, p,p'-DDT, acetamiprid, and thiacloprid, were found to increase CYP3A4 activity. For example, treatment of the cells with 5 μM fipronil resulted in a 10-fold induction of CYP3A4 activity (▶ **Fig. 3**). Subsequently, the effects of these pesticides on the genotoxicity of the PAs lasiocarpine, senkirkine, otosenine, and 7-acetyllycopsamine were determined with the γH2AX/HepaRG assay. In these experiments, HepaRG cells were first treated for 48 h without or with the pesticides, followed by a 24 h exposure to the PAs. Then γH2AX induction was determined using ≥ 1.5-fold above background as cutoff for genotoxicity, as applied previously [22]. Generally, all CYP3A4-inducing pesticides were shown to significantly increase the potencies of the studied PAs except for senkirkine in the γH2AX/HepaRG assay. This is illustrated for fipronil in ▶ **Fig. 4**. Altogether, the results suggest that certain pesticides (and other chemicals that induce CYP3A4) may potentiate the (geno)toxic effects of PAs upon-co-exposure.

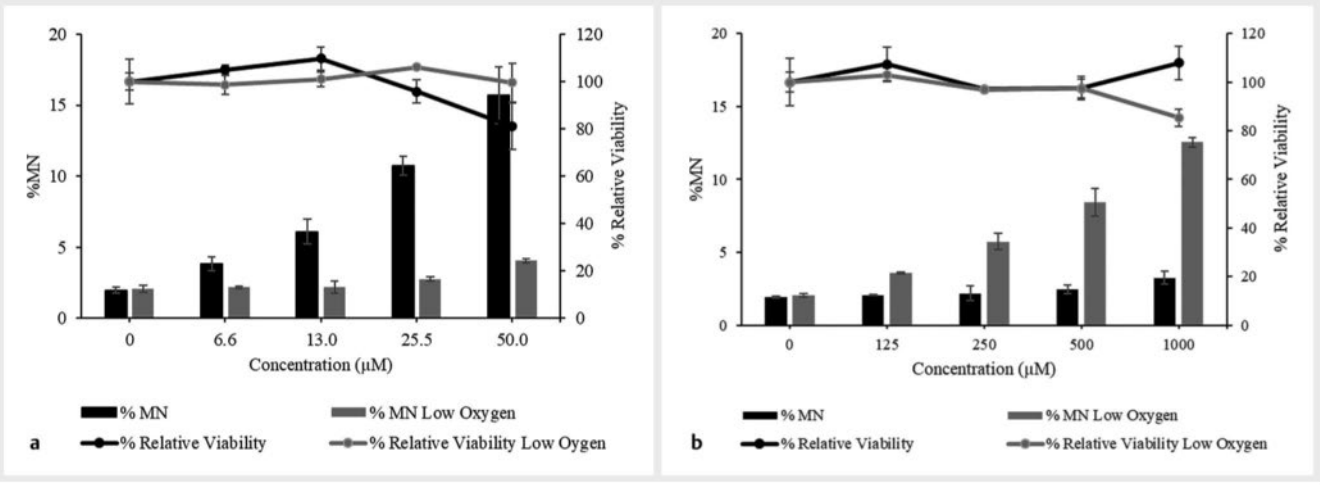
Whether such effects could take place at relevant human exposure levels depends on, amongst other factors, whether the critical internal concentrations required for CYP induction are reached, which requires further study.

Assessment of the Genotoxic Potency of PA *N*-oxides: Role of Oxygen Supply

Using the human hepatic cell line HepaRG, it has previously been demonstrated using the micronucleus assay [3] that the genotoxicity potency of 26 representative PAs varies significantly due to structural differences (▶ **Fig. 5**). In addition, this work demonstrated that PANOs are less potent inducers of DNA damage than their corresponding parent PA when evaluated using standard *in vitro* experimental conditions with ambient air (21 %) oxygen levels. However, one would expect PANOs to be more potent under low oxygen conditions that are representative of *in vivo* liver partial oxygen pressure since such conditions favor reduction reactions. In contrast, the parent PAs are expected to be less potent under these more biologically relevant low oxygen conditions when compared to ambient air, since their metabolic conversion to the DNA-reactive pyrrolic ester requires oxidative metabolism.



▶ **Fig. 4** Effect of fipronil on the induction of γH2AX by PAs. HepaRG cells were exposed to AFB1 (aflatoxin B1; genotoxic control), Lc (lasiocarpine), Sk (senkirkine), AcLy (7-acetyllycopsamine), and Os (otosenine) without (left side) or with (right side) pretreatment with fipronil.



▶ **Fig. 5** HepaRG cells were treated with heliotrine (**a**) and its *N*-oxide (**b**), and the relative viability (lines) and the occurrence of micronuclei (bars) are shown. Grey lines/bars represent results from experiments performed under more physiological low (2%) oxygen (O_2) conditions, while black lines/bars represent results from ambient (21%) oxygen conditions.

To investigate the impact of oxygen status, heliotrine and its *N*-oxide were evaluated under low oxygen (2%) and ambient oxygen (21%) conditions in the HepaRG micronucleus assay using the experimental conditions described [28], and the formation of heliotrine from its *N*-oxide was measured analytically. For the low-oxygen-condition experiments, HepaRG cells were acclimated for one hour in a separate incubator maintained at 2% oxygen. Cells were then exposed to the compounds for 24 h under these low oxygen conditions. After treatment, cells were returned to ambient oxygen conditions and exposed to human epidermal growth factor for 72 h to promote cell division, during which micronuclei may form.

Consistent with the expectation that metabolic activation to the DNA reactive form of the parent PAs will be reduced under low oxygen conditions, heliotrine showed a decrease in micronucleus induction under 2% oxygen conditions when compared to 21% (▶ **Fig. 5a**). The converse was true for heliotrine *N*-oxide, which showed an increase in potential for DNA damage at 2% oxygen compared to 21% oxygen (▶ **Fig. 5b**). These findings are in line with the expectation that low oxygen conditions favor reduction to the parent PAs and are further supported by the analytical results demonstrating that ~ 6× more heliotrine is formed from its *N*-oxide under 2% oxygen conditions compared to 21% oxygen (data not shown). While heliotrine and its *N*-oxide followed the expected pattern, initial data from further experiments seem to show that not all PA and PANO pairs follow the above outlined hypothesis regarding increased reduction under low oxygen conditions. Taken together, these initial data show that the use of phys-

iologically more relevant lower oxygen levels for *in vitro* experiments tend to reduce the potency of PAs, while they tend to increase the potency for PANOs. Further work will be needed to better understand the possible impact of oxygen partial pressure on the PA risk assessment.

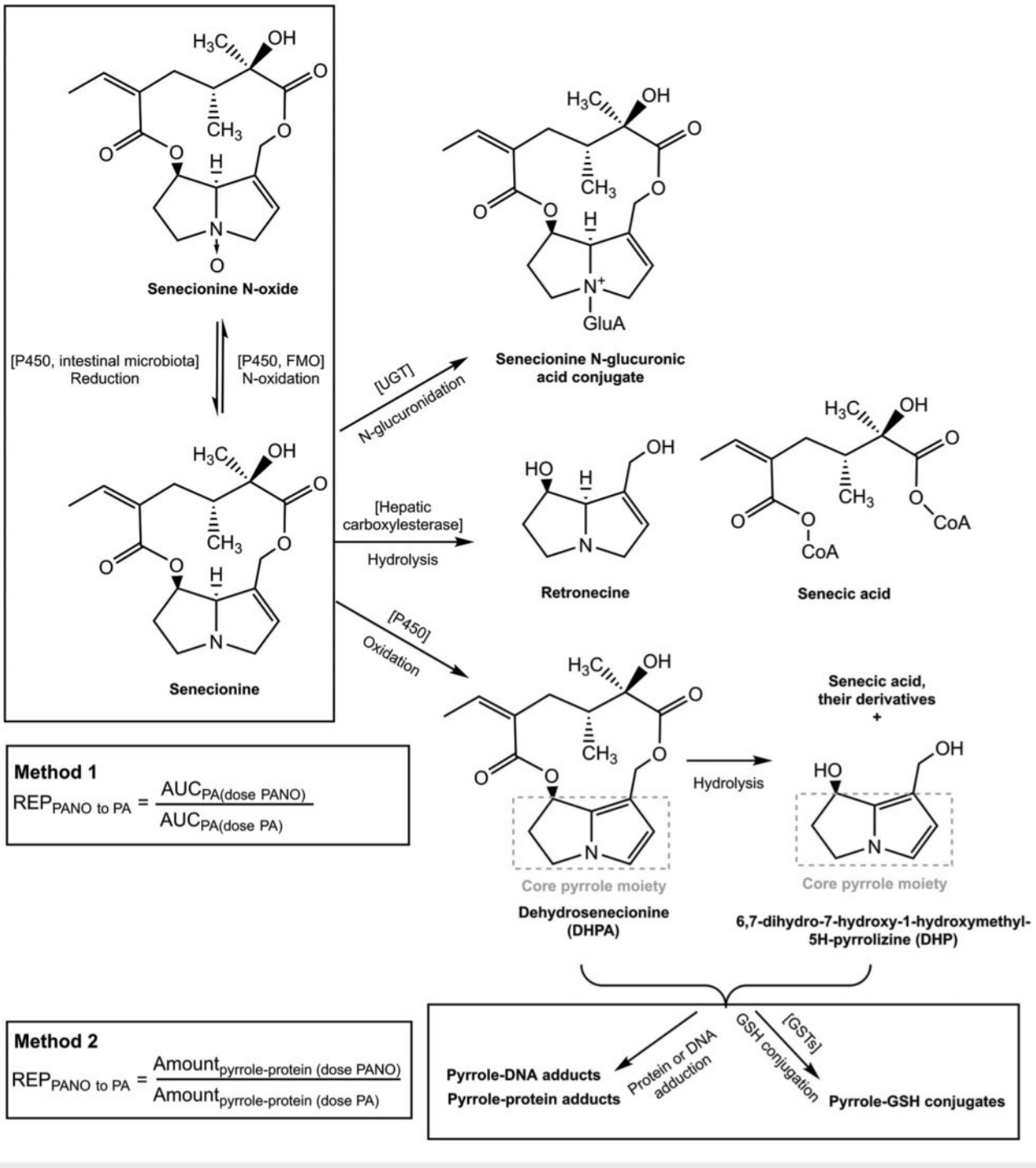
Relative Potency Factors of PA *N*-oxides at Realistic Low Dose Levels

