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Review Article

Pyrrole-protein adducts – A biomarker of pyrrolizidine alkaloid-induced hepatotoxicity

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ABSTRACT

Pyrrolizidine alkaloids (PAs) are phytotoxins identified in over 6000 plant species worldwide. Approximately 600 toxic PAs and PA *N*-oxides have been identified in about 3% flowering plants. PAs can cause toxicities in different organs particularly in the liver. The metabolic activation of PAs is catalyzed by hepatic cytochrome P450 and generates reactive pyrrolic metabolites that bind to cellular proteins to form pyrrole-protein adducts leading to PA-induced hepatotoxicity. The mechanisms that pyrrole-protein adducts induce toxicities have not been fully characterized. Methods for qualitative and quantitative detection of pyrrole-protein adducts have been developed and applied for the clinical diagnosis of PA exposure and PA-induced liver injury. This mini-review addresses the mechanisms of PA-induced hepatotoxicity mediated by pyrrole-protein adducts, the analytical methods for the detection of pyrrole-protein adducts, and the development of pyrrole-protein adducts as the mechanism-based biomarker of PA exposure and PA-induced hepatotoxicity.

1. Introduction

Pyrrolizidine alkaloids (PAs) are naturally occurring toxins produced by over 6000 plant species as secondary metabolites for diverse purposes such as against herbivores [1–3]. Humans are exposed to toxic PAs through the consumption of PA-containing herbal medicines and dietary supplements [4–6], or by the ingestion of PA-contaminated foodstuffs, such as grain crops, honey, milk, and eggs [7–11]. The PA-induced acute toxicity mainly occurs in the liver with typical symptoms of hepatomegaly, jaundice, and ascites, known as hepatic sinusoidal obstruction syndrome (HSOS) [12,13]. Most episodic outbreaks of PA poisoning in humans are related to PA-induced HSOS. In addition to hepatotoxicity, some PAs, such as monocrotaline (MCT), fulvine, and seneciphylline,

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also induce toxicities in extra-hepatic organs, among which MCT-induced pulmonary toxicity is the most studied [14,15].

Since the first documented outbreak of 80 PA-induced liver injury (PA-ILI) cases in South Africa in 1920 [16], numerous PA-poisoning cases have been reported worldwide [5,6,17–21]. The carry-over of PAs through livestock into dietary foodstuffs, such as milk, eggs, and honey, as well as their downstream contamination in the food chain, further increases the incidence of PA exposure in human [7–11]. Prolonged dietary exposure to a low-level of PAs may also contribute to chronic diseases, such as liver cirrhosis, liver cancer, and pulmonary arterial hypertension [11,22,23]. Unlike the acute PA-ILI via a high-level of PA exposure, which shows typical syndromes and often has the chance to be treated immediately, the chronic diseases caused by PAs are latent, often untreated at the early stage, and eventually progress to lethality [23]. Due to the lack of specific and definitive diagnostic method, the determination of whether these chronic diseases are caused by PA exposure is challenging. Therefore, the incidence of PA intoxication and PA-associated diseases could be much higher than the currently recorded cases.

To date, the toxic mechanisms underlying PA-induced toxicity are not fully understood, and the clinical diagnosis of PA-related diseases has not been established. This article reviews the recent advance of the mechanism of PA intoxication mediated by the formation of pyrrole-protein adducts, the analytical methods to qualify and quantitate pyrrole-protein adducts, and the clinical diagnosis of PA exposure and PA-induced toxicities by using pyrrole-protein adducts as the mechanism-based biomarker of PA-ILI.

2. Metabolic activation of pyrrolizidine alkaloids and the formation of pyrrole-protein adducts

PAs are esters of necine bases and the esterifying acids (necic acids) (Fig. 1A and B). Based on different types of necine base, PAs are commonly grouped into three types, retronecine-type (including its 7-α enantiomer, heliotridine-type), otonecine-type, and platynecine-type (Fig. 1A). Retronecine-type and otonecine-type PAs, which possess a double bond at C1 and C2 positions of the necine bases, are highly toxic and carcinogenic, while platynecine-type PAs with a saturated necine base are generally not toxic.

