Recent advances on *Alternaria* mycotoxins

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Abstract

Some of the secondary metabolites produced by *Alternaria* species have been recognized potentially harmful for human health due to their toxicity and occurrence in food commodities. Currently, there are no regulations worldwide for these mycotoxins with the exception of Bavarian health and food safety authority who decided to limit at 500 µg/kg the tenuazonic acid content in sorghum/millet-based infant food. The European Food Safety Authority (EFSA) performed a risk assessment for four of the known *Alternaria* mycotoxins (alternariol, alternariol monomethyl ether, tenuazonic acid and tentoxin) and established the threshold for toxicological concern (TTC) for these mycotoxins.

In this review recent data on the biological activity, toxicokinetics, human exposure, modified forms and reduction strategies of the prevalent *Alternaria* mycotoxins are reported.

Introduction

Within the *Alternaria* genus, *A. alternata, A. tenuissima*, *A. arborescens* and relevant species-group, *A. infectoria* sp.-grp and *A. japonica* have been reported to occur in food products and to produce several toxic secondary metabolites [1]. In 2011, the European Food Safety Authority (EFSA) provided a scientific opinion on the risks for public health related to the presence of *Alternaria* mycotoxins in feed and food [2]. Currently there are no regulations for *Alternaria* toxins worldwide and the EFSA scientific opinion represents the first risk assessment of these mycotoxins. The EFSA Panel on Contaminants in the Food Chain considered the toxins that have been reported to occur in food and feed i.e. alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), iso-tenuazonic acid (iso-TeA), altertoxins (ATXs), tentoxin (TEN), altenuene (ALT) and AAL-toxins (*Alternaria alternata* f. sp. *lycopersici* toxins) [2]. Successively an EFSA scientific report assessed the dietary exposure to AOH, AME, TeA and TEN of the European population [3]. Considering the very limited toxicity data available for these toxins it was decided to use the threshold of toxicological concern (TTC) approach to assess the relative level of concern of these mycotoxins for human health. The value of TTC is 2.5 ng/kg bw per day for AOH and AME, and 1500 ng/kg bw per day for TeA and TEN [3].

In this review recent data on the biological activity, toxicokinetics, human exposure, modified forms and reduction strategies of prevalent *Alternaria* toxins are reported.

Biological activity and toxicokinetics

In the last decade several papers have been published on the *in vitro* toxicity of *Alternaria* mycotoxins. The *in vitro* mutagenicity of altertoxins is well known as well as the genotoxicity of AOH and AME, while no mutagenic activity has been reported for TEN and TeA [2]. The *in vitro* and *in vivo* studies on mechanisms of action and toxicity of AOH has been recently reviewed [4]. The authors concluded that the available mechanistic information obtained from *in vitro* studies is consistent with DNA damage caused by indirect mechanisms, implying that there is a threshold of effect. It was suggested to perform additional *in vivo* genotoxicity studies with focus on gastrointestinal tract [4]. The toxicity of other emerging *Alternaria* mycotoxins has also been recently reviewed [5]. The high *in vitro* mutagenicity of stemphyltoxin III, a perylene chinone with

