In vitro endocrine activity of mycotoxins and their mixtures.

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Introduction

A lot of substances in food and feed can interfere with the hormonal system of humans and animals by influencing the synthesis, metabolism or transport of hormones; or by interacting directly with the hormone receptor (as an agonist or antagonist). Over the past decades there has been a growing concern about the exposure of animals and humans to these endocrine disrupting chemicals (EDCs). EDCs can be chemicals of synthetic (pesticides, industrial chemicals, bisphenols,...) or natural origin (mycotoxins, fytoestrogens,...) (Diamanti-Kandarakis et al., 2009).

Mycotoxins are natural components that can cause adverse health effects in an organism. They include a group of chemically different compounds coming from the secondary metabolism of moulds and are produced mainly by 5 genera of *fungi: Aspergillus, Penicillium, Fusarium, Alternaria and Claviceps* (Steyn, 1995). A substantial part of the crops that are grown in the EU for food and feed contain a detectable amount of mycotoxins (D'Mello et al., 1999). Therefore it is very important to examine the impact of mycotoxins and their metabolites and evaluate the risks related to animal and human exposure. The adverse effects include carcinogenic, nephrotoxic, neurotoxic, hepatotoxic, immunosuppressive and gastrointestinal toxicity (Fung and Clark, 2004). Results also indicate that certain mycotoxins and their metabolites can act as potential endocrine disrupters on the level of nuclear receptor signalling and cause a change in the hormone production. In this context, zearalenone (ZEN) (a *Fusarium* mycotoxin) and its metabolites α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) are oestrogens. α-ZEL exhibits the strongest oestrogenic potency (comparable to 17β-oestradiol). Moreover, these mycotoxins increase the production of progesterone, oestradiol, testosterone and cortisol hormones in the H295R steroidogenesis assay (Frizzell et al., 2011). In vivo, ZEN and its metabolites, α-ZEL and β-ZEL, exhibit endocrine activity in pigs, bovine and sheep causing infertility problems (Fink-Gremmels, 1999; Tiemann and Danicke, 2007). The toxicity of trichothecenes such as deoxynivalenol (DON), nivalenol (NIV) and fusarenon x (FUSx) (*Fusarium* mycotoxins) involve inhibition of protein and DNA synthesis and general cytotoxicity (Fung and Clark, 2004). DON is considered to be responsible for nausea and decreased feed intake in pigs and is an immunosuppressant (Fink-Gremmels, 1999). *In vitro*, there is no evidence that DON can interact directly with steroid hormone receptors to cause endocrine activity, but effects on steroidogenesis and alterations in gene expression indicate its potential as an endocrine disrupter (Ndossi et al., 2012). Alternariol (ALT) is produced by several *Alternaria* species and produces toxic effects in animals, including fetotoxic and teratogenic effects. ALT is mutagenic and clastogenic in various *in vitro* systems (Marin et al., 2013). ALT also exhibits a weak oestrogenic response and results in a significant increase in oestradiol and progesterone production (in H295R cells) (Frizzell et al., 2013; Lehmann et al., 2006). Fumonisins (*Fusarium toxins*) are a group of mycotoxins with a strong similarity to sphinganine, the backbone precursor of sphingolipids. They inhibit ceramide synthase, a critical enzyme for the synthesis of sphingolipids. Fumonisins are involved in acute and chronic toxicity in several animal species, including carcinogenicity (Marin et al., 2013). The IARC has classified fumonisin B1 (FB1) as possibly carcinogenic to humans (Group 2B). Ochratoxine A (OTA) can be produced by several species of *Aspergillus and Penicillium*. OTA inhibits protein, DNA and RNA synthesis. OTA has been shown to induce many toxic effects in animals, the most prominent being nephrotoxicity; and it is also classified as possibly carcinogenic to humans (Group 2B) (Marin et al., 2013). *In vitro,* OTA also shows some endocrine activities, it may be a mild antagonist for the oestrogen, androgen, progestagen and glucocorticoid receptor and increases the production of oestradiol in H295R cells (Frizzell et al, 2013). Citrinin (CIT) is also produced by several species of *Aspergillus and Penicillium*, but is less stable then OTA, it appears to be destroyed by food processing and thus can be more a problem for life stocks than for humans, it mostly acts as a nephrotoxin (Fung and Clark, 2004).

Both *in vivo* and *in vitro* additive, synergistic or antagonistic effects are detected for mixtures of mycotoxins (Alassane-Kpembi et al., 2013; Tatay et al., 2014; Tammer et al., 2007). The impact of all these different components in food and feed is impossible to predict. The potential health risks of mycotoxins require legal limits of maximal permissible concentrations in food and feed, based on toxicological and/or epidemiological data. For a significant number of mycotoxins and a limited number of combinations (like for the fumonisins) the maximal levels have already been determined (in EU, maximal limits are defined in regulation (EC) n°1881/2006 for food and in Directive 2002/32/EC, for feed), but these include far from all, or not even the most common combinations of mycotoxins (i.e. DON + ZEN) (D'Mello et al., 1999).

The aim of the study is to investigate, whether mycotoxins may exhibit other endocrine activities (through receptor interaction) besides those that have already been identified such as the oestrogenic activity of ZEN and its metabolites. For this purpose, the single components were first tested for their agonistic or antagonistic activity on the oestrogen receptor (ER), androgen receptor (AR), thyroid receptor β (TRβ) and peroxisome proliferator-activated receptor type 2 (PPARγ2) using luciferase reporter cell lines. In addition, attention was paid to the response of combinations of mycotoxins including DON + ZEN, ZEN + metabolites α-ZEL and

β-ZEL, DON + acetyl-DONs etc. Subsequently we verified whether the activities of different combinations were an indication for additive effects. We did this by comparing them to the predicted effect using the theory of concentration addition (Loewe and Muischnek, 1926).