PAs are pro-toxins and metabolic activation is a prerequisite for PA-induced toxicities [3,12,24–26]. Catalyzed by hepatic cytochrome P450 (CYP) monooxygenases, the metabolism of retronecine-type and otonecine-type PAs forms the corresponding reactive pyrrolic metabolites, dehydropyrrolizidine alkaloids (dehydro-PAs) [24–27] (Fig. 2A). All of the dehydro-PAs contain an identical pyrrolic moiety regardless of the structures of their parent PAs (Fig. 2A) [27,28]. Dehydro-PAs are chemically and biologically reactive and can rapidly hydrolyze to the less reactive (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine (DHP). Both dehydro-PAs and DHP can covalently bind with cellular proteins and DNA to form pyrrole-protein adducts, pyrrole-DNA adducts, and cross-linking adducts (Fig. 2A) [24–34]. These adducts are correlated with PA-induced cytotoxicity and genotoxicity [26,28,35]. The CYP-mediated metabolism of platynecine-type PAs did not generate dehydro-PAs, and, thus, platynecine-type PAs are generally not toxic (Fig. 2B) [27].

The structures of toxic PAs significantly affect the formation of pyrrole-protein and pyrrole-DNA adducts. Cyclic diester PAs produce higher levels of adducts than mono-ester PAs (Fig. 1B) [27,32,35–37]. Both steric hindrance around the necic acid moiety and the lipophilicity of PAs also affect the adduct formation and the resulting cytotoxicity [32,35,36].

Although the metabolism of PAs, particularly the metabolic activation, has been extensively studied, the characterization of the pyrrole-protein adducts, such as the target proteins and specific binding sites, remains largely unexplored. Evidence supporting the occurrence of protein adduction by pyrrolic metabolites is largely indirect. A series of pyrrole-amino acid adducts, including pyrrole-*N*-acetylcysteine, pyrrole-cysteine, pyrrole-valine, and pyrrole-lysine, and pyrrole-glutathione (GSH) adducts were synthesized by the reaction of pyrrolic metabolites with the corresponding amino acids or GSH [38–44]. These adducts were formed via the binding of the −SH and −NH functional groups of the amino acids and GSH to the 7- and 9-positions of the necine moiety of pyrrolic metabolites (Fig. 2A). These results suggest that reactive pyrrolic metabolites bind covalently to proteins at the −SH and −NH functional groups. In the early 1990's, Mattocks and Juke reported that upon the reaction of liver tissues and blood of PA-treated rats with $AgNO_3$, the pyrrolic moiety was cleaved from pyrrole-bound proteins and was detected via spectrometric analysis [45,46]. In 2016, Li et al. identified 7-lysine-DHP from the proteolytic digestion of liver proteins obtained from MCT-treated mice [43], which further supports the formation of pyrrole-protein adducts *in vivo*. However, the structural characterization of pyrrole-proteins is highly challenging due to the numerous cellular proteins that can bind to pyrrolic metabolites and each protein contains many amino acids. To date, it is not known which are the target proteins that bind to the reactive pyrrolic metabolites, form the pyrrole-protein adducts, and are responsible for the PA-induced cytotoxicity.

3. Mechanisms underlying the pyrrole-protein adducts-induced cytotoxicity

Generally, there are two hypothesized mechanisms for protein adduct-induced cytotoxicity: 1) the covalent modification of proteins results in the impairment of the normal function of the targeted proteins [47,48]; and 2) the protein adducts, serving as haptens, may trigger immune responses, such as cytokine release and autoantibodies, leading to immune-mediated toxicities [47,48]. In addition, the depletion of cellular GSH caused by reactive metabolites, which disrupts cellular anti-oxidative capabilities and leads to oxidative stress in the targeted tissues/organs, is another hypothesized mechanism [49].