PANOs are the predominant version of PAs in plants [32]. Upon consumption, PANOs are reduced mainly by intestinal microbiota and CYP enzymes in the liver to the parent PAs [9], which subsequently give rise to the formation of reactive pyrroles that can form protein- and DNA-pyrrole adducts. A $REP_{PANO\ to\ PA}$ factor can be defined as the ratio between the potency of a PANO and that of the parent PA. Currently, PANOs are assumed to be either equally toxic to the parent PA (i.e., having an $REP_{PANO\ to\ PA}$ factor of 1.0) [11], or to be substantially less potent than the parent PAs ($REP_{PANO\ to\ PA}$ factors < 0.1), the latter mainly based on studies in *in vitro* models [22, 31, 33]. Thus, the toxicity and, thereby, also the $REP_{PANO\ to\ PA}$ value of PANOs depend on the rate and extent of PANO reduction to the parent PA and other kinetic factors such as absorption that would influence what the liver is exposed to. Although the PANO reduction in the liver may be included in the *in vitro* cell model used, these models generally do not consider the PANO reduction by the intestinal microbiota, nor do they accommodate gastrointestinal uptake [34, 35]. Another factor that may influence the $REP_{PANO\ to\ PA}$ values is that the *in vivo* studies from which these $REP_{PANO\ to\ PA}$ values can be derived are generally performed at relatively high equimolar doses of the PANO and PA in order to enable detection of selected biomarkers, while estimated daily dietary intake for the human population occurs at dose levels that may be orders of magnitude lower [1].

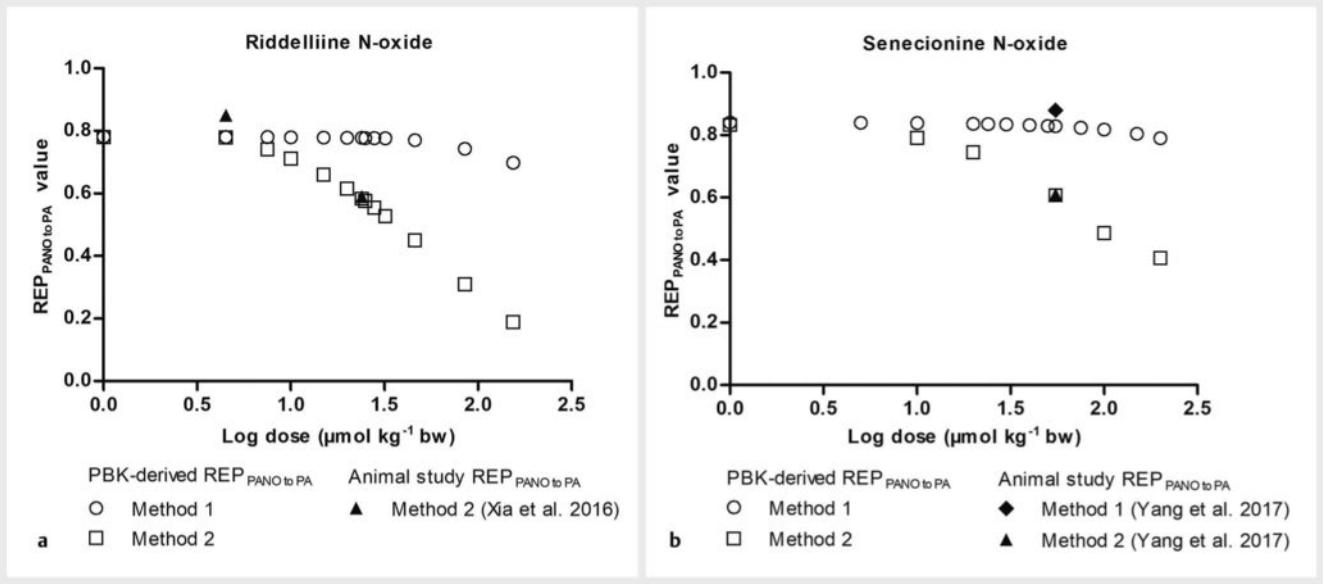
The studies presented here were aimed at defining $REP_{PANO\ to\ PA}$ values and the effect of dose levels on these values using new approach methodologies (NAMs) consisting of physiologically based kinetic (PBK) modeling and *in vitro* and *in silico* models to define the relevant PBK model parameters. The PBK model consists of a model for the PANO describing its kinetics, including the kinetics for PANO reduction by gut microbiota and liver CYP450s, and a sub-model for the kinetics of the corresponding PAs quantifying its general clearance and also 7-GS-DHP (a GSH conjugate formed from a reactive 'pyrrolic' intermediate) formation. Based on the PBK model outcomes, various endpoints can be applied to define the $REP_{PANO\ to\ PA}$ values. In a first method, the $REP_{PANO\ to\ PA}$ values can be determined based on the ratio between the area under the curve (AUC) for the parent PA upon dosing either the PANO (plasma AUC_{PA} (dose PANO)) or an equimolar dose of the PA (plasma AUC_{PA} (dose PA (▶ **Fig. 6**, method 1) [36, 37]. In a second method, $REP_{PANO\ to\ PA}$ values can be calculated based on the ratio of the amount of pyrrole-protein adducts upon dosing either PANO or an equimolar dose of PA (▶ **Fig. 6**, method 2) [38, 39]. The PBK model contained *in silico*-derived parameters and *in vitro* experimentally determined kinetic parameters and considered not only dose levels as high as the ones used in animal studies but also those that are low and more realistic for human exposure. The power of PBK modeling is the ability to predict $REP_{PANO\ to\ PA}$ values at realistic low dose levels, which would be less accessible in *in vivo* studies, and also to predict data for humans by defining a PBK model for the PANOs in humans [37]. *In vitro* studies on 7-GS-DHP formation allowed definition of the kinetic parameters to include this metabolic step in the PBK model and enable calculation of the $REP_{PANO\ to\ PA}$ value by method 2 [38, 39]. Riddelliine *N*-oxide, senecionine *N*-oxide, and their parent PAs were used as the model compounds so that predictions could be compared with available *in vivo* data [32, 40–42].

It was observed that the PBK-model-based predicted $REP_{PANO\ to\ PA}$ values for riddelliine *N*-oxide [36] and senecionine *N*-oxide [37] in rats, calculated based on plasma AUC_{PA} levels (method 1), are close to *in vivo*-derived $REP_{PANO\ to\ PA}$ values (▶ **Fig. 7**) [36, 37]. Although the kinetic constants for PANO reduction and PA clearance in rats and humans were different, the two $REP_{PANO\ to\ PA}$ values at low dose levels were found to be similar for both species [37]. Moreover, it appeared that the $REP_{PANO\ to\ PA}$ values were dose- as well as endpoint-dependent at the oral dose ranges that were examined in this study (approximately 1 to 200 $\mu mol \cdot kg^{-1}$ bw) (▶ **Fig. 7 a** and **b**) [38, 39]. The decrease in the $REP_{PANO\ to\ PA}$ value with increasing dose levels can be ascribed to the saturation of PA clearance upon high-dose PA and saturation of PANO reduction by intestinal microbiota upon high-dose PANO. Saturation of PA clearance increased the plasma AUC_{PA} upon high-dose PA, while saturation of PANO reduction reduced the plasma AUC_{PA} upon high-dose PANO. Together, these effects result in a decrease in the $REP_{PANO\ to\ PA}$ value calculated as the ratio between the AUC_{PA} upon an equimolar dose of the PANO and the PA (▶ **Fig. 6**, Method 1). PBK modeling and *in vitro* studies on saturation of pyrrole glutathione conjugate formation provided insight into the potential mode of action underlying the lower $REP_{PANO\ to\ PA}$ values obtained when calculated based on the amount of pyrrole–protein (method 2) than based on the plasma AUC_{PA} (method 1) [38, 39].

In conclusion, $REP_{PANO\ to\ PA}$ values depend on kinetics (absorption, distribution, metabolism, and excretions (ADME)), dose (low versus high), species (rat versus human), and endpoint (formed PA versus pyrrole protein adducts). In the context of chemical risk assessment, ADME characterization provides critical information that can be applied to evaluate the potential hazards and risks associated with exposure and specific toxicological endpoints. With PBK-based modeling, $REP_{PANO\ to\ PA}$ values at realistic low exposure levels could be defined and a species extrapolation from rat to human could be performed. It is important to note that to define REP values for the PANOs to be used in risk assessment for combined PA exposure, the obtained $REP_{PANO\ to\ PA}$ values still have to be multiplied by the $REP_{PA\ to\ RID}$ values (i.e., REP values of Pas compared to riddelliine as reference PA) to yield the final $REP_{PANO\ to\ RID}$ values, ultimately resulting in $REP_{PANO\ to\ RID}$ values lower than the $REP_{PA\ to\ RID}$ values defined for the parent Pas. Ultimately, this work demonstrated the strength of using NAMs like PBK modeling, to replace, reduce, and refine (3R) the use of animal testing.