Material & Methods

Mycotoxins

The mycotoxins DON, ZEN, OTA, NIV, FUSx and fumonisin B1/B2 (FB1/FB2) (were purchased from Romer Labs Biopure (Food Risk Management, the Netherlands). 3-acetyl-DON, 15-acetyl-DON and CIT were from Cfm Oskar (Germany); and α-ZEL, β-ZEL and ALT were acquired from Sigma-Aldrich (Germany). All mycotoxins were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and the final concentration of DMSO in the culture medium was 1%. Control medium was prepared with 1% DMSO without mycotoxins and showed typically no deviation from untreated controls.

Cell lines

Four different receptors were studied using different cell lines. The CALUX® system (Chemically Actived LUciferase eXpression) of Bio Detection systems (BDS, Amsterdam, the Netherlands) uses U-2 OS cells (human osteoblast) that are stably transfected with human TRβ or human PPARγ2 (BDS, Amsterdam) and a luciferase reporter construct under the control of a receptor specific response element. To determine the oestrogen and androgen activity we used two human breast cancer cell lines MCF-7 (with endogenous ER isoforms 1 and 2) and T47-D (transfected with human AR), again with a luciferase reporter construct developed by (Willemsen et al., 2004). We refer to these cell lines by using the name of the human receptor of interest that they express.

Cell culture and luciferase assay

The cell lines were maintained in T75 culture flasks (Nunc, VWR, Leuven, Belgium) in growth medium DMEM/F12 (Fisher Scientific, Doornik, Belgium) supplemented with 1% Non Essential Amino Acids Solution (NEAA, Fisher Scientific, Doornik, Belgium), 7.5% heat inactivated foetal calf serum (FCS, Fisher Scientific), 10 U/mL penicillin and 10 µg/mL streptomycin (Fisher Scientific). In case of the breast cancer cell lines, 1 µg/mL insulin was also added to the growth medium. The cells were grown in a 5% CO_2 atmosphere at 37°C until confluence.

For the experiments, the cells were seeded in 96-well plates (150.000 cells/ml for ER, 200.000 cells/ml for AR, 80.000 cells/ml for TRβ and 50.000 cells/ml for PPARγ2) with assay medium: phenol red-free DMEM/F12 medium supplemented with 1% NEAA, 10 U/mL penicillin, 10 µg/mL streptomycin and 5% dextran-coated charcoal-stripped FCS (DCC-FCS, BioDetection Systems, The Netherlands) and incubated overnight to allow the cells to attach. Next, the medium was removed and the following 24 hours (48h for the AR cell line), cells were incubated in the presence of a concentration range of the relevant compounds diluted in assay medium. For the assays of anti-hormonal activity, the inhibitory effect of the chemicals was examined in the presence of the reference agonist at EC_{50} concentration: 17β-oestradiol (2.10^{-11} M, anti-estrogenic activity), dihydrotestosteron (DHT, 10^{-9} M, anti-androgen activity), triiodothyronine (T3, 5.10^{-10} M anti-TRβ activity) and rosiglitazone (ROS, 10^{-7}M, anti-PPARγ activity) (all obtained from Sigma-Aldrich, Germany). The reference antagonists were: ICI 18.780 (ER), flutamide (AR), dibutyl phthalate (DBP, TRβ) and GW9662 (PPARγ2) (all obtained from Sigma-Aldrich, Germany). Then the medium was removed and the cells were incubated with 10% of 400 µM resazurin (Sigma-Aldrich, Germany) in PBS for 2h followed by measuring fluorescence (parallel cytotoxicity measurement) (O'Brien et al., 2000). The PBS and resazurin were discarded with a vacuum pump and the cells were lysed by adding 50 µl of lysis buffer. The luciferase activity was measured as relative light units (RLU's), using a luminometer (Berthold Tristar), after adding glowmix (BDS, Amsterdam).

Data Analysis

Data were represented as mean values ± SEM from three independent experiments with triplicate wells for each dose. Dose–response curves were fitted to a non-linear regression (four parameter curve fit) using GraphPad Prism (version 6.00 for Windows, GraphPad Software, San Diego, CA, USA). For the single mycotoxins, the four parameters are determined using GraphPad Prism and used below.

$$y = y0 + m\,\frac{x^h}{K^h + x^h}$$

Mixture design and calculation of predicted mixture effects

Six mixtures were prepared according to table 1.

	Mix 1a (1:1:1)	Mix 1b (EC50 ratio)	Mix 2 (1:1:1)	Mix 3 (1:1:1:1:1)	Mix 4 (1:1)	Mix 5 (1:1:1:1:1:1)
ZEN	33,33%	5,24%	-	-	-	16,66%
α-ZEL	33,33%	0,21%	-	-	-	16,66%
β-ZEL	33,33%	94,5%	-	-	-	16,66%
DON	-	-	33,33%	20%	-	16,66%
3-acetyl-DON	-	-	33,33%	20%	-	16,66%
15-acetyl-DON	-	-	33,33%	20%	-	16,66%
NIV	-	-	-	20%	-	-
FUSx	-	-	-	20%	-	-
ALT	-	-	-	-	-	-
FB1	-	-	-	-	-	-
FB2	-	-	-	-	-	-
OTA	-	-	-	-	50%	-
CIT	-	-	-	-	50%	

Table 1.
List of mycotoxins and test mixtures (in relative proportions (molar))

The joint effects of a mixture with known composition were calculated on the basis of the non-linear regression curve for each compound in the mixture by using the concept of concentration addition (CA). Concentration addition, also called dose addition, was introduced by Loewe and Muischneck (1926). This model is based on a dilution principle, and was designed for chemicals with a similar mechanism of action, and has proven effective in several settings. The corresponding effect concentration ($EC_{mixture}$) is given as:

$$EC_{mixture} = \left(\sum \frac{p_i}{EC_i}\right)^{-1}$$

where EC_i is the concentration of the compound i alone, causing the effect E and pi is the fraction of i present in the mixture, $EC_{mixture}$ causing the same effect E. This calculated effect was compared with the actual effects observed for the mixtures. This method requires knowledge of the mixture composition including mixture ratios of the compounds and concentration-response data for each of the individual compounds. For this reason, these equations cannot be used for predicting mixture effects that exceed the maximal effect of the least efficacious component, because those effect concentrations cannot be defined. To overcome this limitation, we used the pragmatic solution of Evans and co-workers (Evans et al., 2012), where a minimal (no) and maximal (100%) contribution is defined, and thus the CA is a range defined by those two worst case calculations. Differences between predicted and observed effects were deemed statistically significant when 95% confidence belts of the prediction did not overlap with those of the experimentally observed mixture effects.