a chemical structure very similar to ATX II, has been recently demonstrated [6]. However, no data on the natural occurrence of this toxin have been reported [2, 5].
The chemical synthesis of phase I metabolites of AOH and AME i.e. 4-OH-AOH and 4-OH-AME, has been reported and their toxicity was compared to the toxicity of AOH and AME. The four compounds were tested for oxidative stress, DNA damage, topoisomerase inhibition, cellular uptake and metabolism. The effects of 4-OH-AOH and 4-OH-AME were lower as compared to the respective parent compounds AOH and AME [7].
Mixtures of mycotoxins can occur in food products, therefore the *in vitro* toxicity of different mycotoxin mixtures is continuously explored by several authors. AOH and ATX II were tested in constant ratio combination of 1:10 i.e. ATX II in the range of 1-10 µM and AOH in the range of 10-100 µM, or 1:1, i.e. both in the range of 1-10 µM, or 1:1, i.e. both in the range of 1-10 µM, and showed additive cytotoxic effects in HepG2, HT29 and HCEC-1CT cells for the majority of tested combinations [8]. The cytotoxic effects of binary combinations of *Fusarium* toxins (enniatin B, aurofusarin, deoxynivalenol, nivalenol and zearalenone) with TeA was evaluated by the WST-1 assay in the colorectal carcinoma cell-line Caco-2 after 24 h incubation. Unexpectedly, TeA in combination with *Fusarium* toxins reduced the cytotoxicity of these mycotoxins, compared to expected combinatory effects [9]. In particular, a significant reduction of cytotoxicity was observed for the combination of TeA with deoxynivalenol. On the contrary, synergistic estrogenic effects were observed *in vitro* when binary combinations of AOH with zearalenone or α–zearalenol were tested [10].
Toxicokinetic studies are important to assess the metabolism of ingested toxins, the rate of absorption and excretion. These studies are important for risk assessment purpose and for the identification of possible biomarkers of exposure. The results of the toxicokinetics of AOH in NMRI mice were reported in an EFSA supporting publication [11]. The study, conducted with radiolabelled AOH, showed low systemic absorption, with about 90 % of the total dose (radioactive) excreted via faeces and up to 9 % excreted via urine. The blood levels did not exceed 0.06 % of the administered dose (radioactive). In NMRI mice administered with non-radiolabelled AOH four hydroxy-metabolites of AOH were identified in blood and urine. The total urinary excretion of AOH and of the four metabolites during the 72 h collection period accounted for 0.1 to 0.6 % of the administered dose (200 mg/kg BW). This is in contrast with the results obtained with radiolabelled AOH, which suggested that up to 9 % of the applied (radioactive) dose was excreted via urine. A plausible explanation for this discrepancy could be that other metabolites, such as glucuronides and sulfates of AOH or breakdown products, contributed to the radioactivity recovered in urine of mice administered with radiolabelled AOH [11].

Human exposure
A dietary exposure assessment to AOH, AME, TeA and TEN in the European population has been recently estimated by using 15,563 analytical results obtained from 4,249 selected samples collected from 2010-2015 [3]. The report estimated the mean exposure to each mycotoxin for 7 age classes (infants, toddlers, other children, adolescents, adults, elderly, very elderly). The highest exposures to these toxins were estimated for infants and toddlers whereas fruit and fruit products, vegetable oil, cereal-based foods, tomatoes and tomato-based products were the most important contributors to the dietary exposure to these mycotoxins. The report concluded that for AOH and AME the estimated mean chronic dietary exposures at the upper bound and 95th percentile dietary exposures exceeded the TTC value whereas for TeA and TEN the exposure estimates were unlikely to be a human health concern. Vegetarians seem to have higher dietary exposure to the toxins than the general population [3]. A study conducted in Germany roughly estimated the daily exposure to AOH, AME, TeA and TEN from the consumption data and results of the analysis of 96 samples of tomato products, bakery products, sunflower seeds, fruit juices and vegetable oils [12]. The total exposure (µg/day) of each toxin was calculated and compared to their TTC values. Based on mean

consumption data of the analysed samples the percentage of the TTC reached by the average daily exposure were 1400%, 280%, 30% and 1.4% for AOH, AME, TeA and TEN, respectively [12]. Human exposure to AOH and AME and other 46 mycotoxins was assessed in a mycotoxin-dedicated total diet study (mTDS) performed in 2013 in the Netherlands. AOH and AME were detected at levels of 1.0-8.9 µg/kg in a number of composite samples, including tomato products, nuts, cereal products, chocolate and wine [13]. The exposure values were within the ranges estimated by EFSA in 2011 [2] and the high level of exposure (95th percentile) exceeded the TTC for AOH and AME in the two examined populations (2-6 years and 7-69 years) [14]. These results indicate a need for additional toxicity data for the genotoxic AOH and AME and a new risk assessment.

