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# Deoxynivalenol enhances estrogen receptor alpha-induced signaling by ligand-independent transactivation.

M. Drouault<sup>a</sup>, C. Delalande<sup>a</sup>, H. Bouraïma-Lelong<sup>a</sup>, V. Seguin<sup>b</sup>, D. Garon<sup>b</sup>, V. Hanoux<sup>a</sup>

<sup>a</sup> Normandie Univ, UNICAEN, OeReCa, 14000 Caen, France

<sup>b</sup> Normandie Univ, UNICAEN, UNIROUEN, ABTE, 14000 Caen, France

Correspondence should be address to V. Hanoux, UNICAEN EA2608 OeReCa, Esplanade de la Paix, CS 14032, 14032 Caen cedex 5. email: [vincent.hanoux@unicaen.fr](mailto:vincent.hanoux@unicaen.fr)

Abbreviations: **4-OH-TAM**, 4-hydroxytamoxifen; **Akt**, protein kinase B; **CTGF**, connective tissue growth factor; **DON**, deoxynivalenol; **E2**, 17- $\beta$ -estradiol; **EGF**, epidermal growth factor; **ER $\alpha$** , estrogen receptor  $\alpha$ ; **ER $\alpha$  p-Ser<sup>167</sup>**, ER $\alpha$  phosphorylation at serine 167 site; **ER $\beta$** , estrogen receptor  $\beta$ ; **ERE**, estrogen responsive element; **ERK1/2**, extracellular regulated kinase 1/2; **ESR1**, estrogen receptor 1; **ESR2**, estrogen receptor 2; **GAPDH**, glyceraldehyde 3 phosphate dehydrogenase; **GPER**, G protein-coupled estrogen receptor; **JNK**, c-Jun N-terminal kinase; **MAPK**, mitogen-activated protein kinase; **mRNA**, messenger RNA; **p38**, p38 MAPK; **p90-RSK2**, p90 ribosomal S6 kinase 2; **PI3K**, phosphoinositide 3-kinase; **PKR**, protein kinase R; **PPAR $\gamma$ 2**, peroxisome proliferator-activated receptor  $\gamma$  type 2; **PR**, progesterone receptor; **rRNA**, ribosomal RNA; **RT**, room temperature; **RT-qPCR**, quantitative real-time PCR; **siRNA**, small interfering RNA; **TFF1/PS2**, trefoil factor 1; **TGF- $\beta$** , transforming growth factor  $\beta$ ; **ZEA**, zearalenone.

## 1        **1. Introduction**

2        Mycotoxins are natural occurring toxicants, first defined as “fungal metabolites which when  
3        ingested, inhaled or absorbed through the skin cause lowered performance, sickness or death  
4        in man or domestic animals” (Pitt, 1996). Mycotoxic fungi develop on a large variety of crops  
5        (cereals, fruit, nuts), thus resulting in the contamination of the food chain by mycotoxins and  
6        making ingestion the first route of exposure to these compounds (Zain, 2011). Because of  
7        their adverse effects on animal and human health (Richard, 2007) and their widespread  
8        occurrence (Eskola et al., 2020), mycotoxins are raising global concerns. Consequently, in a  
9        recent press release, the World Health Organization (WHO) pointed out the necessity to take  
10       measures against mycotoxin contamination, especially in developing countries (WHO, 2016).

11       Nowadays, over 300 mycotoxins have been identified, but only a few of them exhibit a  
12       concerning toxicological profile, since the toxic effects of most of them are currently not  
13       known. Zearalenone (ZEA) and deoxynivalenol (DON), both produced by *Fusarium* genus,  
14       are also among the most abundant mycotoxins in food and feeds (Garon et al., 2006; Ma et al.,  
15       2018), and at the same time the most concerning ones. Indeed, DON and ZEA have been  
16       described as cytotoxic, genotoxic, immunotoxic, hepatotoxic and could also disrupt the  
17       endocrine system, such as the estrogen signaling pathway (Sobrova et al., 2010; Zinedine et  
18       al., 2007).