▶ **Fig. 6** Metabolic scheme of PANOs with senecionine *N*-oxide as an example and the two methods with different endpoints to calculate $REP_{PANO\ to\ PA}$ values [38].



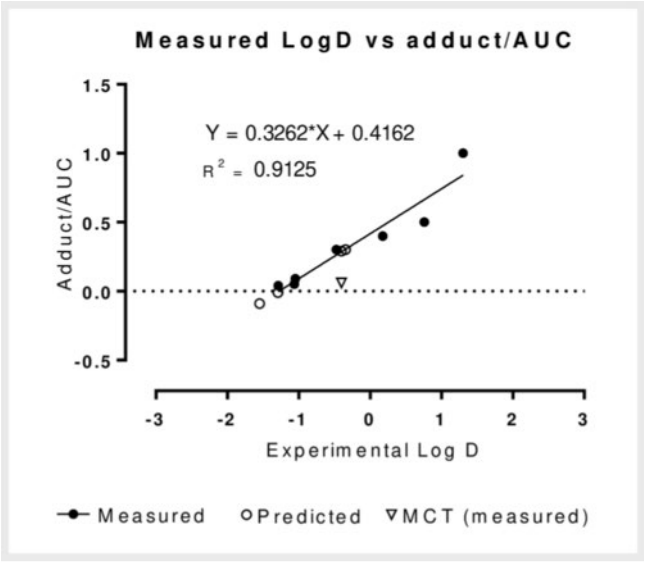
▶ **Fig. 7** Dose-dependent $REP_{PANO\ to\ PA}$ value of (**a**) riddelliine *N*-oxide relative to riddelliine (10, 13) and (**b**) senecionine *N*-oxide relative to senecionine (11, 12) in rat, as obtained by PBK modeling prediction compared to study-derived values [32, 41, 42].

Support for Regulatory Assessment of Percutaneous Absorption of (retronecine-type) PAs through Human Skin

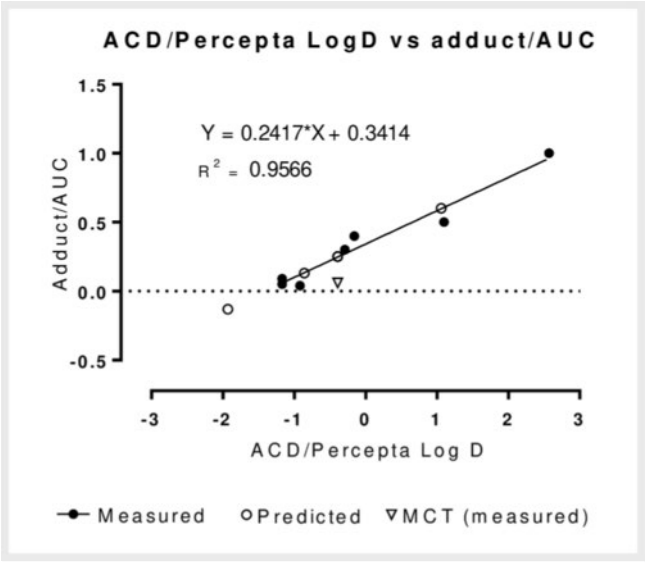
It has previously been demonstrated with PBK modeling how potency differences across a series of PA congeners can be informed for the oral route of exposure. There, *in vitro* measurements of metabolic fate and DNA adduct formation in rat sandwich culture hepatocytes (rSCH) were used alongside measurement of oral absorption and metabolic bio-activation relevant to the gastrointestinal tract [3]. Using this approach, it was confirmed that potency differences of PAs can span several orders of magnitude, as first proposed by Merz and Schrenk [11], with diesters demonstrating higher potency (1–0.1) than monoesters (0.01–>0.001). The respective PANO potency appeared to differ further still, by a factor of 2- to 10-fold, for diesters and monoesters, respectively, but challenges remain to better understand the fraction that reaches the liver due to complexities of a conversion to the free base by gut microbiota [3].

More recently, attention has turned to considering the dermal route of exposure and the extent to which PAs could be absorbed and/or metabolized in the skin. This is necessary for the risk assessment of some topically applied herbal medicines, namely those that naturally contain PAs and where, in spite of additional manufacturing steps to minimize levels, a low-level presence can be expected to remain. However, when individual PAs were taken into consideration for the risk assessment, it became apparent that measured *in vitro* data to understand intrinsic potency did not exist for all compounds of interest. Formerly, a ratio of DHP-DNA adducts/*in vitro* AUC was used, which was experimentally determined using the *in vitro* rSCH model to provide a measure of hepatocyte exposure to reactive metabolites and thereby inform intrinsic potency [43]. These experiments require complex in-house synthesis of DHP-DNA adducts for use as authentic analytical standards for DNA adduct quantification, and measurements can be very challenging, particularly for weakly potent PAs. Given the importance of lipophilicity and ionization state on the kinetic processes of a chemical's absorption and disposition [44], it has been suggested that the degree of ionization and lipophilicity may reflect potency differences between various PAs and respective PANO forms. Further investigation of a series of PAs compared both predicted and experimental LogD (decadic logarithm of the distribution coefficient between octanol and water) values with previously measured DNA adduct/AUC values (▶ **Fig. 8**, manuscript in preparation). The predicted LogD values compared well to the experimental LogD values ($R^2 > 0.9$), which in turn did show a strong relationship to DNA adduct/AUC measurements, in line with the linear relationship that was determined previously (▶ **Fig. 9**). This relationship can be explained by marked differences in physical–chemical properties between these compounds, which are known to influence chemical permeation mechanisms (ionization) and metabolism (lipophilicity), whereby hydrophilic chemicals with the highest basicity demonstrate minimal metabolism and relatively low DNA adduct formation and, in contrast, lipophilic weak bases, which are more likely to permeate through the cell membrane and exhibit higher metabolic loss and DNA adduct formation. The extent of ionization and lipophilicity was therefore confirmed to have an overall influence on hepatocellular disposition and metabolic activation potential.

On the basis of this relationship, a regression model was generated to correlate DHP-DNA adduct/*in vitro* AUC values from the *in vitro* rSCH model and Log D, allowing for the use of LogD values to predict DNA adduct/AUC values for 'untested' compounds and inform as a surrogate for intrinsic potency. When scaled relative to riddelliine, the reference PA used in establishing regulatory limit



▶ **Fig. 8** Regression model correlation between DHP-DNA adduct/*in vitro* AUC from rSCH model and experimentally measured Log D values. MCT = monocrotaline.



▶ **Fig. 9** Regression model correlation between DHP-DNA adduct/*in vitro* AUC from rSCH model and Log D predictions from ACD/Percepta software.

values, both the measured and predicted intrinsic potency values for individual Pas, was applied in a risk assessment example for a topical herbal medicinal product. Based on an assumption of 100% bioavailability via both the oral and dermal routes of exposure, the resulting margin of exposure was calculated to be considerably greater than the minimally accepted 10000-fold. Notwithstanding this critical observation, it was hypothesized that the dermal route of exposure would also serve to limit bioavailability, and so, work was undertaken to assess the penetration of PAs into and through human skin using a GLP and OECD test guideline-compliant protocol [45]. The dermal absorption of the compounds investigated, representative of retronecine-type monoesters (lycopsamine) and open-chained diesters (echimidine), as well as cyclic diesters (retrorsine), revealed a low level of penetration through human skin. Under finite exposure conditions, recovery of each PA at 24 h post-exposure was greatest in stratum corneum > epidermis > receptor fluid > dermis. In the absence of regulatory guidance for the assessment of dermal penetration of herbal medicinal products, the 2017 EFSA guidance for pesticides was used for the quantitative interpretation of the data [46]. Although differences to other guidances exist, e.g., from the OECD [47,48] and SCCS [49], dermal absorption values were derived corresponding to the sum of the receptor fluid, receptor chamber wash, skin (epidermis + clingfilm + dermis), and tape strips (3–20) of the stratum corneum. To address the variability between replicates, the EFSA guidance was followed using a multiple of the standard deviation added to the mean dermal absorption value, determined by the number of replicates used (n = 11). Even with this highly conservative approach to determine skin absorption, low dermal penetration values of < 10%, across all the PAs tested, were derived.

Initial experiments were also conducted to assess metabolic fate in human skin across a series of PAs. These investigations were conducted over a 4 h time period using human skin S9 at a protein concentration of 1.0 mg protein/mL and a substrate concentration of 10 µM, with typical co-factors to aid phase-I-dependent catalytic activity. No apparent losses were detected for the monoesters lycopsamine or intermedine or for the open diesters 7-acetylintermedine, 7-acetyllycopsamine, and echimidine.

Given the observed low penetration and low first-pass effects in skin, further adjustments in the risk assessment of dermal exposure to PAs may be possible. This will require refinement of the reference value used in the risk assessment, in this case the $BMDL_{10}$ for riddelliine. In the case that oral bioavailability of riddelliine is less than 100%, then refinement to the reference value used for risk assessment would be needed to complement the use of human dermal exposure adjusted for bioavailability. Further work is needed to explore this, but in the meantime, an understanding of intrinsic potency differences, measured or modeled using LogD values, may assist in the risk assessment of PAs across different routes of exposure.

