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Short communication

On the masked mycotoxin zearalenone-14-glucoside. Does the mask truly hide?

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ABSTRACT

In the matter of foodborne mycotoxins, beside a number of regulated compounds, regulations are totally missing for phase-II plant metabolites – the toxicological knowledge of which is still in its infancy. Currently, zearalenone-14-glucoside is in the pipeline and its toxicological role is under a glowing scientific debate. In our work it clearly showed high toxicological concerns as it is prone to conversion to well-known toxic compounds (i.e. zearalenone and both zearalenol isomers) when exposed to breast cancer cells culture. The need of future risk assessment studies has been pointed out accordingly.

1. Main text

The tight interconnection between diet and health is known since the ancient time – e.g. Hippocrates of Kos claimed that "Let thy food be thy medicine and thy medicine be thy food" at the turn of the fourth and the fifth century BC (attributed citation). Nowadays the importance of food safety in the maintenance of human health and wellbeing is an unquestionable fact. It has been recently estimated indeed that chemical, physical and biological agents ingested by diet cause more than 200 recognized diseases in human (Kass and Riemann, 2006). The presence of food contaminants has become of increasing concern for consumers, governments and producers, in consideration of the serious implication for food and feed safety, food security, and global trade. In this framework, among natural toxicants, mycotoxicosis plays a major role in the onset of several disorders and diseases (Hussein and Brasel, 2001). Foodborne mycotoxins, secondary metabolites produced by filamentous fungi infecting crops worldwide, rise indeed severe health concerns for several reasons. The widespread diffusion in food and feed and the incapability to void across the food chain the contamination level are among the most prominent. Also, the wide array of adverse effects in mammals and the systematic daily intake – which may lead to chronic toxicity producing in some cases severe illnesses and dysfunctions (Kuiper-Goodman, 1998) – should be considered as well. In spite of this, mycotoxins in food are still a largely underestimated global health issue, as subchronical and/or synergistic effects are often overlooked (Wild and Gong, 2010). In order to ensuring the safety of food and feed, a number of mycotoxins have been objects of extensive investigations along the past years, including deoxynivalenol, fumonisin B1 and B2, aflatoxin B1 and B2, ochratoxin A, patulin and zearalenone. Currently, although not well harmonized, regulations are in force for them in Europe and in many other countries worldwide (for Europe: EC No 1881/2006, EU No 165/2010, EU No 105/2010). Nonetheless, besides parent compounds, a wide range of masked or modified compounds has been described over the last decades. Masked mycotoxins are metabolites produced by phase-II plant metabolism – which are typically conjugated with amino acids, glucoses, sulfate groups and glutathione – that may co-occur as contaminant in addition to parent compounds in food and feed (Berthiller et al., 2013). In spite of an increasing number of studies on masked mycotoxins, neither regulations nor recommendations has been enforced for these compounds, mainly because the decision

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making process is blurred by the lack of toxicological data. Keeping in mind that the fungal infection and the subsequent mycotoxin accumulation is practically impossible to avoid, a rational regulation should be adopted in order to prevent the unjustified rejection of food products and ingredients, thus preventing unreasonable trade barriers and other severe effects on market (Stoev, 2015). On the other hand, the scientific community agrees on the inclusion of masked mycotoxins into a sound risk assessment (Berthiller et al., 2013; EFSA, 2014). To this end, their possible toxicity should be carefully evaluated beforehand. Even if a lower toxicity of masked forms in respect to the parent compounds is commonly argued (Berthiller et al., 2013), the point is actually disputable and it cannot be eligible as general law. Depending on the part of such forms in determining the total toxic load, regulations could change in the near future.

Our work focused on zearalenone-14-glucoside (ZEN-14-Glc) that is an abundant phase-II plant metabolite of zearalenone (ZEN). ZEN is a largely described mycotoxin produced by fungi belonging to *Fusarium* spp. upon infection of small grains and maize (Drzymala et al., 2015). It may contaminate several cereal-based products worldwide including flour, malt, soybean and beer. Besides cytotoxic and genotoxic effects, ZEN poses a health risk for humans and animals on account of its xenoestrogenic activity (EFSA, 2011). Sexual disorders, anabolic effects and development alterations are indeed ascribed to the contamination by ZEN, including, for instance, severe effects on the pubertal timing in human (Massart and Saggese, 2010). The involvement of ZEN in the breast tumorigenesis and tumor maintenance is also under debate (Pazaiti et al., 2012).

Notably, ZEN can be converted in the ZEN-14-Glc up to 30% (Berthiller et al., 2013). Up to now, the understanding of ZEN-14-Glc toxicity is in its embryonic stage, as the hazard of ZEN-14-Glc has been supposed solely on the basis of the hydrolysis to the aglycone observed after digestion in mammals (De Boevre et al., 2015), and upon cleavage by human microbiota (Dall'Erta et al., 2013). Further, the role of ZEN-14-Glc in determining the total ZEN-dependent toxic load is still completely unknown. Nonetheless, glucosidation is considered an effective strategy for mitigating ZEN toxicity since it effectively prevents the interaction with the estrogen receptors (Poppenberger et al., 2006).