Similar to the metabolic activation of many other chemical toxins, the metabolic activation of PAs significantly diminishes cellular GSH levels in different cell lines and animals

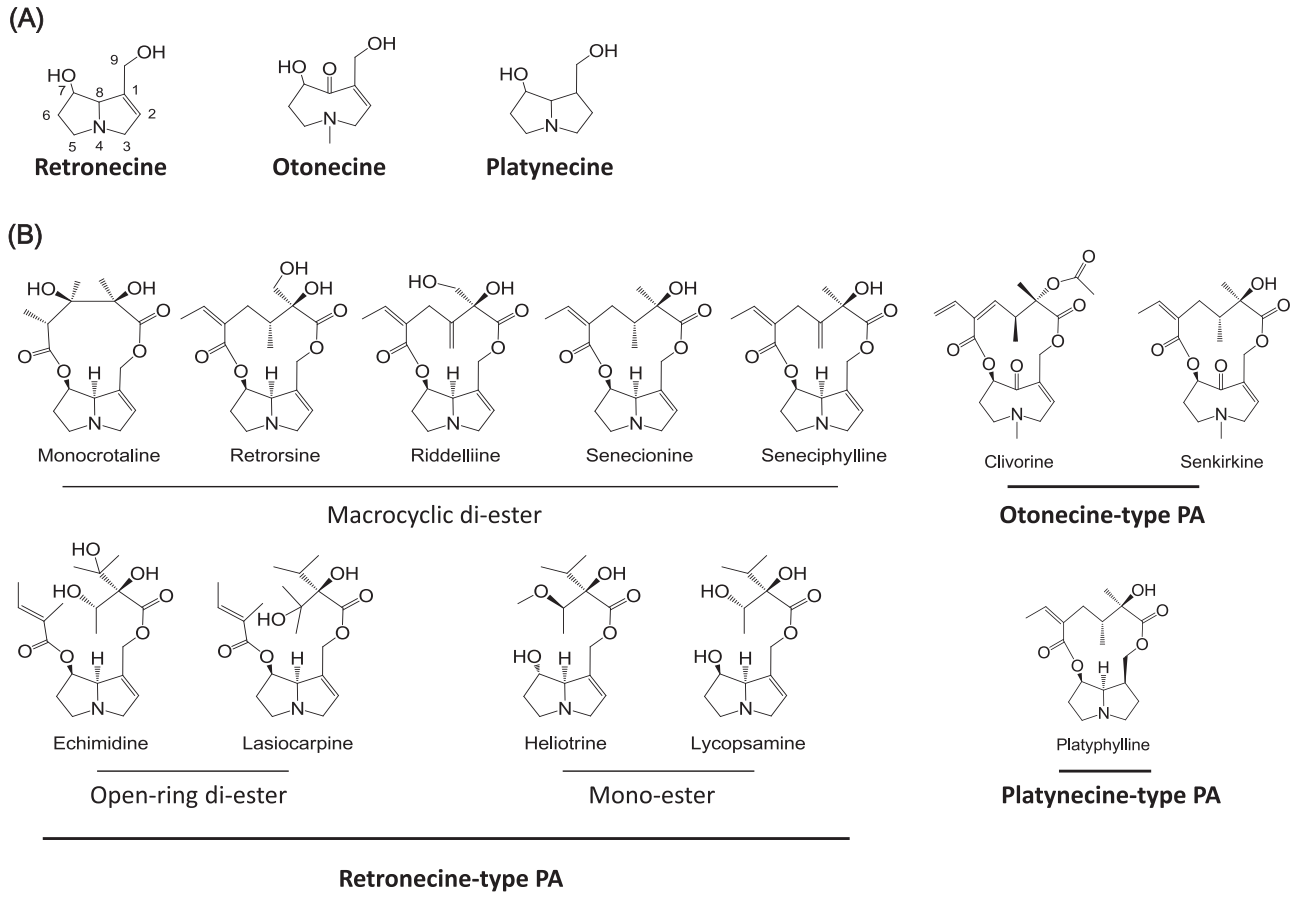

Fig. 1 – Chemical structures of the necine bases (A) and the representative PAs (B). Retrornecine-type PAs can be further divided into three subgroups: macrocyclic di-ester, open-ring di-ester, and mono-ester.

[33,49,50], which causes the disturbance of cellular redox homeostasis and leads to PA-induced cytotoxicity [51]. Restoration of cellular GSH to detoxify the reactive pyrrolic metabolites has proved to be an effective approach for PA detoxification [50,52], although it is only effective with the pretreatment of GSH or its precursors, such as *N*-acetylcysteine and γ-glutamylcysteinylglycyl ethyl ester, or with the treatment at very early stage after PA ingestion.