Control of PA Impurities in Herbal Medicinal Products – Perspective of a National Regulatory Authority

Exposure to naturally occurring PAs has been associated with a risk for adverse health effects in humans and animals, due to their known hepatotoxic and potentially carcinogenic effects. In addition to plants, which produce PAs themselves, presumably as a defense mechanism against insect herbivores, the contamination of plants with PA-containing weeds has been identified as a source for PA uptake [50].

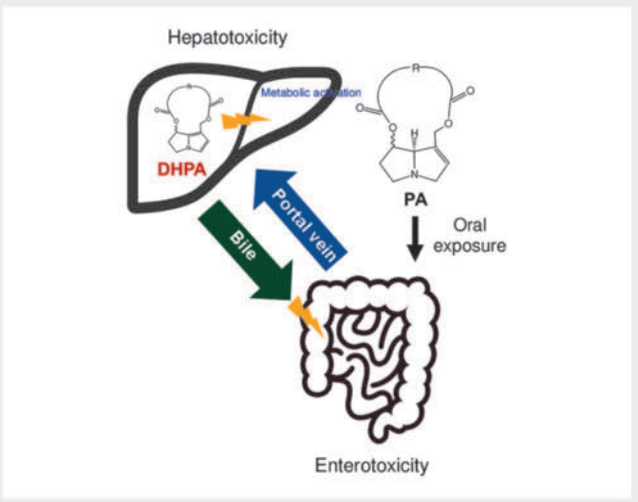
The presence of PAs in plants and derived herbal medicinal products (HMPs) requires action of the pharmaceutical companies and regulatory authorities. From a regulatory perspective, graduated plans and public statements by regulatory authorities and their associated committees have been primarily used to address necessary restrictions and requirements. Guidelines and requirements have been continuously adapted to the prevailing state of scientific knowledge, especially, but not exclusively, concerning contamination of plants without natural PA expression [50–52].

The requirements for HMPs comprise, for example, PA intake limits in release specifications with respect to the daily dosage and age group (e.g., 1.0 µg total PAs/day for adults). In addition, specific requirements for analytical methods and their validation are listed. Guidance documents and monographs have been published to illustrate the approaches and requirements for the challenging trace analysis of PA contaminations [52–54]. In this context, the growing knowledge and experience of different stakeholders can also be considered to modify requirements on the basis of the collected data. Based on the growing knowledge and evaluation of carcinogenicity data, recommendations on the maximum daily intake limits for PAs have been published and adjusted over time [50]. With the end of the previous transitional arrangements concerning the recommended maximum daily PA intake, the required introduction of age- and weight-based PA specification limits for HMPs with pediatric indication can currently be seen as an important challenge [50]. Appropriate adjustments to affected HMPs will, in many cases, require further actions by pharmaceutical companies and regulatory authorities [50]. In conclusion, the publication of more and more relevant PA-specific guidance documents, as well as the increased experience and the ongoing engagement of agricultural producers of medicinal plants, pharmaceutical companies, and test laboratories in the implementation of relevant measures, continues to improve the control of PA contaminations.

The Key Role of the Gut–liver Axis in PA-induced Hepatotoxicity and Enterotoxicity

Outbreaks of acute PA poisoning cases have been reported worldwide due to the ingestion of PA-containing herbal remedies [55] or PA-contaminated food products [56, 57], but the intoxication outcome of long-term exposure to relatively low levels of PAs remains largely unknown. Dehydro-PAs (DHPAs), reactive PA metabolites, bind to proteins and form protein adducts, thereby leading to cytotoxicity [58, 59]. Apart from causing tissue damage in the liver where PAs are primarily metabolized, the extra-hepatic toxicity of PAs has been less investigated.

The gut–liver axis not only refers to the anatomic connection between liver and gut but also to their close coordination in various biological processes. The role of the gut–liver axis in PA intoxication and the underlying mechanism was studied using mice orally treated with retrorsine, a representative toxic PA, at 20 mg/kg bw per day for 14 weeks. Such long-term PA exposure was found to induce intestinal injury manifested by intestinal epithelium damage and disruption of intestinal barrier function. Furthermore, using mice with tissue-selective ablation of CYP activity, it was found that hepatic CYPs, but not intestinal CYPs, predominately catalyzed the PA bio-activation to generate reactive DHPAs, which are further transported via bile excretion into the intestines to exert enterotoxicity (▶ **Fig. 10**). Additionally, the impact of gut-derived pathogenic factors in retrorsine-induced hepatotoxicity was investigated in mice with dextran sulphate sodium (DSS)-induced chronic colitis. Compared to retrorsine-exposed normal mice, DSS-induced colitis mice demonstrated exacerbated retrorsine-induced liver injury manifested by enhanced hepatic vasculature damage, fibrosis, and steatosis. Furthermore, DSS-induced colitis increased the gut permeability; therefore, more gut-derived endotoxins were transported into the liver. On the other hand, DSS treatment did not alter hepatic CYP activity but significantly reduced the hepatic glutathione level, thereby suppressing the PA detoxification pathway.

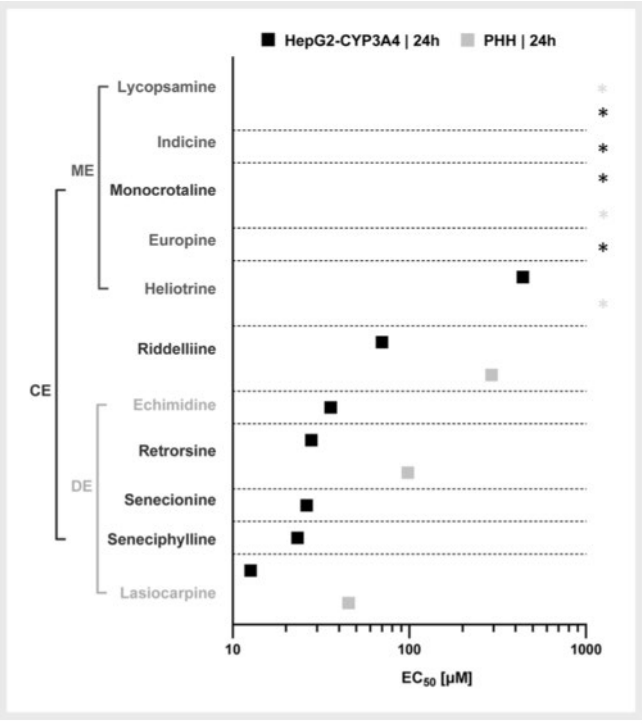


▶ **Fig. 10** Schematic illustration of PA intoxication involving the gut–liver axis. The orally ingested PAs are initially absorbed in the intestines and transported into the liver, where they are metabolized into toxic metabolites, DHPAs, which cause hepatotoxicity. In addition, the hepatic-derived DHPAs are also transported via the bile duct into the intestine, thereby leading to enterotoxicity.

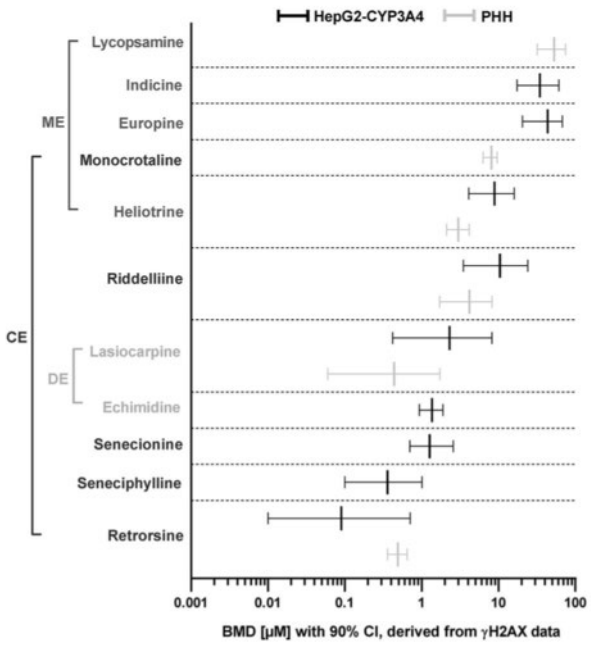
Taken together, these findings provide the first evidence of PA-induced intestinal injury as a consequence of liver metabolism and highlight the importance of gut homeostasis in PA-induced hepatotoxicity [60]. These findings warrant public awareness and further investigations of PA-induced hepatotoxicity associated with chronic intestinal disorders.

Potency Ranking of PAs Using a Genotoxicity Test Battery in Human Liver Cells

The genotoxic action of 1,2-unsaturated PAs comprises DNA adducts, strand breaks, and crosslinks [6, 58, 61, 62]. Previous studies showed that the chemical structure of PAs determines their reactivity and hepatotoxicity (summarized in [3]). However, there is a lack of quantitative genotoxicity data, particularly in primary human hepatocytes as relevant target cells. The objective of this



▶ **Fig. 11** EC_{50} values determined in HepG2-CYP3A4 cells and primary human hepatocytes (PHH) after 24 h incubation with PA monoesters (ME), open-chained diesters (DE), and cyclic diesters (CE) based on cell viability measurements. *indicates that EC_{50} couldn't be determined due to weak cytotoxicity. Please note that 11 PAs were studied in HepG2-CYP3A4 cells, while 6 PAs were tested in PHH. Data were taken from [59].