Results

Endocrine activity of single mycotoxins

In table 2 (agonism) and 3 (antagonism), the results of the single mycotoxins tested on the four different cell lines (ER, AR, TRβ en PPARγ) were shown. Cytotoxicity was always measured before lysis of the cells by using resazurin. Most mycotoxins were not toxic at the highest concentration tested ($1e^{-5}$M). For FUSx cytotoxicity only started at 10^{-5}M. For DON and 15-acetylDON cytotoxicity started at 3.10^{-6}M. Cytotoxic concentrations were excluded from the experiments.

As expected oestrogenic agonist activity was found for ZEN and his metabolites α-ZEL, β-ZEL and ALT. The EC_{50} and fold induction (FI, = ratio of the measured activity to the control activity (1% DMSO)) are shown in table 2. They seem to be full agonists since the response expressed as fold induction was in the same order as the one for 17β-oestradiol and since in the antagonism test (in the presence of the EC_{50} concentration of the reference compound which is expressed

as 100%) they produced an additive effect close to 200%. α-ZEL exhibited the strongest oestrogenic potency. DON had no agonistic effect and was an antagonist with low potency. 3-acetylDON and 15-acetylDON were both partial agonists with an equal potency but the response expressed as FI of 15-acetylDON was quite low (Figure 1). This is partially due to cytotoxicity at the highest concentrations. In contrast to 3-acetylDON, 15-acetylDON did not cause an additive effect in the antagonism test. The potency order for the agonists was: α-ZEL > ZEN >> β-ZEL > 3-acetylDON ~ 15-acetylDON > ALT. We observed that FUSx and OTA acted as inverse agonists, OTA with a very low potency at the highest concentration tested. NIV, FB1, FB2 and CIT had no observable effects on the ER cell line.

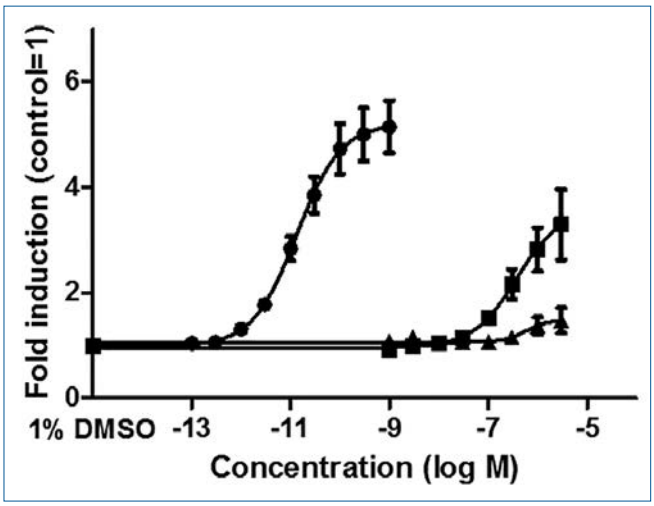

Fig 1.
Oestrogenic response of 17β-oestradiol (●), 3-acetylDON (■) and 15-acetylDON (▲) with the oestrogenic responsive cell line. Data are the mean ± SEM of at least three independent experiments in triplicate.

None of the mycotoxins showed any **androgenic** agonist activity. FB1, FB2 and CIT exhibited no observable antagonistic effect, while all the other mycotoxins demonstrated an antagonistic activity on the AR cell line. The potency order of the anti-androgenic activity was: 15-acetyl-DON > 3-acetyl-DON ~ α-ZEL ~ DON ~ FUSx ~ ZEN ~ ALT ~ OTA > β-ZEL > NIV.

Also, no **TRβ** agonist activity was found for any of the mycotoxins. OTA was found to be an inverse agonist. NIV, 15-acetylDON, FUSx, ALT, FB1/FB2 and CIT showed no effects on TRβ antagonism. The other mycotoxins acted as antagonists. Most of those were only weak partial antagonists with low potency, except for DON and OTA. The potency order for TRβ antagonism is: DON > OTA > α-ZEL > 3-acetyl-DON > ZEN ~ β-ZEL.

On the **PPARγ2** receptor ALT acted as an agonist with a very low potency and both DON and OTA acted as inverse agonists. β-ZEL and FB1 were the only compounds that showed no activity. Only ALT had an additive effect (agonist) in the antagonist test on PPARγ2. All the other tested substances worked as full (DON, OTA and 3-acetylDON) or partial antagonists (ZEN, α-ZEL, NIV, FB2...). The potency order for antagonism was: 15-acetylDON ~ DON > OTA ~ NIV > 3-acetylDON ~ FUS x ~ CIT > FB2 ~ ZEN ~ α-ZEL.

	Cyto-toxicity	ER agonism		AR agonism		TRβ agonism		PPARγ2 agonism	
Compounds tested	Starting from (M)	EC_{50} (M)	FI	EC_{50} (M)	FI	EC_{50} (M)	FI	EC_{50} (M)	FI
Reference	/	17β-oestradiol $1,4\ 10^{-11}$ (± 0,1)	5,1 (± 0,5)	DHT $1,4\ 10^{-9}$ (± 0,6)	13,2 (± 2,1)	T3 $3,7\ 10^{-10}$ (± 0,4)	7,9 (± 1,0)	Ros $6,7\ 10^{-8}$ (± 1,1)	26,0 (± 4,4)
ZEN	/	$8,7\ 10^{-10}$ (± 0,8)	4,8 (± 1,0)	NE		NE		NE	
α-ZEL	/	$3,1\ 10^{-11}$ (± 0,5)	5,6 (± 0,9)	NE		NE		NE	
β-ZEL	/	$1,3\ 10^{-8}$ (± 0,3)	5,0 (± 0,9)	NE		NE		NE	
DON	3.10-6	NE		NE		NE		$4,1\ 10^{-7}$ (± 0,2)	0,1 (±0,02)
3-acetyl-DON	/	$3,8\ 10^{-7}$ (± 1,1)	3,3 (± 0,7)	NE		NE		NE	
15-acetyl-DON	3.10-6	$6,8\ 10^{-7}$ (± 0,5)	1,5 (± 0,2)	NE		NE		NE	
NIV	/	NE		NE		NE		NE	
FUSx	> 10-5	$4,3\ 10^{-7}$ (± 0,5)	0,7 (±0,05)	NE		NE		NE	
ALT	/	$4,7\ 10^{-6}$ (± 1,6)	6,3 (± 1,4)	NE		NE		$> 10^{-5}$	2,3 (± 0,4)
FB1	/	NE		NE		NE		NE	
FB2	/	NE		NE		NE		NE	
OTA	/	NE		NE		$1,6\ 10^{-6}$ (±0,09)	0,6 (±0,07)	$1,4\ 10^{-6}$ (± 0,3)	0,2 (±0,03)
CIT	/	NE		NE		NE		NE	