In addition to the study of these mechanisms leading to cytotoxicity, only a limited number of studies have aimed at identifying the cellular proteins which form the pyrrole-protein adducts associated with PA-induced cytotoxicity [53–55]. One study was reported by Lame et al. in which a number of target proteins of ^{14}C-dehydro-MCT in pulmonary artery endothelial cells were identified [53,54], of which pyrrole-actin adducts were shown to alter the permeability barrier function of pulmonary artery endothelial cells and contributed to the MCT-induced pulmonary toxicity [55].

Most PA-induced cytotoxicity occurs in the liver, known as HSOS, which represents a typical toxicity in hepatic sinusoidal endothelial cells (HSECs) [51,52,56]. DeLeve et al. demonstrated that dehydro-MCT could induce depolymerization of F-actin in HSECs through a covalent modification, leading to the disassembly of F-actin and the rounding up of HSECs [57]. The disassembled F-actin led to enhanced synthesis and activity of matrix metalloproteinases (MMP)-9 and MMP-2, which then digested the extracellular matrix in the space of Disse, causing further dehiscence of HSECs [57]. The morphological changes of F-actin and HSECs, and the degradation of extracellular matrix created the gaps in the endothelial barrier that permitted blood to penetrate into the space of Disse, leading to the dissection of the sinusoidal lining cells, and obstruction of the sinusoids [52,57]. Similarly, despite of the lack of direct evidence of covalent binding, Mingatto et al. proposed that dehydro-MCT covalently bound to the thiol groups of mitochondrial respiratory chain complex I and impaired its normal function [58], contributing to MCT-induced hepatotoxicity. In addition, although it is less related to toxic mechanisms underlying PA-induced toxicities, some drug-metabolizing enzymes, such as CYPs, were also indicated to be covalently modified by dehydro-PAs [59], because these drug-metabolizing enzymes metabolically activated PAs to their reactive pyrrolic metabolites and were also the first enzymes encountered by these reactive metabolites. Currently, studies on the unequivocal identification of proteins targeted by dehydro-PAs, and the mechanistic linkages between the target proteins and PA-induced toxicities are still lacking. Further investigation is required.

Nakayama Wong et al. illustrated that pyrrole-protein adducts *per se* might evoke direct cytotoxicity [60]. The underlying mechanisms probably involved the transport of pyrrolic moiety from the interacted proteins to cellular DNA or the