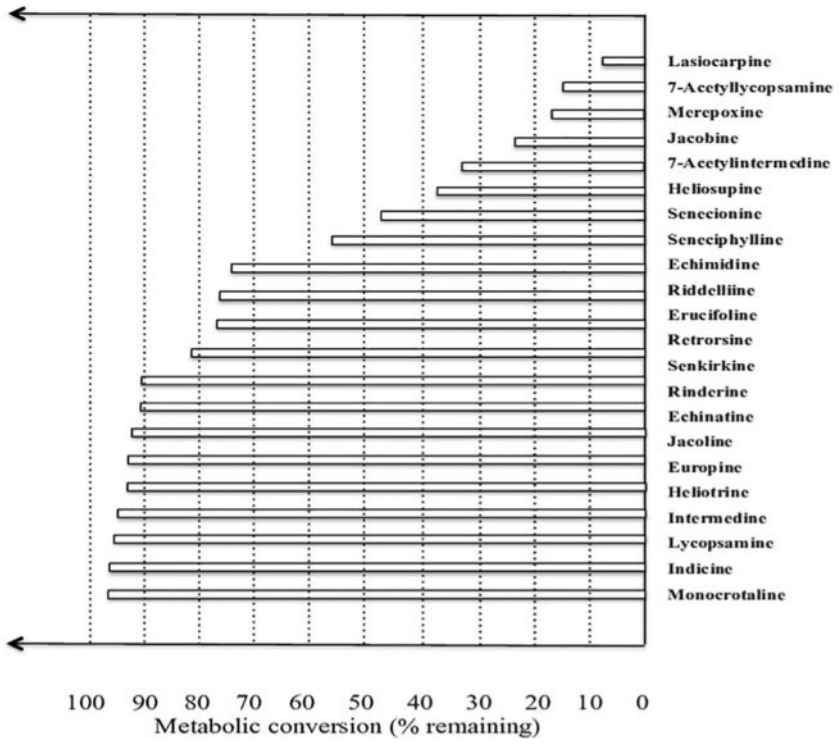
▶ **Fig. 12** Genotoxic potency ranking of PAs in HepG2-CYP3A4 cells and primary human hepatocytes (PHH) using the endpoint γH2AX. Concentration-response data were used to derive BMD values with 90% confidence intervals (CI) by PROAST. PAs were ranked according to their genotoxic potency based on the derived BMD values. Depicted are the BMD confidence interval plots in HepG2-CYP3A4 cells (black) and PHH (green). Please note, 11 PAs were studied in HepG2-CYP3A4 cells, while 6 PAs were tested in PHH. Data were compiled from [64].

study was to analyze the impact of the chemical structure on the genotoxicity and cytotoxicity of PAs and to derive the genotoxic and cytotoxic potency of PAs in different human liver cell models.

A panel of 11 PAs comprising monoesters (heliotrine, lycopsamine, europine, and indicine), open-chained diesters (echimidine and lasiocarpine), and cyclic diesters (riddelliine, senecionine, seneciphylline, retrorsine, and monocrotaline) was selected for our study. To this end, genetically engineered human HepG2 liver cells with CYP3A4 expression [63] and primary human hepatocytes (PHH) were used. As toxicological endpoints, cell viability/cytotoxicity and different genotoxicity markers including DNA strand-break induction (comet assay), accumulation of the p53 tumor suppressor protein, and formation of γH2AX were analyzed. The results showed a clear structure-dependent cytotoxicity for PAs in HepG2-CYP3A4 cells and allowed for cytotoxic potency ranking, which was corroborated in PHH (▶ **Fig. 11**). Furthermore, our genotoxicity test battery consistently revealed the structure-dependent genotoxicity of PAs in HepG2-CYP3A4 cells and allowed for genotoxic potency ranking via BMD modeling, which correlated closely with the determined cytotoxic potency. Importantly, these findings were confirmed in PHH and further provided evidence for genotoxic effects even in the absence of cytotoxicity (▶ **Fig. 12**) [64]. In summary, the work in human liver cell models, including the gold standard PHH, strongly supports the concept of grouping PAs according to their toxicity as first proposed by Merz and Schrenk [11], substantiating the provisional interim relative potency factors [3].

Current Status of the Concept of Relative Potency Factors for PAs: Perspectives and Open Questions

An increasing number of publications provide convincing evidence that the toxicity of PAs differs considerably between congeners. Attempts to take these differences into account led to the derivation of interim relative potency factors (iREP factors) for 15 PA congeners and 3 PANOs [11]. This provisional assignment of factors to PAs was based on their combined genotoxic potency in *Drosophila*, cytotoxic potency *in vitro*, and acute toxicity in adult rodents. This approach also tried to identify certain structural features associated with the iREP factors in order to allow a future classification of congeners with limited or no experimental data, including the differences in potency between and within the major structural classes of monoesters, cyclic diesters, and open-chained diesters.



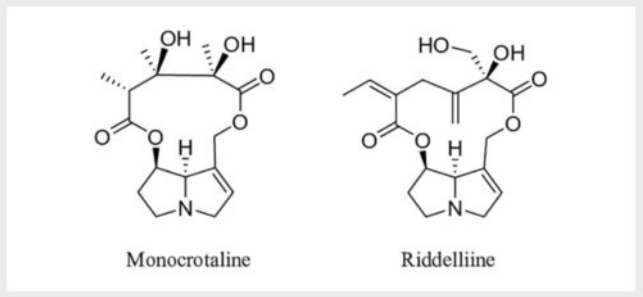
▶ **Fig. 13** Remaining PA concentration (%) after incubation with human liver microsomes and glutathione for 360 min. Taken from [66], where experimental details are provided.

In general, chronic liver damage and liver tumor formation have to be considered as particularly critical endpoints [3]. Furthermore, the assessment should consider both acute toxicity due to accidental intake of PA-plant material and chronic exposure to much lower doses eventually leading to these endpoints in a time- and dose-dependent manner. Currently, large subchronic or chronic animal experiments with a relevant number of congeners to explore their dose-response relationships are not feasible. Thus, there is a need for the application and refinement of relevant NAMs as the major source of additional information. These should comprise models for cytotoxicity, genotoxicity, and toxicokinetics. Data from such studies can be used for the modeling of internal exposure and the resulting risk, as well as structure-potency and mode-of-action studies. However, achieving a consensus among responsible regulatory bodies in order to better define the requirements and conditions for the regulatory use of such data needs to be considered.

From a scientific point of view, investigations into the reasons for the pronounced differences in toxicity between congeners are of utmost importance. Recent *in vitro* metabolism studies in human and rat liver microsomes [65, 66] and in rat hepatocytes and CYP3A4-expressing human HepG2 cells with a broad spectrum of PA congeners revealed that their relative toxic potency is related to the extent and rate of metabolism of the parent PA [67]. Thus, highly potent congeners such as lasiocarpine or riddelliine are extensively metabolized, whereas several monoesters with low toxic potency are particularly resistant to metabolic degradation (▶ **Fig. 13**). These findings were supplemented by detailed analyses of the metabolic patterns showing that those extensively metabolized, highly toxic PAs also form detectable amounts of one or more GSH adducts, while the monoesters did not [67]. Taken together, these studies indicate that the wide differences in relative potency between PAs are structure-dependent and are due to pronounced differences in both the extent and type of the metabolic conversion of the parent PA. Finally, the fraction of reactive metabolites available at the targets is the major driver of damage and thus of toxicity. This conclusion also implies that differences in cellular uptake between congeners seem to play a limited role, since the aforementioned, striking differences were also seen in microsomes, i.e., independent of cellular uptake.

With these data, it appears warranted to take iREP factors into account in the risk assessment of PAs. This is also true for monocrotaline, which exerted a much lower toxic potency in several *in vitro* assays than its cyclic diester structure would suggest [11]. Metabolism data, however, provide evidence that monocrotaline is metabolized as slowly as other PAs with low potency [65]. Although exhibiting a cyclic diester structure, the diester ring is distinctly different from all other cyclic diesters (▶ **Fig. 14**), probably explaining these findings. These data suggest that an adaptation of the iREP factor for monocrotaline (and related structures) to, e.g., a value of 0.1, may be warranted.

Using iREP factors, instead of summing up all PAs under the assumption of equal potencies, for the calculation of total PA levels, human exposure, etc., was reported to lower the calculated levels by 2- to 3-fold [68]. However, there are cases where PAs with low potency are by far the major contributors to the total PA content of certain plants, strongly enhancing the impact of using individual iREP factors. A good example is lycopsamine and related PAs in borage, where the use of iREPs may reduce the calculated risk estimates and, hence, would increase the permitted intake by more than 100-fold [69]. Thus, it is recommended to consider the use of iREP factors for PAs in medicinal and food preparations to avoid possibly inadequate regulatory restrictions for the use of food, feed, and medicinal products contaminated with a large contribution of PAs with low toxicity to the overall PA levels.



▶ **Fig. 14** Chemical structures of two cyclic diester PAs, monocrotaline (11-membered ring) versus riddelliine (12-membered ring).

Novel Approaches in the Risk Assessment of Alkenylbenzenes

Alkenylbenzenes are secondary plant metabolites that occur in various herbs and spices, like basil, fennel, and parsley. Exposure to alkenylbenzenes is a result of a direct consumption of these herbs and spices as food, the use of essential oils as flavoring ingredients, or the use of herbal food supplements with ingredients containing these compounds. In addition, there is an increasing interest in the use of alkenylbenzene-containing essential oils as feed additives, which can potentially lead to transfer from feed to food. Given the genotoxic and carcinogenic effects of various alkenylbenzenes, the presence of these substances in food and feed poses a health concern.