19       Estrogens are steroid hormones involved in various biological functions. Among them,  
20       17 $\beta$ -estradiol (E2) is the most represented and most active type of estrogen. E2 mainly acts  
21       through binding to 3 specific estrogen receptors (ER), namely ER $\alpha$  and ER $\beta$  (nuclear  
22       receptors encoded by *ESR1* and *ESR2* genes respectively) and GPER (a G-protein coupled  
23       receptor associated to the cell membrane; also called GPR30), and consequently activates  
24       transcriptional processes and/or intracellular signaling cascades. Briefly, the different type of

25 estrogen can induce biological effects through four different response mechanisms. The direct  
26 genomic mechanism involves cytoplasmic ER $\alpha$  and ER $\beta$  activated by ligand binding which  
27 dimerize, translocate to the nucleus and bind to the DNA of their target-genes directly on  
28 specific motifs named estrogen responsive elements (ERE). The indirect genomic pathway  
29 requires protein-protein interactions between ligand-activated ER and other transcription  
30 factors for DNA binding. The non-genomic pathway is initiated by ligand binding to the  
31 transmembrane receptor GPER or to some nuclear receptors associated to the cell membrane  
32 by palmitoylation, and activates rapid signaling pathways in the cytoplasm. Lastly, the ligand-  
33 independent transactivation of nuclear ER could occur in the absence of E2 and is mainly  
34 triggered by ER phosphorylation on specific amino acid residues, for the most part serine and  
35 tyrosine (Hewitt and Korach, 2018). The existence of cross-talks between the above-  
36 mentioned pathways makes estrogen signaling an even more complex system.

37 Estrogen signaling can be disrupted by many exogenous compounds including ZEA (formerly  
38 known as F-2 toxin), a non-steroidal estrogenic mycotoxin mainly associated to reproductive  
39 and developmental disorders in males (Tsakmakidis et al., 2006; Zatecka et al., 2014) and  
40 females (Zhao et al., 2014). ZEA exposure compromises fertility (subfertility or infertility),  
41 results in an increase in fetal resorption, a reduced litter size, and gives rise to a  
42 hyperestrogenic syndrome (Kuiper-Goodman et al., 1987; Ropejko and Twarużek, 2021).  
43 These adverse effects are related to the structural similarity between ZEA and estrogen, which  
44 allows its binding to ER $\alpha$  and ER $\beta$  (Kuiper et al., 1998). Thus, ZEA can modulate estrogen  
45 signaling and is therefore recognized as an endocrine disruptor.

46 DON, which is produced by the same fungal species as ZEA, is a trichothecene B mycotoxin,  
47 also referred to as “vomitoxin” because of its emetic effects, particularly severe in porcines.  
48 In humans, the main risks associated to DON exposure involved the intestinal system, the  
49 central nervous system and the immune system. The acute ingestion of DON results in illness

50 characterized by vomiting, anorexia, abdominal pain, diarrhea, malnutrition, headache,  
51 dizziness and fever (Escrivá et al., 2015; Sobrova et al., 2010). Other studies conducted in  
52 rodents also suggest a negative impact of DON on the male reproductive system (Gao et al.,  
53 2019; Sprando et al., 2005). The adverse effects of DON are most commonly a consequence  
54 of its well-known ribotoxic effect. Indeed, at the cellular level, DON binds to the 60S  
55 ribosomal subunit and interferes with the active site of peptidyltransferase. This interaction  
56 leads to the inhibition of peptide bond formation, making DON a potent inhibitor of protein  
57 synthesis (Cundliffe and Davies, 1977; Ueno, 1978). However, it has been demonstrated that  
58 DON causes a severe impairment of reproduction in *Folsomia candida*, a model animal that is  
59 widely used in soil ecotoxicology. This effect was attributed to the alteration of the embryonic  
60 development due to DNA destructive properties of DON (Szabó et al., 2019).