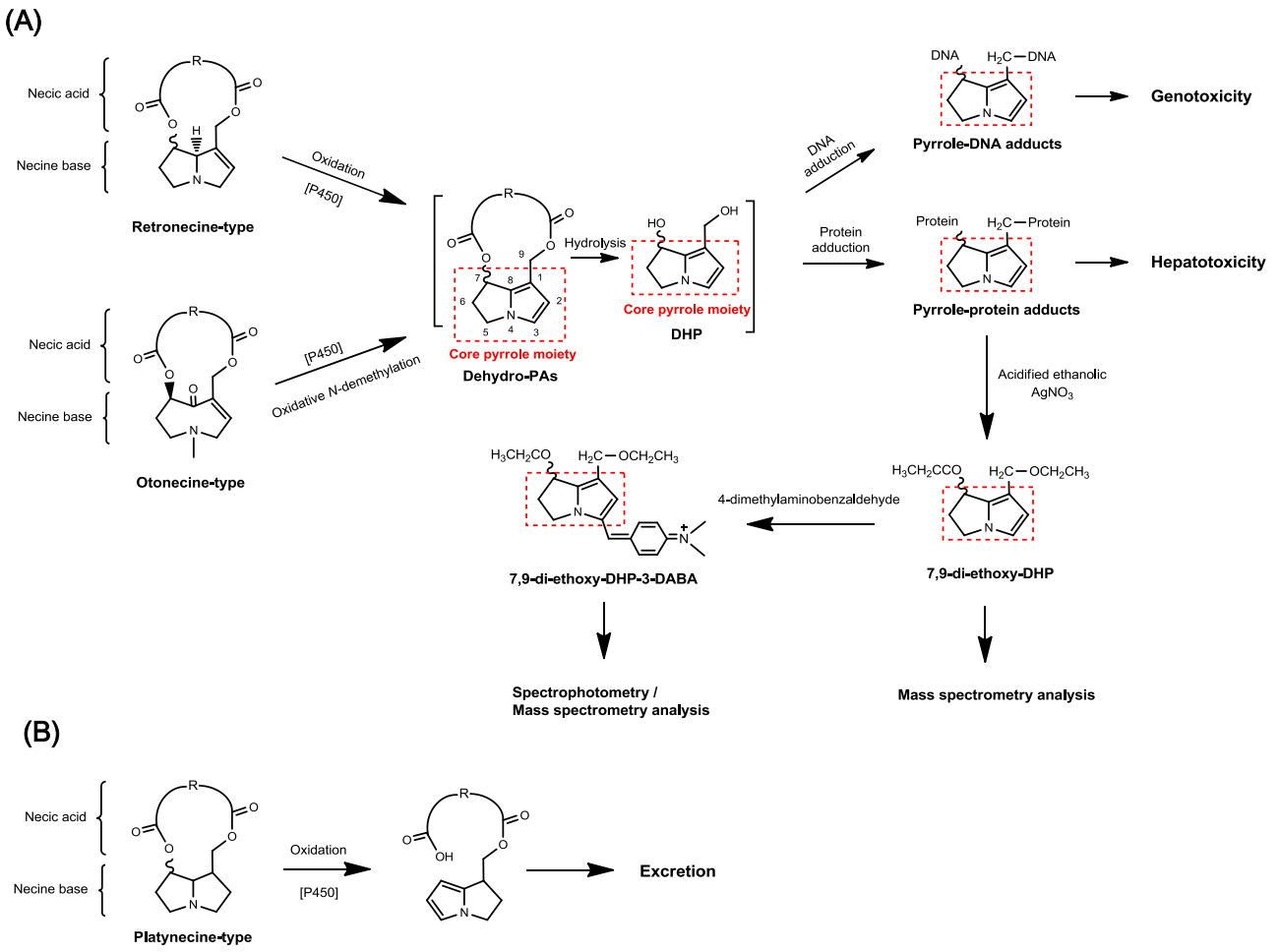

Fig. 2 – Metabolic oxidation pathways of retronecine-type and otonecine-type PAs (A) and platynecine-type PA (B). The derivatization of pyrrole-protein adducts to produce pyrrole-derived analytes, including 7,9-di-ethoxy-DHP and 7,9-di-ethoxy-DHP-3-DABA, by acidified ethanolic $AgNO_3$. DHP: (±)-6,7-dihydro-7-hydroxy-1-hydroxymethyl-5H-pyrrolizine; DABA: 4-dimethylaminobenzaldehyde.

formation of pyrrole-protein-DNA crosslinking, and the modified DNA would initiate apoptosis [60–64]. Very recently, Fu and co-workers reported that several pyrrole-amino acid adducts, such as 7-cysteine-DHP, 7-N-acetylcysteine-DHP, and 7-GSH-DHP, were identified secondary pyrrolic metabolites that could bind to DNA leading to the formation of pyrrole-DNA adducts [39–42]. All these findings suggest that since secondary pyrrolic metabolites of PAs can bind to cellular DNA to form pyrrole-DNA adducts, these secondary pyrrolic metabolites may also lead to the pyrrole-protein adduct formation and associated with PA-induced cytotoxicity. This warrants further investigation.

4. Detection and quantitation of pyrrole-protein adducts

To date, the study of pyrrole-protein adducts formed *in vivo*, *in vitro*, or chemically, has focused mainly on the identification of the covalent binding locations between the pyrrolic moiety and the nucleophilic sites of the interacted proteins. This approach is based on the understanding that reactive pyrrolic metabolites are alkylating agents possessing bi-functional electrophilic positions at the C7 and C9 positions of the necine moiety, which are capable of alkylating to the nucleophilic sites of the target proteins, leading to the formation of pyrrole-protein adducts (Fig. 2A).