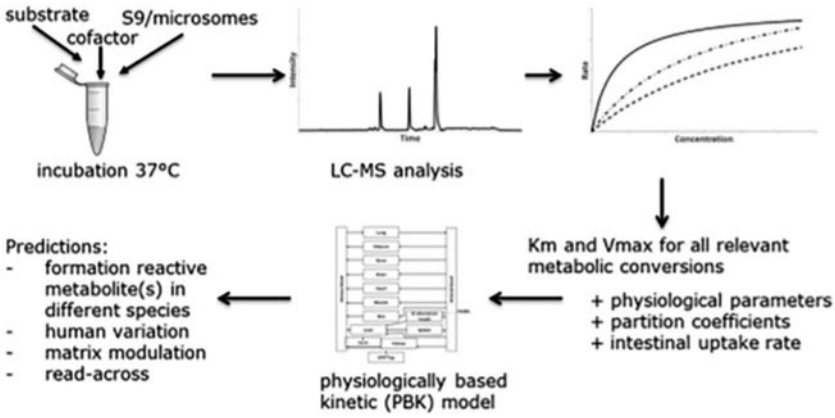
Several challenges exist with the risk assessment of alkenylbenzenes. Carcinogenicity studies are, for example, performed at high dose levels in rodents, and questions can be raised with respect to how these results are best extrapolated to low-dose human exposure scenarios. In addition, human exposure to the pure chemical does not occur, but it occurs in a complex matrix of substances. Furthermore, while estragole, safrole, or methyleugenol are known to be genotoxic and carcinogenic based on animal experiments, less information is available for other alkenylbenzenes like myristicin or elemicin.

NAMs play an important role in filling up data gaps in the risk assessment of alkenylbenzenes (▶ **Fig. 15**). Given that the genotoxicity and carcinogenicity depend on the metabolic activation of these substances, particularly NAM-derived information on metabolism can be used to investigate the relevance of the observed effect(s) across species and to inform and/or refine the risk assessment of alkenylbenzenes.

▶ **Fig. 15** shows the NAM that has been set up at Wageningen University to study species differences, human variation, and matrix modulation of the bio-activation and detoxification of different alkenylbenzenes. *In vitro* measurements are performed to derive the kinetic constants for the different bio-activation and detoxification pathways. Upon integration of these kinetic data in a physiologically based kinetic (PBK) model, one can simulate the bioactivation and detoxification in an animal species of interest. With this approach, it could, for example, be determined that dose-dependent effects and species differences in bio-kinetics cannot be used as arguments to reduce the default uncertainty factors in the risk evaluation of alkenylbenzenes [70–73]. Also, differences in the extent of bio-activation between different alkenylbenzenes could be established [73]. Furthermore, the flavonoid nevadensin was identified to inhibit sulfotransferase (SULT)-mediated bio-activation and DNA adduct formation. However, this matrix-based interaction appears to be dose-dependent and predicted by the PBK models to be absent at realistic low dietary human intake [74, 75]. Overall, *in vitro* kinetic experiments in combination with PBK modeling provide a relevant framework to explore the dose-dependent effects, species differences, human variation, and matrix modulation of alkenylbenzenes.

Alkenylbenzenes and Medicinal Products

Alkenylbenzenes occur in a large number of plant families with relevance for medicinal products, especially in *Apiaceae, Asteraceae, Rosaceae, Solanaceae, Piperaceae, Rutaceae*, or *Poaceae*. Because of the volatility of the majority of alkenylbenzenes, they are found in the respective essential oils, although they may also occur as nonvolatile [76, 77]. The scientific interest in the toxicology of alkenylbenzenes was stimulated mainly by the discovery of toxic properties of safrole in the 1950s and 1960s [78]. Because of their favorable sensory properties, pure alkenylbenzenes or the essential oils containing them have been used in industrial food production for decades. The increasing knowledge of their toxicological profiles prompted regulators in the USA to prohibit the use of safrole already in 1960, of beta-asarone in 1968, and, only recently, of methyleugenol in 2018. In the EU, the use of pure safrole, asarone, methyleugenol, and estragole in food was prohibited by regulation (EC) 1334/2008, effective since January 2011 [79]. With this same regulation, permissible concentrations in certain food categories are defined for these alkenylbenzenes if naturally present in flavorings and food ingredients with flavoring properties and in certain compound foods as consumed, to which flavorings and/or food ingredients with flavoring properties have been added. This regulatory action was preceded by safety assessments of the respective compounds by the Scientific Committee on Food [80] and the Council of Europe [81]. The Herbal Medicinal Products Committee (HMPC) elaborated statements on the use of methyleugenol and estragole in HMPs [82–85]. Between 2005



▶ **Fig. 15** NAMs to study species difference, human variation, and matrix modulation of bio-activation and detoxification of different alkenylbenzenes.

and 2013, numerous publications on estragole metabolism, toxicity, and mode of action, as well as estragole occurrence levels, particularly for fennel teas, appeared in the scientific literature, and the European ban of four pure alkenylbenzenes in food was published in 2008. In 2011, an NTP study on sub-chronic toxicity and carcinogenic effects (of note: no full carcinogenicity study) of estragole was published [86]. This prompted the HMPC to start an assessment in September 2013. The draft revision document as of November 2014 proposed an acceptable daily dose of 0.5 mg/person per day (adult, 50 kg bw), derived with reference to a $BMDL_{10}$ of 10 mg/kg bw/d, as previously discussed by EFSA and considering that a NOAEL would be lower, by application of an additional safety factor of ten [87]. In the further course of the revision process, the HMPC considered newly published regulatory guidance relevant to the issue [88]. In addition, the awareness increased that estragole-containing preparations are not only relevant as active pharmaceutical ingredients (APIs) of HMPs but also as excipients of many HMPs, as well as of numerous chemically defined medicinal products, necessitating further alignment with the European Medicines Agency (EMA) standard procedures. The second draft (Feb. 2020) no longer defined a limit dose for the general population but recommended the use of the ALARA principle, except for children under 12 y and pregnant/breastfeeding women. Furthermore, the principles of ICH M7 (less-than-lifetime principle, food background exposure, and the TD_{50} approach in the absence of a robust BMD basis) were fully applied, and estragole-containing excipients were also considered. The final public statement corroborated these approaches and was published on 1 March 2022 [89–90].

On 20 July 2022, the HMPC has drafted revisions of the monographs on bitter fennel fruit and sweet fennel fruit, as well as of bitter fennel oil. The HMPC no longer endorsed the use of bitter fennel oil in HMPs due to the high dosage specified in the hitherto existing HMPC monograph and the resulting high estragole intake. As regards the fennel fruit monographs, the HMPC proposed a reduction in the single/daily doses to the minimum levels indicated in the previous versions, reflecting the ALARA approach promoted in the public statement on estragole [91–93].

While limited to a max. 5% of fennel essential oil by the respective Ph. Eur. monograph, estragole is typically present in the essential oil portion of bitter fennel fruit at levels > 2.5%. The HMPC public statement on estragole suggests selection of "*low estragole cultivars*" as an option for minimizing the estragole content of fennel fruit. However, despite extensive breeding and selection efforts, it was not possible until now to establish fennel cultivars with estragole levels significantly lower than 2.5% (with reference to the essential oil portion) without serious impairment of vitally important properties (e.g., pest resistance toward *Mycosphaerella* and frost resistance). In addition, a strong positive correlation was observed in these studies between the content in estragole (Ph. Eur.: max. 5%) and anethole (Ph.Eur.: min 60% content in the essential oil). Once the estragole content undercut a level of 2.0 (2.2%, respectively), no single plant yielded fruit compliant with the pharmacopoeial requirement for anethole [94].

Although the HMPC public statement on estragole and the respective monograph revision drafts continue to principally allow the use of estragole-containing HMPs and fennel fruit in children, it will be extremely difficult if not impossible for many existing products to meet the guidance value.

Owing to the widespread occurrence of estragole in food plants (for examples, see ▶ **Table 2**), estragole background exposure can be considered significant.

However, robust quantitative data on background exposure to estragole via food is not available today. The EFSA's assessment, as recently mandated by the European Commission, will hopefully improve the information basis about estragole exposure from food intake [95]. The EFSA must provide the output of its assessment by 15 May 2025. For the coming years, the challenge will be for both regulatory authorities and applicants, as well as for academia, to further elucidate the unresolved scientific questions.

▶ **Table 2** Occurrence of selected alkenylbenzenes in plants used as medicine and/or food.