61 Nonetheless, a few *in vitro* studies suggested other mechanisms of action. Notably, DON  
62 could modulate the expression of most steroidogenic genes in the H295R cell line,  
63 consequently leading to an altered steroid hormone production (Ndossi et al., 2012).  
64 Interestingly, the latter point has been identified as one of the ten key characteristics of  
65 endocrine disruptors (La Merrill et al., 2020). The ability of DON to act as an agonist on ER  
66 has been studied, but no estrogenic activity of this mycotoxin has been demonstrated.  
67 However, DON has been reported to be an antagonist of peroxisome proliferator-activated  
68 receptor  $\gamma$  type 2 (PPAR $\gamma$ 2) (Demaegdt et al., 2016). Since PPAR $\gamma$ 2 activation is known to  
69 inhibit estrogen signaling through the proteasome-mediated degradation of ER $\alpha$ , DON could  
70 indirectly modulate estrogen signaling through its inhibitory effect on PPAR $\gamma$ 2 activity (Qin  
71 et al., 2003; Suzuki et al., 2006).

72 The aim of this study was to characterize the effects of DON on estrogen signaling and also to  
73 investigate its mechanisms of action. We conducted an *in vitro* study using MCF-7 (ER $\alpha$ -  
74 positive) and MDA-MB-231 (ER $\alpha$ -negative) cells, two breast carcinoma cell lines commonly

75 used to characterize the disruption of estrogen signaling (Hanoux et al., 2018). The cytotoxic  
76 properties of DON were evaluated in both cell lines by crystal violet and ATP luminescent  
77 cell viability assays after 6 or 72 h of exposure. The effect of DON on the expression of  
78 estrogen-responsive genes was analysed by RT-qPCR after a 6 h of culture, and its action  
79 mechanism on estrogen signaling was characterized using ER specific antagonists, silencing  
80 of *ESR1* and *PPAR $\gamma$ 2* gene expression and by assessing ER $\alpha$  phosphorylation level. The  
81 above-mentioned endpoints were investigated with DON alone or combined with E2 or ZEA.

82

83           **2. Material and methods**

84           **2.1 Chemicals and material.**

85 Culture plates and flasks were provided by Falcon. DMEM/Ham's F12 ( $\pm$  phenol red), Fetal  
86 Bovine Serum (FBS) and OptiMEM were obtained from Gibco. Dimethyl sulfoxide (DMSO),  
87 deoxynivalenol (DON), zearalenone (ZEA), 17- $\beta$ -estradiol (E2), Epidermal Growth Factor  
88 (EGF), 4-hydroxitamoxifen (4-OH-TAM), crystal violet, sodium dodecyl sulfate (SDS), Tris-  
89 HCl, dithiothreitol (DTT), bromophenol blue, Tween 20,  $\beta$ -mercaptoethanol and RNase free  
90 water were purchased from Sigma Aldrich. G36 was provided by Tocris, phosphate buffered  
91 saline (PBS) by PAN (Dutscher), Lipofectamin RNAiMax by Invitrogen, acrylamide/bis 37:1  
92 40% (w/v) solution by Eurobio, and Pink Prestained Protein Marker by Nippongenetics.  
93 Nitrocellulose membrane, enhanced chemiluminescence (ECL) and hyperfilm ECL were  
94 obtained from Amersham. NucleoZOL was purchase from Macherey-Nagel. Murine leukemia  
95 virus reverse transcriptase (M-MLV RT), deoxynucleotide triphosphates (dNTPs), random  
96 primers, ribonuclease inhibitor (RNasin®), GoTaq qPCR master mix and CellTiter-Glo® 2.0  
97 were purchased from Promega. The primers used for qPCR as well as small interfering RNA  
98 (siRNA) targeting *ESR1* mRNA (*ESR1*-siRNA) or *PPAR $\gamma$ 2* mRNA (*PPAR $\gamma$ 2*-siRNA) were  
99 obtained from Eurogentec. Control siRNA-A and mouse monoclonal anti-ER $\alpha$  (F-10) (sc-  
100 8002) antibody were obtained from Santa Cruz Biotechnology. Mouse monoclonal anti-actin  
101 (MAB1501) antibody and rabbit polyclonal anti-phospho-ER $\alpha$ -S167 (AP0508) antibody were  
102 purchased from Merck Millipore and Diagogmics respectively. The secondary antibodies (goat  
103 anti-mouse IgG HRP-linked (#7076) and goat anti-rabbit IgG HRP-linked (#7074)) were  
104 obtained from Cell Signaling Technologies.