For the first time, in this work it has been observed that ZEN-14-Glc is converted in a time-dependent manner in well-known xenoestrogens by human breast cancer cell culture.

As first, the cytotoxic activity of ZEN-14-Glc has been investigated in MCF7 by using MTS assay, but no cytotoxic effect has been observed up to 1 μM (Fig. 1).

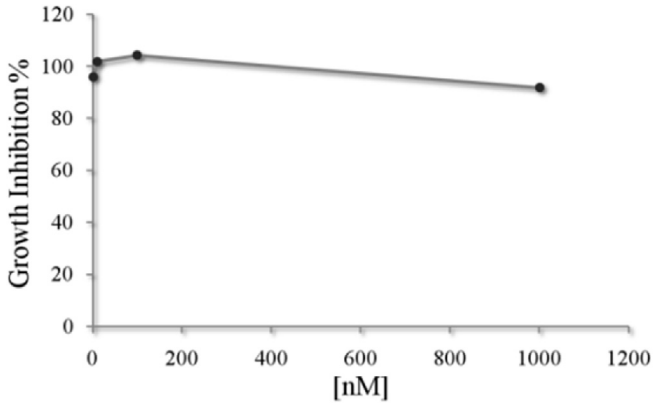

Fig. 1. Growth Inhibition curve in MTS assay. No effects on cell viability were found up to 1 μM.

In details, MCF7 cells have been incubated with ZEN-14-Glc 1 μM for 2 and 6 h and the presence of ZEN, α-zearalenol and β-zearalenol has been monitored by using LC-MS analysis in both cell lysate and growth medium (results are reported in Fig. 2, while experimental details are reported in Supporting Information). The MCF7 cell line was chosen because: i) It is a solid benchmark for a wide number of bioactivity assessment assays (e.g. ref. (Santos et al., 2013; Wang et al., 2014)). ii) In the view of the possible role of ZEN in breast cancer development and maintenance, it is important to note if ZEN-14-Glc can be converted into known xenoestrogens by an estrogen-sensitive cell culture.

In both the cell lysate and growth medium the presence of ZEN, α-zearalenol and β-zearalenol has been observed at each incubation time, with the exception of β-zearalenol that was under the limit of detection in the growth medium at 2 h. The repartition of toxic metabolites between cells and growth medium has been thus pointed out clearly (see Supporting Information for further details).

Furthermore, it is worthy to note the presence of an unknown metabolite (ringed in Fig. 2) in the growth medium at each incubation time responding to the same transitions recorded for zearalenols but with a reduced retention time (likely due to a an increased polarity). On the contrary the presence in cells was under the limit of detection. This compound is formed only when the cell culture is fed with ZEN-14-Glc, and not its parent form (data not shown). As the formation of more toxic metabolites cannot be excluded, future investigations should be devoted to elucidate the formation of further unknown metabolites for a better understanding of ZEN-14-Glc metabolism.

Our work showed that breast cancer cells culture – which is widely used in bioactivity assays – can convert ZEN-14-Glc to several well-known xenoestrogenically active aglycones. It is worth noting that the chosen assay asked for a growth medium including fetal bovine serum among other components. Although serum-free media are indeed available, the addition of serum is actually crucial to provide essential transport proteins, fatty acids, growth factors and hormones. On the other side, this enzymatically active extracellular environment, which strongly resembles the *in vivo* like conditions and preserves from severe metabolic alterations in the cell behavior, may actually play a critical role in the bioavailability and transformation of compounds. The outcome of the assay are thus the result of possible transformation due to the whole cell culture, without the extrapolation of the contributions of the extracellular medium and the cell itself.

The findings presented herein raise a major issue also from methodological point of view. As aforementioned, the bioactivity/cytotoxicity of masked mycotoxins is a thorny problem. Decision making in the matter of food safety must rely on a precise knowledge in terms of chemo-types that truly mediate the effects. Therefore, methodological biases in attributing structure-activity relationship must be avoided. In this framework, the use of MCF7 and other cancer cell lines may affect the bioactivity statement of ZEN-14-Glc and other masked mycotoxins *per se*. To better understand the experimental outcomes of masked mycotoxins in cell-based assays, our results pointed out the need for checking the possible reversion of these compounds as a common practice in their bioactivity assessment.

In conclusion, ZEN-14-Glc appeared to be prone to deglycosylation in a mammalian cell culture. Keeping in mind that the cooperativity of the whole spectrum of metabolites ultimately causes the toxic action, the evidence of partial hydrolysis of this plant glycoconjugate suggests the existence of additional – and actually still overlooked – actors in determining the total ZEN-dependent xenoestrogenic load. However, the concern for human health should be claimed cautiously, at least as regard the xenoestrogenic activity, because deglycosylation phenomena have to