The detection of soluble and tissue-bound pyrrole-protein adducts was first reported by Mattocks et al. who developed a spectrophotometric analytical method to detect pyrrolic metabolites. This method used the Ehrlich reagent, using the freshly prepared *p*-dimethylaminobenzaldehyde (DABA), to react with the pyrrolic moiety of the pyrrole-protein adducts to form the products that had a specific UV absorbance [65,66]. Before the Ehrlich reaction, mercuric chloride was employed to cleave the pyrrolic moiety from the tissue-bound pyrrole-protein adducts [67]. However, by this approach, after the reaction, a portion of the pyrrolic mioety was still bound with the proteins/tissues resulting in complex matrixes, or formed insoluble complexes with mercury ions, making the further isolation and identification of the pyrrolic products difficult [45]. In the early 1990's, Mattocks

et al. improved the detection method by using $AgNO_3$, which was more effective in releasing the pyrrolic mioety from the sulfur (S)-linked pyrrole-protein adducts formed in various biological samples [45,46,68]. The detached pyrrolic moiety then reacted with ethanol to produce 7,9-di-ethoxy-DHP, which could be readily separated from biological matrices and detected by chromatography and mass spectrometry (Fig. 2A) [45,46,68,69].

Following this strategy, Lin et al. [6] developed a specific and sensitive method for the qualitative determination of pyrrole-protein adducts in the blood of PA-ILI patients. This novel analytical method involved: (i) the reaction of pyrrole-protein adducts in the blood with acidified ethanolic $AgNO_3$ to form 7,9-di-ethoxy-DHP; (ii) upon treatment with Ehrlich reagent, 7,9-di-ethoxy-DHP-3-DABA was formed; and (iii) the formed product was subsequently analyzed by ultrahigh pressure liquid chromatography-mass spectrometry (UHPLC-MS) (Fig. 2A). Using this method, the first reported detection of pyrrole-protein adducts in humans was achieved. Lin's group further improved this method by using 7,9-di-GSH-DHP to construct a calibration curve for the quantitative analysis of pyrrole-protein adducts [5,36]. In 2016, Xia et al. synthesized the deuterium isotope 7,9-di-C_2D_5O-DHP as an internal standard for the absolute quantitation of pyrrole-protein adducts, and used this method to quantify pyrrole-protein adducts in the plasma and hemoglobin samples from PA-treated rats [37].

In addition to these chemical analysis methods, recently Lin's group also developed a polyclonal antibody to recognize specifically pyrrole-protein adducts [70]. Using this antibody, pyrrole-protein adducts were detected in biological specimens from PA-treated rats. However the method for quantitative analysis of pyrrole-protein adducts needs to be further developed.

5. Pyrrole-protein adducts – A biomarker of PA exposure and PA-induced liver injury in humans

Clinical diagnosis of human liver injury specifically caused by exposure to toxic PAs continues to be highly challenging due to the lack of reliable and specific diagnostic method. The development of specific biomarkers associated with the exposure to toxic PAs would be very useful for the clinical confirmative diagnosis of PA-ILI.

Routine clinical liver tests provide a series of serum biomarkers of liver injury, such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and bilirubin. In addition, some serum biomarkers have been proposed specifically for assisting the diagnosis of drug-induced liver injury (DILI), including microRNAs, glutamate dehydrogenase, macrophage colony stimulating factor receptor, paraoxonase 1, cytokeratin-18, and high mobility group box protein 1 [71–73]. Nevertheless, among these biological modulations, most of these biomarkers are not drug specific. On the contrary, for PA-ILI, pyrrole-protein adducts have been demonstrated to be a reliable mechanism-based biomarker of PA exposure and PA-induced hepatotoxicity. Most of the research findings for using this biomarker are from our recent studies [5,6,20,36,37,74,75].

In 2011, Lin et al. reported the first identification of pyrrole-protein adducts in the blood of a patient who was diagnosed as HSOS and confirmed to intake a PA-containing herb, *Gynura segetum* [6]. In the subsequent studies, using quantitative LC/MS/MS analysis, Lin and co-workers identified blood pyrrole-protein adducts in 15 patients, each of whom consumed the PA-containing *G. segetum*, but not in healthy subjects [5,20]. These PA-ILI patients, including 9 men and 6 women with ages ranging of 35–73 years old with the intake of the herb from 5 days to 200 days via self-medication, had detectable blood pyrrole-protein adducts ranging from 0.14 to 74.4 nM (Table 1) [5]. In addition, the same pyrrole-protein adducts were detected in the liver and blood of rats and mice treated with the same PA-containing herb (*G. segetum*) taken by PA-ILI patients [5,6,75].