Following the EFSA suggestion to continue to generate more analytical data on the occurrence of *Alternaria* toxins, several papers have been recently published on this topic. AOH was found in all 44 commercial beers available in Germany with a median concentration of 0.45 µg/L [15]. TeA, AOH and AME were measured in mouldy samples of sweet pepper produced in Argentina at median levels of 96, 26 and 14 µg/kg, respectively with positives samples of 50%, 26% and 14%, respectively [16]. AOH, AME, TeA and TEN were found to occur regularly in cereals, tomato sauces, figs, wine and sunflower seeds in the Netherlands, whereas ALT was not detected in any of the 95 samples analysed [17]. A focused survey on 3 food categories (figs, sunflower products and tomato products) confirmed the high incidence of samples positive to TeA (>60%), whereas AOH and AME occurred in 26 and 7% of analysed samples, respectively [18]. Despite the occurring of high levels of TeA in sunflower seeds (up to 1350 µg/kg) and figs (up to 1728 µg/kg), the authors concluded it is unlikely that the population in the Netherlands is exposed to levels of concern. However, it was also reported that in the worst case situation, consumption of these products may result in exposure at levels above the TTC of 2.5 ng/kg body weight/day for AOH [18].

Within the 370 wheat samples collected in 2015 in Anhui province of China, TeA (100% positives, median 150 µg/kg) was the predominant toxin detected followed by TEN (77% positives, median 77 µg/kg), AOH (47% positives, median 7.9 µg/kg) and AME (15% positives, median 4.2 µg/kg). Moreover, 95% of the wheat samples were positive for more than one type of *Alternaria* mycotoxins. Authors indicated the need to set tolerance limits for these mycotoxins in China, and to produce more data on their occurrence also in other agro-products [19].

It is known that TeA is produced by *Alternaria spp.* as well as *Phoma sorghina*. The natural occurrence of TeA in 100 Brazilian sorghum samples collected at four grain maturity stages was found strictly associated with the presence of *P. sorghinia* that was the only *Phoma* specie isolated. TeA was detected in 100% of samples, from 20 to 1234 µg/kg, with the exception of those collected at milk stage (36% positive) [20]. High levels of TeA have been recently confirmed in sorghum/millet-based infant food consumed in Germany, therefore the Bavarian health and food safety authority decided to limit at 500 µg/kg the TeA content in these foods. This is the first official decision of an authority regarding *Alternaria* mycotoxins worldwide [21]. The occurrence of AOH, AME and TEN was evaluated in strawberries samples stored at different temperatures ranges and periods. AOH and AME but not TEN were detected in stored samples and no difference in levels and incidence of mycotoxins production was observed among both ranges of temperatures studied [22].

An alternative way to measure human exposure to mycotoxins at individual level is the measurement of the mycotoxin or its metabolite in biological fluids, mainly urine. It was previously reported that about 90% of the ingested TeA (30 µg) was excreted in 24 h urine by two volunteers [23]. Human exposure to TeA was determined in 48 adults in Germany by measuring urinary concentration of this mycotoxin. TeA was detected in all samples and its urinary concentrations were used to estimate the provisional mean daily intake (PDI) that was 183 ng/kg body weight. This value is about 8 times lower the TTC value established by EFSA for this mycotoxin (1500 ng/kg body weight). However, for one individual the PDI (1583 ng/kg body weight) exceeded the TTC value of this mycotoxin [24].