Table 2.

Agonistic activity of single mycotoxins.

Results are expressed as EC_{50} and fold induction (FI i.e. normalization to the control (1% DMSO)). Data are represented as mean values ± SEM from three independent experiments with triplicate wells for each dose.

NE: no effect measured at highest concentration (10^{-5} M or last not cytotoxic concentration).

	Cyto-toxicity	ER agonism		AR agonism		TRβ agonism		PPARγ2 agonism	
Compounds tested	Starting from (M)	IC_{50} (M)	% of half maximal activity	IC_{50} (M)	% of half maximal activity	IC_{50} (M)	% of half maximal activity	IC_{50} (M)	% of half maximal activity
Reference	/	ICI 2,5 10^{-9} (±0,04)	30,6 (± 4,2)	FLU 7,3 10^{-7} (± 0,9)	19,5 (± 8,0)	DBP 1,5 10-4 (± 1,0)	21,8 (± 4,0)	GW9662 7,6 10^{-10} (± 1,0)	4,5 (± 1,1)
ZEN	/	1,2 10^{-9} (± 0,6)	170,6 (± 5,6)	3,2 10^{-6} (± 1,3)	42,4 (± 5,4)	> 10^{-5}	72,5 (± 8,1)	9,3 10^{-6} (± 5,7)	57,3 (± 2,3)
α-ZEL	/	1,8 10^{-10} (± 1,2)	206,8 (± 25,5)	1,6 10^{-6} (± 0,7)	42,2 (± 20,4)	6,3 10^{-6} (± 2,4)	64,5 (± 3,7)	1,9 10^{-5} (± 1,2)	38,5 (± 2,4)
β-ZEL	/	1,5 10^{-7} (± 0,9)	186,4 (± 17,5)	6,1 10^{-6} (± 0,3)	21,4 (± 6,9)	> 10^{-5}	76,4 (± 8,0)	NE	
DON	3.10^{-6}	4,3 10^{-6} (± 1,2)	37,9 (± 3,3)	1,3 10^{-6} (± 0,8)	29,2 (± 11,8)	3,3 10^{-7} (± 2,0)	16,3 (± 2,4)	3,5 10^{-7} (± 0,3)	2,8 (± 0,2)
3-acetyl-DON	/	2,2 10^{-6} (± 0,4)	139,0 (± 28,4)	1,3 10^{-6} (± 0,2)	28,7 (± 9,9)	2,8 10^{-5} (± 1,7)	41,4 (± 4,4)	3,1 10^{-6} (± 0,2)	7,4 (± 1,4)
15-acetyl-DON	3.10^{-6}	NE		4,4 10^{-7} (± 1,0)	28,6 (± 11,8)	NE		3,4 10^{-7} (± 0,3)	23,3 (± 3,3)
NIV	/	NE		> 10^{-5}	68,1 (± 10,8)	NE		1,6 10^{-6} (± 0,5)	51,3 (± 4,0)
FUSx	> 10-5	3,4 10^{-6} (± 2,5)	61,5 (± 9,7)	2,5 10^{-6} (± 0,5)	22,0 (± 5,9)	NE		3,2 10^{-6} (±0,06)	12,4 (± 1,8)
ALT	/	5,2 10^{-6} (± 1,4)	216,2 (± 26,7)	3,8 10^{-6} (± 0,4)	27,1 (± 4,9)	NE		> 10^{-5}	157,5 (± 35,5)
FB1	/	NE		NE		NE		NE	
FB2	/	NE		NE		NE		8,3 10^{-6} (± 2,1)	45,9 (± 3,9)
OTA	/	> 10^{-5}	33,7 (± 5,0)	4,7 10^{-6} (± 0,9)	40,4 (± 3,7)	1,6 10^{-6} (±0,06)	19,3 (± 4,6)	1,2 10^{-6} (± 0,2)	2,4 (± 0,6)
CIT	/	NE		NE		NE		4,7 10^{-6} (± 1,3)	38,4 (± 1,7)

Table 3.

Antagonistic activity of single mycotoxins.

Results are expressed as IC_{50} and % of half maximal activity (100% is the control (=1%DMSO in the presence of EC_{50} concentration of reference agonist)). Data are represented as mean values ± SEM from three independent experiments with triplicate wells for each dose. NE: no effect measured at highest concentration (1 10^{-5} M or last not cytotoxic concentration).

Endocrine activity of mycotoxin mixtures

The composition of the mixtures is given in table 1. Only for ZEN and its metabolites an EC_{50} ratio was used on the ER receptor. In the other cases, only an equimolar ratio was used because one or more compounds in these mixtures had no or hardly any effect on the receptors (EC_{50} could not be determined). For all mixtures, cytotoxicity was measured as described above. Mixtures 1a, 1b and 4 showed no cytotoxicity at the highest total concentration (10^{-5} M). For mixture 2 and 3, and mixture 5, cytotoxicity started at 3.10^{-6} M and 10^{-5} M respectively. Cytotoxic concentrations were excluded from the calculations.