Plant	ES	ME	SAF	ASA	MYR	HMP	Food
Acorus calamus				x		x	
Anthriscus cerefolium	x						x
Artemisia dracunculus	x	x				x	x
Boswellia serrata	x					x	
Citrus aurantium	x						x
Cymbopogon sp		x					
Foeniculum azoricum	x						x
Foeniculum vulgare	x	x				x	x
Fragaria vesca	x						x
Glycyrrhiza sp.	x					x	x
Hippophae rhamnoides							
Illicium verum	x	x	x		x	x	x
Malus domestica	x						x
Mangifera indica	x						x
Musa sapientium	x						x
Myristica fragrans		x	x		x		x
Ocimum basilicum	x	x			x		x
Pastinaca sativa					x		
Petroselinum crispum			x				x
Pimenta dioica	x						x
Pimpinella anisum	x	x				x	x
Piper nigrum	x	x	x		x		x

ES = estragole; ME = methyleugenol; SAF = safrole; ASA = β-asarone; MYR = myristicin; HMP = herbal medicinal products

Dose-response Studies on the Genotoxic Potential of Estragole and its Metabolite 1′-hydroxyestragole in Human Liver Cells

Due to its known genotoxicity and hepatotoxicity, estragole and structurally related phenylpropenes such as methyleugenol are of concern [96]. Recently, the HMPC has recommended a reduction in the contents of estragole in HMPs to below the guidance value of 0.05 mg per person and day [81]. Following its oral uptake, estragole is transported to the liver, where it undergoes extensive phase I metabolism to form different products. O-demethylation of estragole to 4-allylphenole (chavicol) is a detoxification step mediated by CYPs [97]. Its epoxidation to estragole-2′,3′-epoxide and subsequent hydrolysis to estragole-2′,3′-diole catalyzed by epoxide hydrolase are also considered as detoxification reactions [98]. Moreover, estragole can be bio-activated to 1′-hydroxyestragol, which is catalyzed by CYP1A2 and CYP2A6 [99]. The hydroxylated metabolite is then converted to 1′-sulphooxyestragole by sulfotransferase 1A1 (SULT1A1) and SULT1C2 [100, 101]. The formed intermediate spontaneously decomposes, giving rise to a reactive carbenium ion and subsequent DNA damage, with *trans*-E3′-N^2-1′-desoxyguanosine (E-3′-N2-dG) and E3′-N^6-2′-desoxyadenosine (E-3′-N6-dA) as the main DNA adducts [102, 103]. DNA adduct formation was recently shown to occur in a concentration- and time-dependent manner in primary rat hepatocytes, with maximum adduct levels after 6 h [104]. At later time points, the adduct levels moderately decreased, which may be attributable to DNA repair. In line with this notion, a recent study provided evidence for the involvement of DNA repair in the removal of E-3′-N2-dG adducts, albeit with limited efficiency [105]. Another explanation for the decreased adduct levels found at later time points could be cytotoxicity, which is indeed observed at high estragole concentrations [104]. As mentioned before, estragole is structurally closely related to methyleugenol, and both share a common bio-activation pathway. Interestingly, higher levels of methyleugenol-derived DNA adducts cause replication stress in liver cells, which triggers p53-dependent mitochondrial apoptosis [106]. The concentration-response studies with estragole in primary rat hepatocytes further indicated a point of departure (PoD) for DNA adduct formation [104]. Such non-linear concentration-response curves can be caused by DNA repair

pathways [107], as previously shown for DNA alkylation damage [108–111]. With regard to human metabolically competent liver cell models, there are little quantitative genotoxicity data available so far for estragole. Therefore, current research investigates the concentration-dependent cytotoxicity and genotoxicity of estragole and its main phase I metabolite 1′-hydroxyestragole in human liver cell models, including genetically engineered HepG2 cells and primary human hepatocytes. The concentration-response data will be used for benchmark concentration modeling and hockey-stick modeling, in order to determine whether a 'virtually no effect' PoD for the genotoxic mode of action exists in human liver cells or not. These results are eagerly awaited and will hopefully contribute to refining the risk assessment for estragole in food and HMPs.

Summary and Conclusions

This chapter is aimed at summarizing the contributions and results from the workshop and presenting conclusions based on these results. A broader discussion, taking into account all relevant data and drawing overall conclusions from those, would have been beyond the scope of this report.

PAs are found in approximately 2–3% of all flowering plants worldwide. A major source of contamination of feed and food is the occurrence in weeds not sufficiently removed during the harvest of the cultured plants. Uptake from soil and subsequent distribution into various parts of the plant represents another pathway of contamination. The risk of adverse health effects in humans and livestock has triggered intense research on the occurrence, exposure, and risk assessment over the last decade. Studies on the transfer of PAs from farm animals to food products indicate, e.g., limited overall transfer of PAs into cow's milk or into eggs, although strong differences seem to prevail between congeners. In particular in the rumen, the reductive metabolism of PAs results in the conversion of PA *N*-oxides into their parent PAs. In insects raised for food or feed, e.g., in mealworm larvae, much higher transfer rates from the feed into the larvae were found for a number of congeners. Taken together, these data indicate that a transfer from farm animals into food occurs, usually to a low extent, depending, however, on the congener. For insects used as food or feed, substantially higher transfer rates of parent PAs are possible.

In order to assess the relative genotoxic potencies of PAs *in vitro*, the metabolically competent human HepaRG liver cell line using the γH2AX assay is a suitable tool. In combination with LC-MS/MS analysis, less-investigated congeners with a substantial genotoxicity can be identified. Furthermore, this methodology has been used successfully to address the issue of possible interactions between PAs and pesticides with respect to the genotoxic outcome. Recent data suggest that co-exposure in humans to PAs and DDT may enhance hepatotoxicity. In HepaRG cells, it was found that several pesticides can induce CYP3A4 with a subsequent increase in PA genotoxicity. This suggests that these pesticides (and other chemicals that induce CYP3A4) may potentiate the (geno)toxic effects of PAs upon co-exposure if sufficient internal concentrations for CYP induction are reached.

More research has been carried out with respect to the role of metabolism in PA toxicity. The three major pathways are hydrolysis of the ester group(s), *N*-oxidation to form PANOs, and hydroxylation of the necine bases followed by spontaneous dehydration to produce dehydro-PAs (DHPAs). The latter together with their hydrolytic product 1-hydroxymethyl-7-hydroxy-6,7-dihydropyrrolizine (DHP) are considered to be the reactive metabolites responsible for toxicity. In experimental animals, CYP3A and 2B subfamilies were the major CYPs catalyzing the metabolisms of several Pas, while in humans, CYP3A4 and 3A5 are critical in the activation of retronecine-type PAs, with the exception of monocrotaline being mainly bio-activated by CYP2A6. In lymphoblastoid TK6 cell lines expressing individual human CYPs, the dominant role of CYP3A4 in bio-activation was confirmed for the metabolism of prototype congeners from heliotridine-, retronecine-, and otonecine-type PAs. CYP3A5 and 3A7 were found to play a minor role. The results were confirmed in the micronucleus assay and in cell cycle analysis. A relative-potency analysis for genotoxicity revealed a broad spectrum of potencies with lasiocarpine being the most potent and lycopsamine the least potent in a row of 13 congeners. The relative potencies spanned several orders of magnitude and were in striking agreement with published results from other human and rat cell models. In particular, the low potencies of monoesters together with the cyclic diester monocrotaline were confirmed. The data indicate that relative potency estimates for a key endpoint in the risk assessment of PAs, i.e., for genotoxicity, are converging between *in vitro* studies making the basis for risk-assessment decisions more solid.

Likewise, in HepaRG cells, the genotoxic potencies of a broad spectrum of representative PAs differ significantly due to the different structures. Under standard *in vitro* conditions with ambient air (21%) oxygen levels, *N*-oxides are less potent inducers of DNA damage than their corresponding parent PA. With heliotrine, it was found that, under these conditions, the *N*-oxide was a less potent genotoxicant than under low oxygen (2%), whereas the opposite was found for the parent PA. Chemical analysis revealed that several-fold more potent PA is formed under low oxygen when compared to a standard ambient air oxygen concentration. These data indicate that the oxygen level has a significant impact on the genotoxic potency of heliotrine *N*-oxide, which should have a future impact on the design and interpretation of respective *in vitro* studies.

Since PANOs are often the predominant form of PAs in plants, their risk assessment is of special importance. Reduction into the parent PAs can occur via the intestinal microbiota but also via enzymes in the host's tissues. Using PBK modeling based on *in silico* parameters and parameters derived from *in vitro* experiments, it is possible to predict the relative potency factors using toxicokinetic parameters for parent PAs vs. PANOs for both rats and humans. The predictions can then be compared to the amount of pyrrole–protein adducts as an effect marker. PBK-based predictions of toxicokinetics for riddelliine *N*-oxide, senecionine *N*-oxide, and their parent PAs in rats were close to *in vivo*-derived values. Furthermore, the models suggest that the relative potencies are dependent on dose, species, and endpoint. Modeling at low, relevant dose levels may allow the derivation of estimates relevant for risk assessment with an extrapolation to humans.

PBK models are usually based on *in vitro* data on metabolism, DNA adduct formation, etc., together with measures of oral absorption and metabolic bio-activation relevant to the gastrointestinal tract. Similar efforts are warranted considering the dermal route of exposure and the extent to which PAs could be absorbed and/or metabolized in the skin. The ratio of DHP-DNA adducts/*in vitro* AUC (determined in rat hepatocyte cultures) was found to be correlated to the intrinsic toxic potency of congeners. Furthermore, both predicted and experimental lipophilicity parameters were correlated with DNA adduct/AUC values. On this basis, it can be shown that LogD values are useful to predict DNA adduct/AUC values and relative potency estimates for PAs with insufficient data. Even under the assumption of complete dermal bioavailability, these considerations result in margin-of-exposure estimates for topically applied HMCs well above 10 000, suggesting a low concern for human health. Furthermore, actual measurements with *in vitro* models for dermal absorption revealed low dermal penetration values of < 10 % of the applied dose for lycopsamine, echimidine, or retrorsine. Incubations with human skin S9 preparations did not show any metabolic losses with lycopsamine, intermedine, 7-acetylintermedine, 7-acetyllycopsamine, or echimidine, suggesting marginal or negligible metabolic activation in the skin.

Since PAs may be found in plant-derived herbal medicinal products (HMPs), there is a requirement for adequate action by pharmaceutical companies and regulatory authorities. These requirements have been addressed, e.g., by European authorities, which published mitigation plans and public statements including recommendations for restrictions and requirements. The latter have been adapted to the evolving scientific knowledge. Examples are requirements for specification limits, analytical methods, and their validation. A current challenge is the introduction of adjusted specification limits for PAs in HMPs for children. A further improvement in the control of PA contaminations in HMPs can be expected from future guidance documents and the progress made by agricultural producers, pharmaceutical companies, and test laboratories in the implementation of relevant measures.