All the currently available results demonstrate that pyrrole-protein adducts are mechanism-based and specific in PA-ILI, and therefore, pyrrole-protein adducts in the blood can be developed as a potential biomarker of PA exposure and PA-ILI in humans [5,6,20,74,75].

6. Perspectives

To date, the diagnosis of PA-ILI mainly relies on the clinical features of HSOS and the exclusion of other etiologies of liver injury coupled with the retrospective identification of possible PA-producing or PA-contaminated natural products and/or foodstuffs ingested by the patients. Lin and co-workers were the first to establish a highly sensitive and specific UHPLC-MS method for the detection of pyrrole-protein adducts in the blood of PA-ILI patients. The studies conducted by Lin and co-workers represent a pioneering start on the development of blood pyrrole-protein adducts as a reliable and specific biomarker for the confirmative diagnosis of PA exposure, and also revealed a potentially high prevalence of PA exposure in DILI patients in China.

However, the currently available detection of blood pyrrole-protein adducts is by LC-MS, which requires a sophisticated and expensive instrument and well-trained personnals to conduct the analysis, and thus is unlikely to be adopted as a routein test in most hospitals. Therefore, it is necessary to develop more practical and doable methods, such as antibody-based ELISA assay to measure blood pyrrole-protein adducts, for routine clinical tests.

On the other hand, the application of blood pyrrole-protein adducts in the risk assessment of PA exposure is also highly challenging and far from being fully characterized. Many factors may affect the level of blood pyrrole-protein adducts, such as the amount and duration of PA consumption and the blood sampling time. Moreover, the correlation between the levels of pyrrole-protein adducts in blood and target tissues have not been fully established. Studies on the association of the formation of pyrrole-protein adducts to PA-ILI are still limited. Well-designed studies addressing the formation and elimination kinetics of blood pyrrole-protein adducts and linking the level of blood pyrrole-protein adducts with the toxicological events would facilitate future development of blood pyrrole-protein adducts as a biomarker for PA-ILI and risk assessment of PA exposure.

Table 1 – Information of 15 PA-ILI patients due to exposure of PA-containing herb, *Gynura segetum* (Lour.) Merr.[a]

Patients code	Sex	Age	Herb intake period	Toxic PAs content in the herb (mg/g)	Blood level of pyrrole-protein adducts (nM)	Jaundice/ascites/ hepatomegaly
1	M	72	5 days	0.274 ± 0.005	6.07	N/Y/N
2	F	57	3 weeks	0.829 ± 0.105	12.90	Y/Y/Y
3	F	54	2 years	7.886 ± 0.656	11.80	Y/Y/N
4*	F	72	2 months	2.283 ± 0.268	15.90	Y/Y/N
5	F	63	2 months	7.452 ± 1.049	4.70	Y/Y/Y
6	M	35	22 days	12.765 ± 2.313	74.40	N/Y/N
7	M	46	1 week	0.554 ± 0.197	11.50	N/Y/N
8	F	72	4 months	8.188 ± 0.460	0.33	Y/Y/Y
9	M	53	200 days	4.237 ± 0.790	10.70	Y/Y/N
10	F	73	10 days	5.746 ± 0.077	20.02	Y/Y/N
11	M	44	44 days	10.094 ± 0.051	32.11	N/Y/Y
12*	M	42	10 days	13.645 ± 0.059	0.65	Y/Y/Y
13	M	49	70 days	1.828 ± 0.018	0.14	Y/Y/Y
14	M	50	1 week	6.240 ± 1.004	8.50	Y/Y/Y
15	M	46	10 days	11.445 ± 0.524	0.14	Y/Y/N

*Patient died with liver failure.
[a] Data summarized from Reference [5].

Nevertheless, considering the current unavailability of specifically diagnostic method for PA-ILI and a high prevalence of PA exposure in DILI patients, as a mechanism-based biomarker, blood pyrrole-protein adducts should be a good candidate to be developed as confirmative and specific biomarkers to diagnose definitively PA-ILI in DILI patients especially those with unknown etiology and to more accurately uncover the incidence of PA-ILI in the population.

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Notes

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