With the **oestrogenic** responsive cell line, we tested mixture 1 (ZEN, α-ZEL and β-ZEL) in an equimolar ratio and an EC_{50} ratio. In both cases, testing revealed a good agreement with CA, as shown by the experimental data overlapping the predicted CA range (within the 95% confidence band for the concentration-response curve for the observed values, Figures 2A and 2B). Figure 2 includes an extrapolation to extend the predictive range of the CA model. Without extrapolation, the CA model equations limit the predicted effect to the lowest maximal effect of any of the tested compounds, which can be as low as 25% of the maximal observed effect of the mixture (ex. Figure 2E). Mixtures 2 and 3 also revealed a good agreement with CA, as shown by Figures 2C and 2D. Mixture 4 was not tested on the ER cell line because both OTA and CIT showed no agonistic activity. For mixture 5, the CA overestimated the potency compared to the observed values (CA was situated to the left of the real curve, Figure 2E).

Since most single mycotoxins showed some androgenic antagonism, we tested the CA theory on antagonism with the androgenic responsive cell line. Mixture 1 (Figure 3A) seemed to be less potent than predicted by the CA curve, it did not fall in the 95% confidence band and it was shifted to the left. For mixtures 2 and 5 additive effects were found, the predicted CA corresponded well to the tested mixture (Figures 3B and 3E). Mixtures 3 and 4 both deviated a little from the CA curve, the predicted curve shifted to the right of the observed one (Figures 3C and 3D).

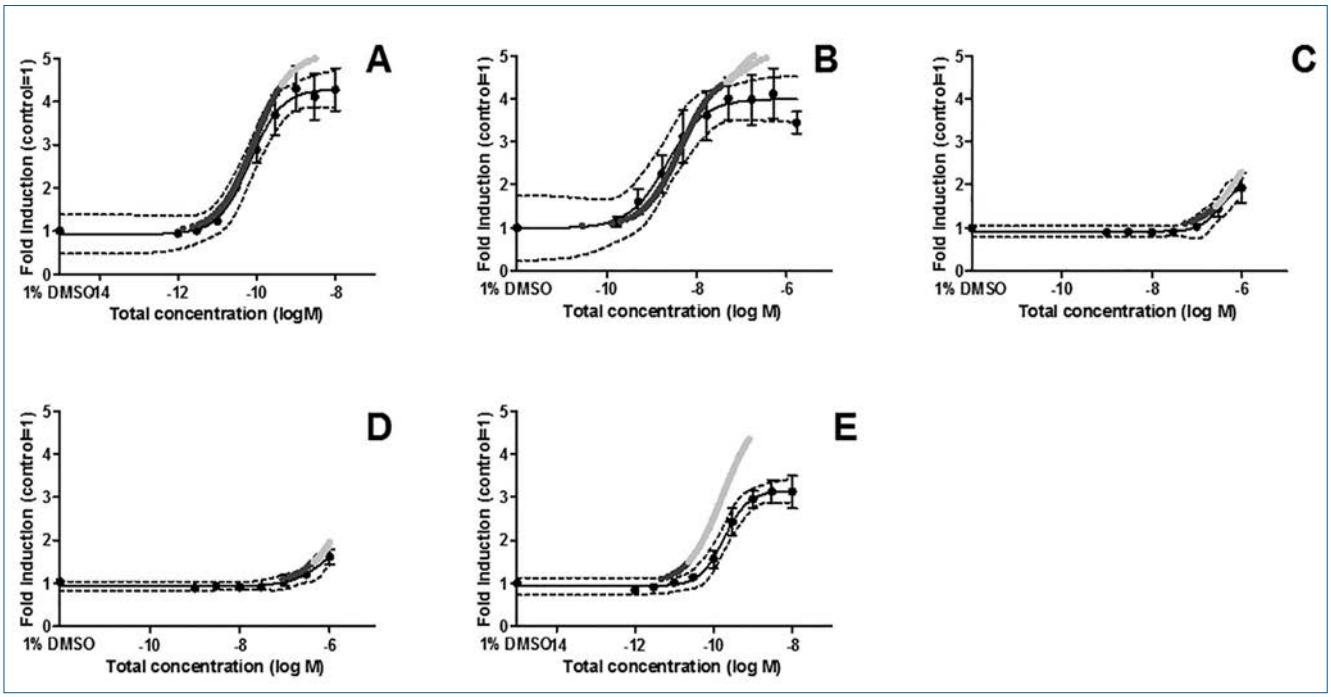

Fig 2.
***Predicted and observed agonistic effects of mixtures on the oestrogenic responsive cell line**. A) Mixture 1a containing ZEN and its metabolites (ZEN, α-ZEL, β-ZEL) in an equimolar ratio. B) Mixture 1b containing ZEN and its metabolites in an EC50 ratio. C) Mixture 2 containing DON and its metabolites (DON, 3-acetylDON, 15-acetylDON) in an equimolar ratio. D) Mixture 3, a 5 component mixture, containing DON, 3-acetylDON, 15-acetylDON, NIV, FUSx in an equimolar ratio. E) Mixture 5, a 6-component mixture, containing ZEN, α-ZEL, β-ZEL, DON, 3-acetylDON, 15-acetylDON in an equimolar ratio. Each graph shows experimental data (dots) with best fit regression curves (solid black) and their 95% confidence belts (dotted black lines). All data are normalized to the control (1% DMSO) and are the mean ± SEM of three independent experiments in triplicate. Prediction curves according to concentration addition are shown as dark grey solid line. The use of extrapolation to extend the range of CA is shown by light grey lines (worst case upper and lower assumptions).*

