Detailed research report

We performed most of the studies planned. However, due to the COVID19 pandemic, certain experiments have not been completely finished yet, because some of our laboratories were closed for months and the acquisition of certain chemicals become very difficult during the last 1.5 year. Nevertheless, the ongoing studies will be finished during the next few months, then the results will be published (indicating the grant number in the funding/acknowledgements section).

During the 4 years of the grant, we published 18 research articles (ΣIF = 78.889; Q1: 13; Q2: 5) as a part of this research project. In addition, one article is under review and the preparation of one further manuscript is in progress. We explored and characterized many molecular interactions, and their potential utilization were also examined, considering the toxicological importance as well as the analytical, toxin extraction, and detoxification applications. Our main results are summarized in the following six points.

1. Interactions of mycotoxins with serum albumin:

We examined and characterized the interactions of 25 mycotoxins and mycotoxin metabolites with serum albumin, including the binding constants of the formed complexes and the binding sites of mycotoxins on the protein. The following mycotoxins were tested: aflatoxins (B1, B2, G1, G2, and M1) [1], alternariol (AOH) [2], beauvericin (BEA) [manuscript under review], cyclopiazonic acid (CPA) [manuscript under review], deoxynivalenol (DON) [3], dihydrocitrinone (DHC) [4], ochratoxins (OTA, OTB, OTC, and 2'R-OTA) [3,5,6], patulin (PAT) [3], sterigmatocystin (STC) [manuscript under review], T-2 toxin [3], zearalenone (ZEN) [7], α- and β-zearalenol (α- and β-ZEL) [7], zearalanone (ZAN) [8], α- and β-zearalanol (α- and β-ZAL) [8], zearalenone-14-glucoside (Z14G) [8], and zearalenone-14-sulfate (Z14S) [8].

Herein, the decimal logarithmic values of the binding constants (*K*; unit L/mol) of mycotoxins with human serum albumin are listed as well as the binding sites of mycotoxins on the protein: aflatoxins (B1: logK = 4.5, B2: logK = 4.4, G1: logK = 4.4, G2: logK = 4.0, M1: logK = 4.3; binding site: Sudlow's Site I), AOH (logK = 5.6; binding site: Sudlow's Site I), BEA (no interaction observed), CPA (logK = 4.4; binding site: Sudlow's Site I), DON (no interaction observed), DHC (logK = 5.5; binding site: FA1), ochratoxins (OTA: logK = 7.3, OTB: logK = 6.0, OTC: logK = 7.0, 2'R-OTA: logK = 6.3; binding site: Sudlow's Site I), PAT (logK = 4.1), STC (logK = 4.0; binding site: FA1), T-2 toxin (no interaction observed), ZEN and its metabolites (ZEN: logK = 5.1, α-ZEL: logK = 4.7, β-ZEL: logK = 4.3, ZAN: logK = 4.6, α-ZAL: logK = 4.6, β-ZAL: logK = 4.4, Z14G: no interaction observed, Z14S: logK = 4.7; binding site: non-conventional site between subdomains IIA and IIIA). Based on these results, the complex formation of AOH, DHC, ochratoxins, and ZEN (and some of its metabolites) with albumin can be considered as high stability interactions [2-8], thus their albumin binding likely have significant toxicological importance.

The species differences regarding the albumin binding of mycotoxins were also evaluated employing human, bovine, porcine, and rat serum albumins. Similarly to ochratoxin A, ZEN and its metabolites showed very high species dependent alternations, where even more than one magnitude differences were observed between the binding constants of albumin complexes [7,8]. The significant importance of these interactions is also suggested by recent toxicokinetic animal studies: the affinities of albumin-ligand complexes determined by our research group show good correlation with the elimination speed of these mycotoxins. Therefore, the species dependent differences in the albumin binding of ZEN and its metabolites should be considered during the evaluation of animal studies and during the extrapolation of the results to other species.

2. In vivo investigation of mycotoxin-albumin interactions (rat experiments):

We tested the potential effects of Sudlow's Site I markers on the toxicokinetics of OTA. OTA and Site I markers were administered i.v., after which blood samples were collected (2, 6, and 24 h). After 24 h, rats were euthanized and kidney samples were collected. Some competitors (e.g. phenylbutazone and chrysin-7-sulfate) were able to induce a slight decrease in the plasma concentrations of OTA at each time point tested, however, the concentrations of OTA in kidneys remained unchanged [unpublished results]. These data highlight that the displacement of OTA from albumin is very difficult *in vivo*. Our *in vitro* results suggest that the application of some FA1 ligands can be also considered [6], based on the non-competitive mechanism of these interactions. Similar investigations are in progress regarding citrinin (CIT) and ZEN.

3. Interactions of mycotoxins with cyclodextrins (CDs) and soluble CD polymers:

We examined the interactions of the following mycotoxins with native and chemically-modified CDs: AOH, DHC, ZEN, α-ZEL, β-ZEL, Z14G, and Z14S.

DHC formed the most stable complexes with beta-CDs, the quaternary ammonium beta-CD proved to be the most effective binder (logK = 3.2) [9].

Regarding AOH, gamma-CDs (logK = 3.0 to 3.2) formed more stable complexes with the mycotoxin than beta-CDs [10]. Furthermore, cooperativity of beta-CD rings was noticed with beta-CD polymers, which bound AOH with approximately ten-fold higher affinity than the beta-CD monomer [10]. In addition, our recent investigation highlighted that SUGAMMADEX forms highly stable complexes with AOH (logK = 4.4) [manuscript is under preparation].