105           **2.2 Cell lines and cell culture.**

106 Human breast carcinoma cell lines MCF-7 (ATCC-HTB-22) and MDA-MB-231 (ATCC  
107 HTB-26) were routinely maintained in DMEM/Ham's F12 supplemented with 10% FBS at  
108 37°C in a 5% CO<sub>2</sub> humidified atmosphere. To overcome any estrogenic activity, the cells

109 were deprived from steroid 48 h before the experiments started by culturing them in a phenol  
110 red-free DMEM/Ham's F12 supplemented with 10% charcoal-stripped FBS. All experiments  
111 were conducted in this steroid-depleted culture medium. The tested compounds were  
112 dissolved in ethanol (DON, E2 and ZEA) or DMSO (4-OH-TAM and G36) and  
113 extemporaneously diluted in the steroid-depleted culture medium at suitable concentrations.

### 114 **2.3 Cell viability assay.**

115 The cytotoxic properties of DON in MCF-7 and MDA-MB-231 cell lines were determined by  
116 the crystal violet cell proliferation assay. Both cell lines were seeded at the density of  
117  $4 \times 10^3$  cells/well in 96-well plates. In order to determine the number of cells after the  
118 treatment ended, a cell range ( $4 \times 10^3$ ;  $8 \times 10^3$ ;  $1.6 \times 10^4$ ;  $3.2 \times 10^4$  cells/well) was performed. In  
119 parallel, the cells were pre-incubated for 45 min in presence or absence of G36 ( $10^{-5}$  M) and  
120 then treated with DON ( $10^{-6}$  M) or incubated with E2 ( $10^{-10}$  M) and increasing concentrations  
121 of DON ( $10^{-13}$  to  $10^{-6}$  M) for 72 h. The cells were then fixed with 4% formaldehyde (20 min,  
122 RT), washed twice with PBS and stained with 0.2% crystal violet (30 min, RT). The staining  
123 step was followed by two washings with deionized water, and crystal violet dye was then  
124 extracted using 10% acetic acid. The absorbance was read at 600 nm using a microplate  
125 reader (Multiskan GO, Thermo Scientific). The results are expressed as a percentage of cells  
126 number relative to the control condition. The IC<sub>50</sub> of DON was calculated using CellTiter-  
127 Glo 2.0 assay according to the manufacturer's instructions. The cells were cultured as  
128 described above with or without increasing concentrations of DON ( $10^{-9}$  to  $10^{-4}$  M) for 6 or  
129 72 h. The luminescence was read using the FlexStation 3 (Molecular Devices) and the IC<sub>50</sub>  
130 were determined using the GraphPad Prism software.

### 131 **2.4 Silencing of *ESR1* and *PPAR $\gamma$ 2* gene expression.**

132 MCF-7 cells were harvested and transfected with siRNA targeting *ESR1* mRNA  
133 (ESR1-siRNA) (5'-UCAUCGCAUCCUUGCAAAdTdT-3'; 5'-