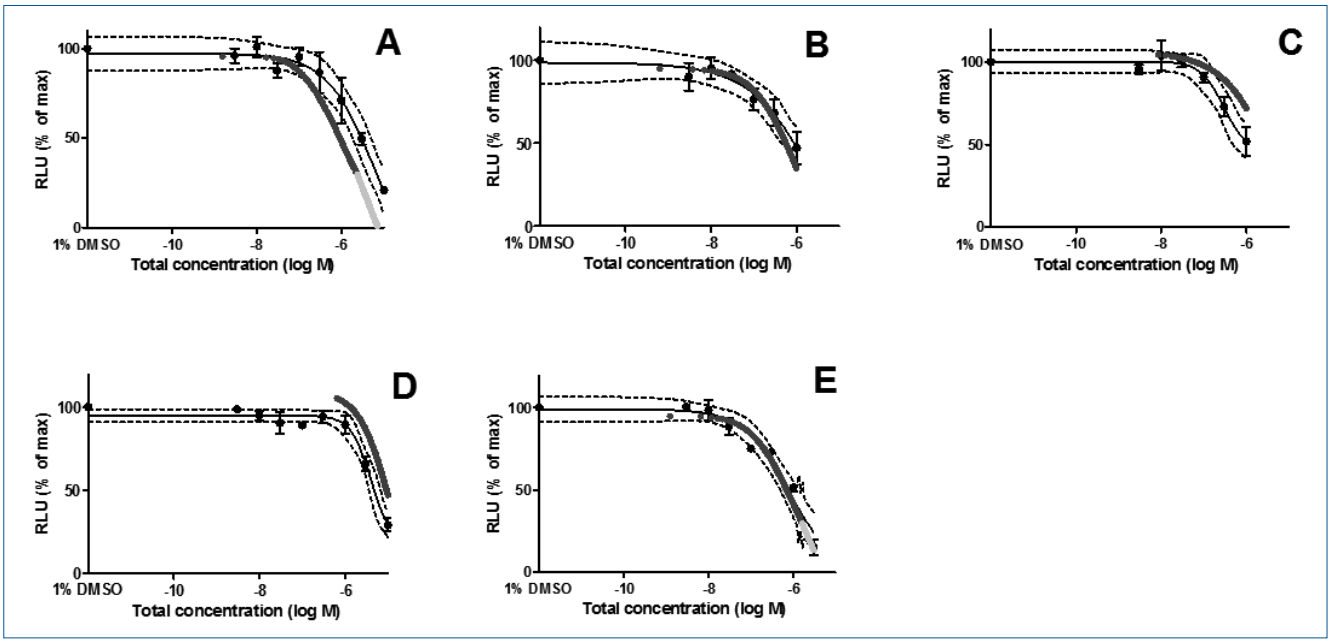

Fig 3.

Predicted and observed antagonistic effects of mixtures on the androgenic responsive cell line. *A) Mixture 1a containing ZEN and its metabolites (ZEN, α-ZEL, β-ZEL) in an equimolar ratio. B) Mixture 2 containing DON and its metabolites (DON, 3-acetylDON, 15-acetylDON) in an equimolar ratio. C) Mixture 3, a 5 component mixture, containing DON, 3-acetylDON, 15-acetylDON, NIV, FUSx in an equimolar ratio. D) Mixture 4 containing OTA and CIT in an equimolar ratio E) Mixture 5, a 6 component mixture, containing ZEN, α-ZEL, β-ZEL, DON, 3-acetylDON, 15-acetylDON in an equimolar ratio. Each graph shows experimental data (dots) with best fit regression curves (solid black) and their 95% confidence belts (dotted black lines). All data are normalized to the control (=100%; 1% DMSO in the presence of 10^{9} M DHT) and are the mean ± SEM of three independent experiments in triplicate. Prediction curves according to concentration addition are shown as dark grey solid line. The use of extrapolation to extend the range of CA is shown by light grey lines (worst case upper and lower assumptions).*

On the **TRβ** responsive cell line, mixture 1 was not tested because both ZEN and β-ZEL showed almost no antagonistic activity, thus it was not possible to determine the parameters needed for the CA calculation. Mixtures 2 and 3 both deviated a little from the CA curve, the predicted curve was situated to the right of the observed one (Figures 4A and 4B). For mixtures 4 and 5 additive effects were found, the predicted CA corresponded well to the tested mixtures (Figures 4C and 4D).

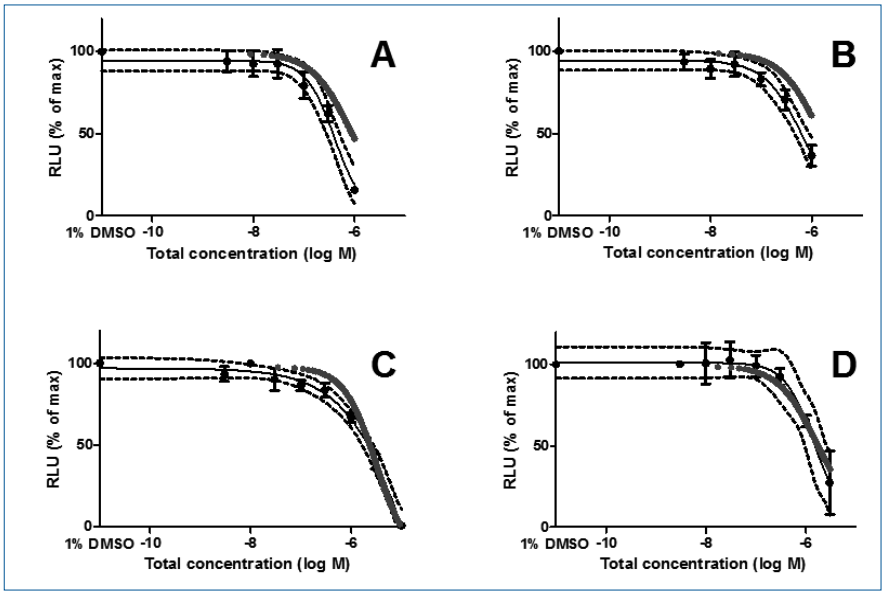

Fig 4.