Modified forms

Mycotoxins can be partially conjugated, mainly with glucose or sulfate, either by living plants or their producing fungi. The conjugated mycotoxins can be found in foods together with unconjugated (free) mycotoxins. These compounds are currently referred as "modified mycotoxins" and are potentially toxic because they may be hydrolysed in the digestive tract to form the native mycotoxin [25]. Hildebrand et al., [26] demonstrated that AOH and AME are efficiently conjugated, mainly with glucose, in cultured tobacco BY-2 cells [26]. Successively, the same group demonstrated that sulfate conjugates of AOH and AME were produced by the fungus *A. alternata* together with unconjugated AOH and AME [27]. They also demonstrated that the fungus, in addition to sulfate conjugates, produces a mixed sulfate/glucoside diconjugate of AOH and AME when cultured on tomato [27].

Modified forms of AOH (AOH-3-glucoside (AOH-3G), AOH-3-sulfate (AOH-3S)) and AME (AME-3-glucoside (AME-3G), AME-3-sulfate (AME-3S)) were chemically synthesized and used as standards for method development and occurrence studies. The natural occurrence of AOH-3S and AME-3S was investigated in 83 commercially available tomato products and 11-26% of samples were found positives for AOH-3S and 32-78% were positives for AME-3S. All investigated samples were negative for AOH-3G and AME-3G [28]. In a previous study involving 31 rice and 16 oat flakes samples commercially available in Belgium none of AOH and AME conjugates (AOH-3G, AOH-3S, AME-3G, AME-3S) were detected [29]. AME-3G was analysed and not found in 159 samples of South African sunflower seeds although overall, over 80% of the samples were positive for one or more analytes. Interestingly, the most occurring toxin was TEN followed by TeA, AME and AOH [30]. No modified forms of AOH and AME were detected in selected food categories (dried figs, sunflower products and tomato products) commercially available in the Netherlands [18]. These data suggest further studies on the natural occurrence of modified *Alternaria* mycotoxins and on the identification and chemical characterization of conjugated forms of other *Alternaria* mycotoxins such as TeA and TEN.

Reduction of exposure and toxicity

There is a continuous need of effective prevention measures and reduction strategies to reduce the levels and toxicity of mycotoxins in agricultural products in order to minimize human and animal exposure to these important natural contaminants.

Extracts of *Eucalyptus globulus* and *Calendula officinalis* showed fungistatic activity against *A. alternata* and *A. arborescens* and a good reduction of mycotoxins (TeA, AOH, AME) biosynthesis when tested on tomato fruits. Before their practical application the authors suggested to evaluate the impact of these plant extracts on flavour of tomatoes and the residual level of volatiles after storage [31].

The levels of mycotoxins during food processing can be affected depending of the specific process and the stability of the mycotoxin. Clarification process of pomegranate juice naturally contaminated with TeA, AOH and AME produced a significant increase of mycotoxin concentrations in the clarified juice. The increase was explained by the possible presence of modified forms of TeA, AOH and AME in pomegranate juice that were cleaved to form free mycotoxins by the proteolytic enzymes that are used during clarification. The stability of the three toxins was also evaluated during pasteurization of juices. As expected, no change of mycotoxin concentrations was observed [32].

Extrusion process can reduce mycotoxin levels in treated products and the processing conditions can be optimized to maximize mycotoxin reduction. Under the optimal extrusion conditions a reduction of 65.6%, 87.9% and 94.5%, for TeA, AOH and AME, respectively, was achieved in naturally contaminated whole wheat flour [33].

It was reported that the polyphenols genistein and delphinidin antagonize the genotoxic effects of AOH in human colon carcinoma cells and might aid to protect against genotoxic damages caused

by AOH [34]. On the other hand, the polyphenol quercetin showed no protective effect on the cytotoxicity of AOH and AME when they were tested simultaneously in Caco-2 cells [34].
A review on possible strategies that can be applied in post-harvest to reduce mycotoxin biosynthesis in fruits and vegetables has been recently published [36]. Arginine and urea, a metabolite of Arginine catabolism, inhibit the biosynthesis of TeA, AOH and AME in cultures of *A. alternata* on PDA and wheat medium. Although these results cannot be generalized, the Authors emphasized that Arginine has a great potential to control the biosynthesis of several mycotoxins under certain conditions [37].

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