134 UUUGCAAGGAAUGCGAUGAdTdT-3'), *PPAR $\gamma$ 2* mRNA (*PPAR $\gamma$ 2*-siRNA) (5'-  
135 GGGUGAAACUCUGGGAGAUdTdT-3'; 5'-AUCUCCCAGAGUUUCACCCdTdT-3') or an  
136 off-target siRNA used as negative control (control-siRNA) (control siRNA-A, Santa Cruz).  
137 Briefly, siRNAs were diluted in OptiMEM medium to a final concentration of 6 pM and  
138 transfected into MCF-7 cell suspension using Lipofectamine RNAiMax according to the  
139 manufacturer's instructions. The cells were then seeded at the density of  $7.5 \times 10^5$  cells/well in  
140 24-well plates for 24 h (*PPAR $\gamma$ 2*-siRNA-MCF-7 cells) or 48 h (*ESR1*-siRNA-MCF-7 cells)  
141 prior to the experiments.

## 142 **2.5 RNA extraction and RT-qPCR.**

143 The cells were seeded at the density of  $1.8 \times 10^5$  cells/well in a 24-well (MCF-7) or 6-well  
144 (MDA-MB-231) plate and were left to adhere for 24 h. The cells were pre-incubated for 45  
145 min in presence or absence of the tested agents (4-OH-TAM at  $10^{-6}$  M or G36 at  $10^{-5}$  M) and  
146 then cultured for 6 h with various concentrations of DON and/or E2 ( $10^{-10}$  M) or ZEA ( $10^{-7}$   
147 M), as described in the results section. RNA extraction was performed using NucleoZOL  
148 reagent according to the manufacturer's instructions. 250 ng of total RNA was reverse  
149 transcribed in a final volume of 20  $\mu$ L using 100 U of M-MLV reverse transcriptase in the  
150 presence of 1 mM of dNTPs, 100 ng of random primers and 12 U of RNasin®, for 1 h at  
151 37°C. The enzyme was then heat denatured for 10 min at 72°C to stop the reaction. A  
152 negative control was performed by omitting M-MLV reverse transcriptase. The cDNAs  
153 obtained were diluted (1:10 in RNase free water) and qPCR was performed with the C1000  
154 Thermal Cycler CFX96 Real Time System (Biorad) using GoTaq qPCR master mix. Each  
155 reaction was prepared according to the manufacturer's protocol in a final volume of 20  $\mu$ L.  
156 For negative control, the cDNA template was replaced by RNase free water. The primers used  
157 and the size of the PCR products are listed in Table 1. The amplification program consists in  
158 an initial step of 3 min at 95°C followed by 40 cycles at 95°C for 30 s and 60°C for 1 min.

159 The specificity of PCR products was assessed by running a dissociation curve after the  
160 amplification reactions. The expression levels of target genes were normalized with the  
161 *GAPDH* gene. The relative expression levels were determined using the  $2^{-(\Delta\Delta Ct)}$  method. The  
162 expression levels of 3 estrogen-responsive genes were analysed: trefoil factor 1 (*TFF1/PS2*)  
163 under nuclear ER control (direct genomic pathway) (Brown et al., 1984); progesterone  
164 receptor (*PR*) under nuclear ER control (indirect genomic pathway) (Read et al., 1988);  
165 connective tissue growth factor (*CTGF*) under GPER control (Pandey et al., 2009).