Predicted and observed antagonistic effects of mixtures on the thyroid β responsive cell line. *A) Mixture 2 containing DON and its metabolites (DON, 3-acetylDON, 15-acetylDON) in an equimolar ratio. B) Mixture 3, a 5 component mixture, containing DON, 3-acetylDON, 15-acetylDON, NIV, FUSx in an equimolar ratio. C) Mixture 4 containing OTA and CIT in an equimolar ratio D) Mixture 5, a 6 component mixture, containing ZEN, α-ZEL, β-ZEL, DON, 3-acetylDON, 15-acetylDON in an equimolar ratio. Each graph shows experimental data (dots) with best fit regression curves (solid black) and their 95% confidence belts (dotted black lines). All data are normalized to the control (=100%; 1% DMSO in the presence of $5*10^{-10}$ M T3) and are the mean ± SEM of three independent experiments in triplicate. Prediction curves according to concentration addition are shown as dark grey solid line.*

On the **PPARγ2** responsive cell line, mixture 1 was not tested because both ZEN and its metabolites showed very low antagonistic activity. For mixture 2, 4 and 5 additive effects were found, the predicted CA corresponded well to the tested mixture (Figures 4A, 4C and 4D). Mixture 3 deviated a little from the CA curve, the predicted curve was situated to the right of the observed one (Figure 4B).

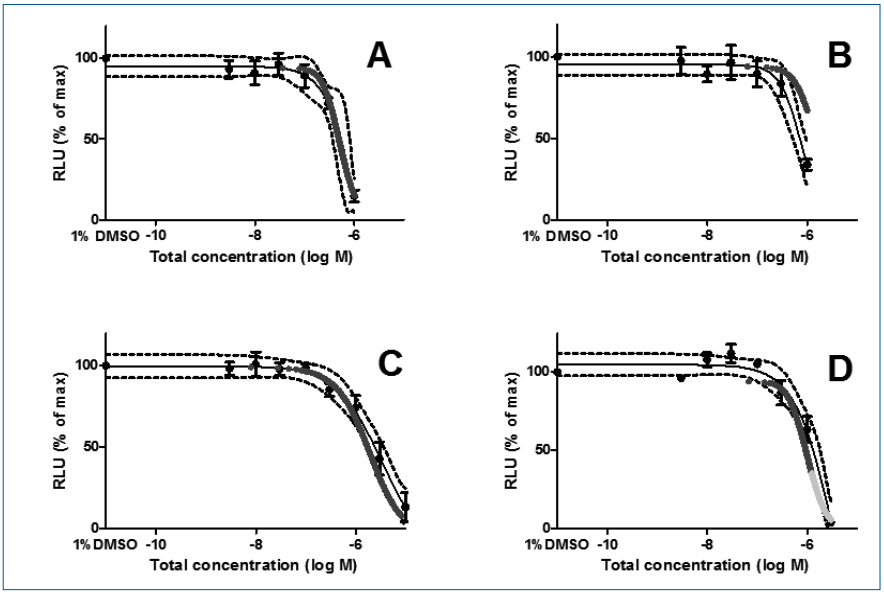

Fig 5.
Predicted and observed antagonistic effects of mixtures on the PPARγ2 responsive cell line. *A) Mixture 2 containing DON and its metabolites (DON, 3-acetylDON, 15-acetylDON) in an equimolar ratio. B) Mixture 3, a 5 component mixture, containing DON, 3-acetylDON, 15-acetylDON, NIV, FUSx in an equimolar ratio. C) Mixture 4 containing OTA and CIT in an equimolar ratio D) Mixture 5, a 6 component mixture, containing ZEN, α-ZEL, β-ZEL, DON, 3-acetyl-DON, 15-acetyl-DON in an equimolar ratio. Each graph shows experimental data (dots) with best fit regression curves (solid black) and their 95% confidence belts (dotted black lines). All data are normalized to the control (=100%; 1% DMSO in the presence of 10^{-7} M Ros) and are the mean ± SEM of three independent experiments in triplicate. Prediction curves according to concentration addition are shown as dark grey solid line. The use of extrapolation to extend the range of CA is shown by light grey lines (worst case upper and lower assumptions).*

Discussion

In the present study, 13 mycotoxins were tested for their potential endocrine activity through interaction with nuclear oestrogen, androgen, thyroid β or PPARγ2 receptors. All single tested compounds showed interactions with multiple receptors, except for FB1 and FB2 for which no effects were found and CIT which is only a weak PPARγ2 antagonist. As expected, **ZEN and its metabolites α-ZEL and β-ZEL** showed strong estrogenic activity, α-ZEL being the most potent one. **ALT** was also **a full ER agonist** but with low potency. These activities and their EC_{50} values were in good agreement with the results of Frizzell and co-workers (2011; 2013) This was expected since these authors use the same oestrogen responsive (MMV-Luc) cell line. Not DON, but both **3-acetylDON and 15-acetylDON** show **partial agonism** on the oestrogen receptor. Their potency is modest (about 25 times less potent than β-ZEL and 25000 times less potent than 17β-oestradiol, Figure 1) but not negligible, so it could contribute to endocrine activity in mixtures, especially since DON is usually found in food at 10-100 times higher concentrations than ZEN (Marin et al., 2013). Moreover, it is in the same order of magnitude as that of bisphenol A (a well known endocrine disrupter used in polycarbonate and epoxyresins) for which a range of adverse effects in animals is detected and major concerns have been raised about its impact on reproductive systems (Molina-Molina et al., 2013; Richter et al., 2007). To our knowledge, the results of the acetylDONs on the oestrogen receptor are new.

Of all mycotoxins tested on the androgen receptor, **15-acetylDON, 3-acetylDON and α-ZEL** have the highest potency as **antagonists on AR**, around the same order as the reference compound flutamide, a non-steroidal antiandrogen drug primarily used to treat prostate cancer.

Our results for OTA and DON were in accordance with those in literature. No agonistic effect was observed at any concentration of OTA and DON on the ER and AR. On the other hand, an antagonistic effect on the ER and AR was seen by us and by another research group (Ndossi et al., 2012; Frizzell et al., 2013). This group did not consider this to be a true antagonistic effect because, despite no significant or small effects in the cytotoxicity test for OTA and DON respec-

tively (in correspondence with this study), this response was observed in all their transactivation cell lines and suggests a parallel change in cell morphology. It may be that toxicity is occurring earlier at some level but further investigation would be required. In another publication (Frizzell et al., 2013) , these authors explained the antagonistic effect of ALT on the AR (and two other cell lines) in the same way, however in our study antagonism for this compound only occurred on the AR and very different effects were seen on the other cell lines. Hence, our results suggest that in this case there is a true antagonistic effect.