ZEN (logK = 4.2 to 4.8), α-ZEL (logK = 4.6 to 4.9), and β-ZEL (logK = 3.6 to 3.9) produced highly stable complexes with methylated and sulfobutylated beta-CDs [11]. We also tested the potential interactions of modified (Z14S) and masked (Z14G) mycotoxins with CDs. Z14S formed the most stable complexes with dimethyl-beta-CD (logK = 4.6), showing similarly high affinity towards CDs than the parent mycotoxin [12]. In contrast, Z14G preferred gamma-CDs (logK = 3.0 to 3.3) and produced less stable complexes with CDs than ZEN [13].

4. Extraction of mycotoxins from aqueous solutions and beverages using beta-CD bead polymer:

Beta-CD bead polymer (BBP) proved to be an effective binder of AOH [10] and ZEN [14] in aqueous buffers. Importantly, the polymer can extract the metabolites of ZEN, including ZELs, Z14S, and Z14G [12-14]. Our observation that CDs can also entrap masked and modified ZEN metabolites may have high importance, because it suggests that even these derivatives can be effectively removed from solutions employing CD technology.

Moreover, we tested the extraction of AOH from wine and tomato juice [15], and ZEN from beer samples [14]. The removal of AOH from wine and ZEN from corn beer were similarly effective than from buffers, while the extraction of AOH from tomato juice was less successful. We also examined the changes in certain quality parameters of beverages (e.g. color and polyphenol content). For wine and beer, the quality parameters showed much lower relative decrease than the mycotoxin content. These observations also underline that the utilization of CD polymers as mycotoxin binders seems to be promising. Furthermore, BBP can be regenerated with the washing of ethanol-water mixture, and thereafter the polymer can be used more times again with similar mycotoxin binding ability. Therefore, further investigations are reasonable to confirm the applicability of CD polymers as mycotoxin binders. For example, in one of our ongoing study, we examine the effect of BBP on wine quality, employing electronic tongue.

Recently, we also tested the binding ability of BBP vs. further 12 mycotoxins. BBP removed high amounts of CIT, DHC, OTA, STC, ZAN, and ZALs from aqueous buffers [16]. These interactions may be worthy for further investigations in complex matrices.

5. Testing the protective effects of CDs vs. mycotoxin-induced toxicity:

The entrapment of certain mycotoxins by the CD cavity may be properly strong to inhibit the cellular toxin uptake, and consequently CDs may be able to alleviate the toxic impacts of some mycotoxins. To test this hypothesis, *in vitro* experiments and *in vivo* studies were performed on cell cultures and zebrafish embryos, respectively [17]. Our results demonstrated that certain CDs can strongly alleviate or even completely abolish the ZEN-induced toxic effects on HeLa cells and zebrafish embryos. In addition, certain CDs also show similarly strong protective impacts vs. AOH-induced toxicity [manuscript is under preparation].

In vivo investigations in rats did not show changes in the toxicokinetics of OTA after the i.v. administration of quaternary ammonium beta-CD [unpublished results], which is not surprising considering the extremely strong interaction of this mycotoxin with serum albumin (it is very difficult to be disrupted by CDs). Nevertheless, the rat experiments are in progress to test the impact of sulfobutyl-beta-CD on the toxicokinetics of ZEN.

6. Analytical methods used and developed, including sample preparation:

During this project, HPLC methods for the following mycotoxins were optimized in our laboratory [10,12-16]. HPLC-UV: aflatoxin B1, aflatoxin M1, CPA, DON, PAT, STC, ZAN, α-ZAL, and β-ZAL. HPLC-FLD: AOH, CIT, DHC, OTA, ZEN, α-ZEL, β-ZEL, Z14G, and Z14S.

Among the mycotoxins tested, certain CDs induced a more than tenfold increase in the emission signal of the following mycotoxins: AOH, DHC, ZEN, α-ZEL, β-ZEL, and Z14S [9-12]. Therefore, these interactions may be suitable for the sensitization of the fluorescence detection of mycotoxins listed. We tried to develop more sensitive HPLC-FLD methods, where CDs were applied in the eluent or added post-column. Nevertheless, the presence of CDs did not cause strong elevation in the signal, which can likely be explained by the followings: (1) organic solvents (acetonitril/methanol) in the HPLC eluents also induce enhancement in the emission signals of these mycotoxins; (2) the relatively high concentration of organic solvents in HPLC eluents may disrupt the formation of mycotoxin-CD complexes.

Thus, our results suggest that CDs are not suitable to considerably increase the sensitivity of HPLC-FLD methods; however, other analytical applications of CD polymers in sample preparation were demonstrated. The fact that AOH and ZEN were effectively displaced from the CD cavity (using ethanol-water mixture) regarding BBP demonstrates that CD polymers bound to insoluble beads can be used for extraction and sample enrichment from complex matrices [14,15].

In addition, albumin can also be utilized in sample preparation as an affinity protein, as it has been demonstrated in our study [15]. Since no antibody-based products are available for sample enrichment regarding the emerging mycotoxin AOH, we used bovine serum albumin to extract and purify AOH from tomato juice samples. Thus, the strong albumin binding makes possible this kind of application of the protein. The selectivity of albumin toward mycotoxins is obviously lower (compared to antibodies); however, it can be used as a cheap alternative.

Finally, we performed a detailed investigation regarding the effects of microenvironmental changes on the fluorescence signal of AOH. These studies demonstrated that magnesium ions can strongly increase the emission intensity of the mycotoxin. Based on this principle, we successfully developed a novel magnesium-sensitized HPLC-FLD method, which is suitable for the highly sensitive analytical detection and quantification of this emerging mycotoxin [18].

In conclusion, our studies provided basic data regarding the molecular interactions of mycotoxins with serum albumin and CDs, and give a good starting point for the development of new CD-based mycotoxin binders for mycotoxin removal and detoxification purposes.

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