## 166 **2.6 Protein extraction and Western blot.**

167 MCF-7 cells were seeded at the density of  $6 \times 10^5$  cells/well in a 6-well plate and left to adhere  
168 for 24 h. The cells were incubated for 30 min in presence of the tested compounds (DON at  
169  $10^{-5}$  M; EGF at 10 ng/mL), washed twice in PBS and lysed in SDS-lysis buffer (62.5 mM  
170 Tris-HCl pH 6.8; 2% (w/v) SDS, 10% (v/v) glycerol; 50 mmol/L DTT; 0.01 % (w/v)  
171 bromophenol blue). Total proteins were sonicated for 10 s using Vibra-Cell™ ultrasonic  
172 processor (Bioblock Scientific). Equal-volume samples were heat-denatured, loaded on a 12%  
173 SDS-polyacrylamide gel and separated by electrophoresis. Thereafter, proteins were  
174 transferred to hybond ECL nitrocellulose membranes. Nonspecific binding sites were blocked  
175 for 1 h at room temperature with Tris-Buffered Saline supplemented with 0.1% Tween 20  
176 (TBST) and 5% non-fat dried milk. The membranes were then incubated with anti-phospho-  
177 ER $\alpha$ -S167 primary antibody diluted in TBST-5% non-fat dried milk (1:500) overnight at 4°C.  
178 After 3 washings with TBST, the membranes were incubated with goat anti-rabbit IgG-HRP  
179 secondary antibody (1:2000 in TBST-5% non-fat dried milk) for 1 h at room temperature. The  
180 membranes were washed again in TBST and the signals were developed by incubating the  
181 membranes with ECL and exposing them to hyperfilm ECL. The membranes were stripped  
182 with 100 mM  $\beta$ -mercaptoethanol, 2% (w/v) SDS, 62.5 mM Tris-HCl pH 6.7 for 30 min at  
183 50°C, washed twice with TBST and then reprobed with anti-ER $\alpha$  primary antibody (1:1000 in

184 TBST-5% non-fat dried milk) or anti-actin primary antibody (1:5000 in TBST-5% non-fat  
185 dried milk) as described above. The quantitative results were obtained using the Image J  
186 software and normalized against the corresponding total ER $\alpha$  value.

## 187 **2.7 Statistical Analysis.**

188 The results are shown as means  $\pm$  SEM of at least 3 independent experiments. The statistical  
189 analysis of the data was conducted using the GraphPad Prism software. The statistical  
190 comparisons were performed using a paired *t*-test (comparison between 2 groups) or a one-  
191 way analysis of variance (ANOVA) (comparison between 3 or more groups) followed by a  
192 Bonferroni post-hoc test. The *p* values that were lower than 0.05 were considered to be  
193 statistically significant.

194

195 **3. Results**

196 **3.1 DON exposure affects cell viability of MCF-7 and MDA-MB-231 cell lines.**

197 In order to first investigate the effect of DON on estrogen signaling, a crystal violet cell  
198 proliferation assay was conducted in MCF-7 and MDA-MB-231 cell lines after 72 h of  
199 culture. In MCF-7 cells, DON at concentrations ranging from  $10^{-13}$  M to  $10^{-7}$  M had no effect  
200 on cell viability. However, DON at  $10^{-6}$  M induced a decline of about 40% in the number of  
201 MCF-7 cells compared to the control (Fig. 1A). By contrast, in MDA-MB-231 cells, DON  
202 exerted a negative impact on cell viability at all tested concentrations (excepted for DON at  
203  $10^{-12}$  M) (Fig. 1B). The effect of DON on cell viability was also characterized using an ATP  
204 luminescent cell viability assay. After 6 h of culture in the presence of DON, no effect on cell  
205 viability was observed in MCF-7 and MDA-MB-231 cell lines, whatever the concentration  
206 used (Fig. 1C). However, the culture of the two cell lines in the presence of DON for 72 h led  
207 to a dose-dependent decrease in cell viability (Fig. 1D). Thus, IC<sub>50</sub> of DON was determined  
208 at approximately  $2 \times 10^{-6}$  M for MCF-7 and MDA-MB-231 cell lines after 72 h culture  
209 (Fig.1D). The effect of DON was also investigated on the E<sub>2</sub>-induced proliferation of MCF-7  
210 cells. Thus, as expected, E<sub>2</sub> ( $10^{-10}$  M) alone induced a great 6-fold increase in MCF-7 cells  
211 number. The addition of DON at concentrations ranging from  $10^{-12}$  M to  $10^{-7}$  M did not  
212 modulate the E<sub>2</sub>-induced increase in the number of MCF-7 cells. However, the stimulatory  
213 effect of E<sub>2</sub> on the proliferation of MCF-7 cells was abolished in presence of DON at  $10^{-6}$  M  
214 (Fig. 1E).