Studying the interactions with the thyroid β and PPARγ2 receptor of these mycotoxins is new to our knowledge. Agonism was only seen on the PPARγ2 receptor for ALT, but with very low potency. The most interesting **antagonist for TRβ** was **DON**, it was 500 times more potent than the reference compound DBP (Shi et al., 2011). However, this reference antagonist is a very weak antagonist and was used only due to the lack of commercially available potent antagonists for the TRβ receptor. For **PPARγ2, DON and 15-acetylDON** were **the most potent antagonists**, they were about 500 times less potent then the reference antagonist, GW9662 (Leesnitzer et al., 2002).

Before we can interpret the results of the mixtures, we have to make sure the CA model is applicable. The model relies on the assumption that the mixture components share a common mechanism of action (Loewe and Muischnek, 1926). Since we only look into one effect at a time (i.e. ER agonism, AR antagonism etc.) and all cell lines are designed to study the interaction with one specific receptor, we assume this is the case.

The mixture studies reported in this paper showed that the **CA model provided good predictions** of multicomponent mixture effects in the responsive cell lines used. The dose-response curves of the predicted CA are close to the observed ones. For most of them, the CA is within the 95% confidence interval. However, some small deviations were detected. In the oestrogenic responsive cell lines, the mixtures were tested on their potential agonistic activity. For mixture 5 (ZEN, α-ZEL, β-ZEL, DON, 3-acetylDON, 15-acetylDON), the CA overestimated the potency compared to the observed values (CA is situated to the left of the real curve, Figure 2E). A possible explanation could be the presence of DON in this mixture, which is a weak antagonist and could counteract some of the agonist activity. We would expect the same for mixtures 2 and 3, however these mixtures reacted with a much lower potency than mixture 5 and thus the effect of DON is probably not visible in this case. For mixture 5 (Figure 2E), the extrapolation of the CA showed a big difference in maximum level compared to the curve of the observed values. This might be due to the presence of partial agonists with lower individual maximum responses in this mixture (i.e. 3-acetyl- and 15-acetylDON). After all, one of the principles behind the CA concept is the requirement that components can replace each other to generate the same level of effect. Partial agonists only partially fulfil this requirement. The difficulty of predicting maximum effect levels of mixtures containing partial agonists is also discussed by others (Loewe and Muischnek, 1926; Houtman et al., 2006; Payne et al., 2000).

For the androgenic, thyroid β and PPARγ2 responsive cell lines, the mixtures were tested on their antagonistic activity. Two kinds of deviations from the CA model arose (CA did not fall in the 95% confidence band of the observed curve). The first kind was where CA overestimated the observed effect, it was situated to the left of the observed curve (i.e. this is the case for mixture 1 on the AR, Figure 3A). An interaction between two or three substances could be a possible explanation (as in Ermler et al., 2011). This kind of interaction should be further investigated (i.e. affecting each other's solubility or metabolisation,...). The second kind was when CA underestimated the observed effect, it was situated to the right of the observed curve (i.e. this is the case for mixture 3 and 4 on the AR, Figure 3C and 3D; mixtures 2 and 3 on the TRβ, Figure 4A and 5; and mixture 3 on the PPARγ2, Figure 5B). In these cases, there were one or more components present (for example NIV in mixture 3 on the AR) in the mixture that show none or hardly any anti-activity individually at the concentrations tested (but possibly at higher concentrations). For these compounds, it was not possible to determine the parameters needed for the CA calculation (thus no contribution) which could be the reason why the CA underestimated the effect slightly. These kind of deviations were also seen in previous studies (Kjaerstad et al., 2010).

For a number of mycotoxins and a limited number of combinations (like for the fumonisins) the limits for regulatory control (cf. European and National legislation) have already been determined, but these include far from all, or not even the most common combinations of mycotoxins (i.e. DON + ZEN). For example for ZEN and DON (separately!) a TDI of 0,25 µg/kg and 1 µg/kg body weight respectively was determined (EFSA, 2011; EFSA, 2013). These do not account for the possible presence of other mycotoxins. For some metabolites, a TDI is missing due to the lack of toxicological data. This is the first time that mycotoxin mixtures are tested on their endocrine activity in receptor luciferase reporter cell lines. Particularly, the additive effects on ER agonism are important. To illustrate, the maximum agonistic effect of mixture 1 (ZEN, α-ZEL, β-ZEL) was achieved at 10^{-9} M, this is 10 times

lower than in the case of ZEN alone. As, in general, animals and humans are exposed to several mycotoxins at the same time, the results are important and should be investigated in depth both *in vitro* and *in vivo*. *In vivo,* ZEN and its metabolites already exhibit endocrine activity in pigs, bovine and sheep causing infertility problems (Fink-Gremmels, 1999; Tiemann and Danicke, 2007). It should be investigated if other active mycotoxins (like 3-acetylDON) can contribute to these problems. Moreover, most of the mycotoxins and their mixtures show anti-androgenic effects which could add up to the reproductive disruptions.

In conclusion, in this study we showed that **mycotoxins not only interact with the oestrogen receptor**, but also with other nuclear receptors. We found that 3-acetylDON is also an oestrogenic agonist. Furthermore, most of the mycotoxins and their mixtures show anti-androgenic effects, which could increase the reproductive effects in livestock even further. Also, especially DON and 15-acetylDON act as antagonists for the PPARγ2 receptor. Combined toxicity of mycotoxins is difficult to predict *in vivo*. However in this study, *in vitro* we showed that **most of the mixtures of mycotoxins and their metabolites react as predicted by the concentration addition theory** (in the cases where they did not, CA is even an underestimation). This may have repercussions in vivo and for the legislation of the mycotoxins in food and feed, especially for ZEN. Current procedures that determine regulation should move toward accounting for mixture effects based on concentration addition. In addition, it would be important to confirm the here obtained results in vivo (especially combinations of ZEN and its metabolites and other mycotoxins on reproductive effects).

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