The fact that pyrrolic PA metabolites can bind to proteins, thus forming detectable adducts, offers a possibility to analyze these as a biomarker not only of exposure but also of effect. For example, this method allowed the study of the role of the gut–liver interplay in PA toxicity. Upon long-term treatment of mice with retrorsine, the intestinal mucosa can be damaged with a subsequent disruption of the intestinal barrier. When intestinal CYP activity is suppressed, the formation of pyrrolic metabolites was not affected substantially, however, indicating that hepatic metabolism and biliary excretion of metabolites but not intestinal metabolic activation are mainly responsible for the observed enterotoxicity. When chronic colitis was induced with DSS, the hepatotoxicity of retrorsine was enhanced. Although CYP activity in the liver was unaltered under these conditions, depletion of hepatic glutathione was observed. Furthermore, damage of the intestinal barrier upon colitis may enhance endotoxin transport to the liver and may also contribute to the enhanced liver damage observed. These findings illustrate the role of the intact intestine in PA toxicity in the liver and suggest a possibly higher susceptibility to PAs in patients with intestinal affections.

An increasing number of publications provide convincing evidence that the toxicity of PAs differs considerably between congeners. Establishing relative potency (REP) factors would be an important tool for the refinement of the risk assessment of complex PA mixtures. Such mixtures are usually present in plants and, consequently, in food, feed, and herbal preparations. Their current assessment is based on the assumption that all 1,2-unsaturated PAs are as toxic as the most toxic congeners(s), i.e., lasiocarpine or riddelliine, and thus may lead to an inadequate overestimation of the risk.

Recent *in vitro* metabolism studies in human and rat liver microsomes, in rat hepatocytes, and in CYP3A4-expressing human HepG2 cells using a broad spectrum of PA congeners revealed that their relative toxic potency is related to the rate and extent of the metabolism of the parent PA. Highly potent congeners were extensively metabolized, whereas several monoesters with low toxic potency were not. A detailed analysis of the GSH adducts in human liver microsomes revealed that those extensively metabolized, highly toxic PAs also form detectable amounts of one or more GSH adducts, while the monoesters did not. Thus, extensive metabolism and formation of detectable amounts of GSH conjugates are obviously markers for high toxic potency. Since relative toxic potencies in intact cells for a few congeners were in accordance with the measured GSH conjugate formation in a subcellular metabolically active fraction (liver microsomes), differences in uptake and distribution seem to play a limited role for the relative toxic potency in these cases. Further studies on this issue are warranted.

The concept of iREPs was supported by a number of recent publications on *in vitro* models of both human and rodent origin. The structure–potency relationship was also in very good agreement with the published concept with the exception of monocrotaline. *In vitro* human metabolism data confirm, however, that monocrotaline is a weak substrate for microsomal metabolism and does not form detectable amounts of GSH conjugates. This exception is likely due to the peculiar ring structure of monocrotaline.

Alkenylbenzenes are secondary plant metabolites that occur in various herbs and spices, like basil, fennel, and parsley. Exposure to alkenylbenzenes is a result of a direct consumption of these herbs and spices, as well as of the use of essential oils as flavoring ingredients or the use of herbal supplements. In addition, alkenylbenzene-containing essential oils are used more and more as feed additives. Given the potential genotoxic and carcinogenic effect of various alkenylbenzenes, the presence of these substances in food and feed poses a health concern. The fact that only a few rodent studies at relatively high dose levels are available makes the risk assessment of these compounds difficult. Furthermore, no data are available on their possible carcinogenicity in humans. The toxicological data focus on estragole, safrole, or methyleugenol, while much less information is available for other alkenylbenzenes.

Upon integration of toxicokinetic (transport and metabolism) data in a PBK model, one can simulate the bio-activation and detoxification in a species of interest. This approach, for example, allowed the determination that dose-dependent effects and species differences in bio-kinetics cannot be used as arguments to reduce

default uncertainty factors in the risk evaluation of alkenylbenzenes.

Also, differences in the extent of bio-activation between different alkenylbenzenes could be established. Furthermore, the flavonoid nevadensin was identified to inhibit sulfotransferase (SULT)-mediated bioactivation and DNA adduct formation. However, these matrix-derived interactions appear to be dependent and predicted by the PBK models to be absent at realistic low dietary human intake. Overall, *in vitro* kinetic experiments in combination with PBK modeling provide a relevant framework to explore dose-dependent effects, species (human vs. animal) differences, human variation, and matrix modulation of alkenylbenzenes.

Alkenylbenzenes occur in a large number of plant families, e.g., *Apiaceae, Asteraceae, Rosaceae, Solanaceae, Piperaceae, Rutaceae*, or *Poaceae*. Because of their volatility, they are mainly found in the respective essential oils. However, alkenylbenzenes may also occur in the form of nonvolatile glycosides. Because of their favorable sensory properties, pure alkenylbenzenes or essential oils containing them have been used in industrial food production for decades. The increasing knowledge of the genotoxic and carcinogenic effects prompted regulators in the USA to prohibit the use of safrole, beta-asarone, and methyleugenol. In the EU, the use of pure safrole, asarone, methyleugenol, and estragole in food has been prohibited since 2011. Permissible concentrations in certain food categories are defined for these alkenylbenzenes naturally present in flavorings and food ingredients with flavoring properties and in certain compound foods, as consumed, to which flavorings and/or food ingredients with flavoring properties have been added. The HMPC adopted additional statements on the use of methyleugenol and estragole in HMP in 2005. At that time, with reference to interspecies differences of metabolic activation steps, the probable nonlinear dose-response curve at low doses, and the low doses and short treatment duration typically found in the medicinal use of relevant herbal products, no dose limits were imposed, but the committee stipulated the need for further data, including data on background exposure levels from food intake. On 1 March 2022, the HMPC published a revised public statement on the use of HMPs containing estragole. This document sets a “guidance value” of 1 µg/kg bw as a maximum daily dose for children and pregnant and breastfeeding women and demands reducing the estragole content in other HMPs as much as practicable. The necessity, with reference to the ICH M7 guideline, to consider the duration of use and background exposure from food is stipulated.

In 2022, the HMPC drafted revisions of the monographs on bitter fennel fruit and sweet fennel fruit, as well as of bitter fennel oil. The HMPC no longer endorses the use of bitter fennel oil in herbal medicinal products due to the high dosage specified in the hitherto existing HMPC monograph and the resulting high estragole intake. It will be on the part of marketing authorization/registration holders to elaborate on whether other measures are reasonably practicable to further reduce the content of estragole in their products. Although these documents continue to principally allow the use of estragole-containing HMPs and fennel fruit in children, it will be a challenge for many existing products to meet the guidance value since robust quantitative data on background exposure to estragole via food are not available today.

Upon oral uptake, estragole is transported to the liver, where it undergoes extensive phase I metabolism. This includes detoxification steps such as O-demethylation or epoxidation to estragole-2′,3′-epoxide and subsequent hydrolysis to estragole-2′,3′-diole. Estragole can also be bioactivated to 1′-hydroxyestragol and catalyzed by CYP1A2 and CYP2A6. The hydroxylated metabolite is then converted to 1′-sulphooxyestragole by sulfotransferases and may spontaneously decompose, giving rise to a reactive carbenium ion. The latter can form various DNA adducts in a concentration- and time-dependent manner. Higher levels of methyleugenol-derived DNA adducts cause replication stress in liver cells, which triggers p53-dependent mitochondrial apoptosis. Concentration-response studies on DNA adduct levels with estragole in primary rat hepatocytes further indicated a point of departure (PoD) for DNA adduct formation. Such nonlinear concentration-response curves can be caused, e.g., by detoxification mechanisms and DNA repair pathways, as previously shown for DNA alkylation damage. Concentration-response modeling is warranted in order to determine whether a ’virtually no effect’ PoD for the genotoxic mode of action exists in human liver cells or not.

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Contributors’ Statement

All authors:

- supervision of generation of data and/or concepts presented/discussed in the individual chapters
- design of the concept of the paper
- drafting of chapters
- drawing of figures (if appropriate)
- designing of tables (if appropriate)
- response to reviewers
- final reading

Dieter Schrenk:

- all of the above-mentioned plus coordination of the drafting process

Acknowledgements

The authors want to acknowledge the work of their colleagues and collaborators who have contributed substantially to the original work (which has been published or is in the publication process) discussed in this review: Gabriel Ackermann, Yasser Alhejji, Marc Audebert, Greg Dameron, Mona Delagrange, Andrea Edginton, Peter Fu, Lan Gao, Ina Geburek, Arjen Gerssen, Manuel Haas, Yesheng He, Wendy Jansen Holleboom, Mike Karb, James Keating, Jan-Heiner Küpper, Cathy Lester, Jochem Louisse, Jiang Ma, Nan Mai, Karl-Heinz Merz, Cindy Obringer, Ivonne Rietjens, Deborah Rijkers, Lukas Rutz, Milou van de Schans, Bernd Schäfer, Geert Stoopen, Anja These, Sebastiaan Wesseling, Jamyang Yangchen, Liang Zheng, and Rubing Zou. The help of Arie van der Bent and Deborah Rijkers with Figures 3 and 4 is gratefully acknowledged.

Conflict of Interest

The authors declare that they have no conflict of interest.

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