215 To further investigate if GPER could be involved in the effect of DON on cell viability, the  
216 selective GPER antagonist G36 was used. As described above, DON at  $10^{-6}$  M led to a decline  
217 of about 50% in the number of MCF-7 cells. G36 ( $10^{-5}$  M) alone had no effect on cell  
218 viability. Moreover, G36 ( $10^{-5}$  M) did not modulate the DON-induced decline in the number  
219 of MCF-7 cells (Fig. 1F).

220 **3.2 DON exposure induces an increase in the expression of estrogen-responsive**  
221 **genes in MCF-7 cell line.**

222 In order to finely assess the effect of DON on estrogen signaling, the expression levels of  
223 three estrogen-responsive genes (*TFF1/PS2*, *PR* and *CTGF*) were analysed by RT-qPCR after  
224 exposure of MCF-7 and MDA-MB-231 cell lines with DON for 6 h. As expected, in MCF-7  
225 cells, E2 ( $10^{-10}$  M) induced a 5- and 14.5-fold increase in *TFF1/PS2* and *PR* mRNA  
226 respectively (Fig. 2A and B). An increase was observed in the expression of these mRNA  
227 when MCF-7 cells were cultured in presence of DON, although to a lesser extent than with E2  
228 (Fig. 2A and B). Indeed, DON at  $10^{-5}$  and  $10^{-4}$  M led to, respectively, a 1.5- and 2.9-fold  
229 induction of *TFF1/PS2* mRNA expression (Fig. 2A), while only DON at  $10^{-4}$  M was able to  
230 induce a 7.9-fold higher expression of *PR* mRNA compared to the control (Fig. 2B). For  
231 *CTGF* mRNA expression, DON and E2 exhibited differential effects. Indeed, E2 ( $10^{-10}$  M) led  
232 to a down-regulation of *CTGF* mRNA expression whereas DON at  $10^{-5}$  and  $10^{-4}$  M induced a  
233 9- and 46-fold increase in *CTGF* mRNA level respectively (Fig. 2C).

234 In MDA-MB-231 cells, E2 ( $10^{-10}$  M) had no effect on the mRNA expression of any of the  
235 estrogen-responsive genes (Fig. 2D-F). Similarly, in contrast to its stimulatory effect in ER $\alpha$ -  
236 positive cells, DON had no effect on the expression of *TFF1/PS2* mRNA, and *PR* mRNA was  
237 not detected in MDA-MB-231 cells (Fig. 2D and E). However, the highest concentrations of  
238 DON still increased *CTGF* mRNA expression in this cell line, with a 5.1- and 19-fold  
239 induction after exposure to DON at  $10^{-5}$  and  $10^{-4}$  M respectively when compared to the  
240 control (Fig. 2F).

241

242           **3.3 DON exposure in combination with estrogenic compounds exhibits an**  
243           **additive effect on the expression of estrogen-responsive genes in MCF-7 cell**  
244           **line.**

245 Since DON alone stimulated the expression of estrogen-responsive genes in MCF-7 cells, the  
246 effect of its mixture with estrogenic compounds (*i.e.* E2 or ZEA) was then investigated in this  
247 cell line. As described above, E2 ( $10^{-10}$  M) alone increased the level of *TFF1/PS2* and *PR*  
248 mRNA (Fig. 3A and B). Moreover, E2 ( $10^{-10}$  M), in the presence of DON at  $10^{-6}$  and  $10^{-5}$  M,  
249 led to a respectively 1.4- and 1.6-fold induction of *TFF1/PS2* mRNA expression compared to  
250 E2 alone (Fig. 3A). This effect was slightly more pronounced regarding the level of *PR*  
251 mRNA, with a 2.3- and 2.1-fold induction after an exposure with E2 ( $10^{-10}$  M) in the presence  
252 of DON at respectively  $10^{-5}$  M and  $10^{-4}$  M (Fig. 3B).

253 As expected, ZEA ( $10^{-7}$  M) alone increased the expression of *TFF1/PS2* and *PR* mRNA to a  
254 similar extent than E2 ( $10^{-10}$  M) (Fig. 3.C and D). MCF-7 cells cultured for 6 h in the presence  
255 of ZEA ( $10^{-7}$  M) and DON also exhibited a greater stimulatory effect on the expression of  
256 estrogen-responsive genes than ZEA alone, following the same trend as those observed for E2  
257 ( $10^{-10}$  M) in combination with DON (Fig. 3.C and D).

258           **3.4 4-OH-TAM, unlike G36, partially reverses the DON-induced expression of**  
259           **estrogen-responsive genes in MCF-7 cell line.**

260 To further explore the mechanisms by which DON modulates the expression of estrogen-  
261 responsive genes in MCF-7 cell line, selective antagonists of ER were used. 4-OH-TAM  
262 ( $10^{-6}$  M), a non-steroidal antagonist of ER $\alpha$ , partially reversed the stimulatory effect of DON  
263 on the level of *TFF1/PS2* and *PR* mRNAs. The effect was only observed for the condition  
264 DON at  $10^{-4}$  M in which pre-incubation with 4-OH-TAM led to a 2- and 3.8-fold decrease in  
265 *TFF1/PS2* and *PR* mRNA levels respectively (Fig. 4A and B). On the other hand, pre-

266 incubation with G36 ( $10^{-5}$  M) did not cause any difference in the DON-induced expression of  
267 *CTGF* mRNA compared with the relevant control condition (Fig. 4C).

268 **3.5 DON exposure does not affect the expression of nuclear ER in MCF-7 cell**  
269 **line.**

270 To characterize the stimulatory effect of DON on the expression of genes regulated by nuclear  
271 ER, the effect of DON on the level of *ESR1* and *ESR2* mRNAs in MCF-7 cells was quantified  
272 by RT-qPCR after a 6 h exposure. Thus, DON did not significantly affect the levels of *ESR1*  
273 and *ESR2* mRNAs, whatever the concentration of DON used compared to the control (Fig. 5A  
274 and B). Moreover, the level of *ESR2* mRNA was barely detectable in all conditions studied  
275 when compared to that of *ESR1* mRNA, whose expression was more than 1000-fold higher  
276 (Fig. 5C).

277 **3.6 siRNA-mediated *ESR1* silencing partially reverses the DON-induced**  
278 **expression of estrogen-responsive genes in MCF-7 cell line.**

279 In order to complement the investigations carried out on the action mechanisms of DON on  
280 estrogen signaling, a siRNA-mediated *ESR1* silencing was performed in MCF-7 cell line.  
281 *ESR1* silencing was confirmed by the total abolition of E2 stimulation on *TFF1/PS2* and *PR*  
282 gene expression (Fig. 6A and B). The DON-induced up-regulation of *TFF1/PS2* mRNA level  
283 was reversed for all concentrations of DON in *ESR1*-siRNA-MCF-7 cells compared to  
284 control-siRNA-MCF-7 cells (Fig. 6A). However, in *ESR1*-siRNA-MCF-7 cells, DON at  $10^{-4}$   
285 M still led to an increase in *TFF1/PS2* mRNA level compared to the control condition (Fig.  
286 6C). On the contrary, the potent induction of *PR* mRNA expression by DON  $10^{-5}$  M and DON  
287  $10^{-4}$  M was abolished in *ESR1*-siRNA-MCF-7 cells compared to both the corresponding  
288 condition in control-siRNA-MCF-7 cells and the control condition in *ESR1*-siRNA-MCF-7  
289 cells (Fig 6B and D).

290 **3.7 siRNA-mediated *PPAR* $\gamma$ 2 silencing does not reverse the DON-induced**  
291 **expression of estrogen-responsive genes in MCF-7 cell line.**

292 To investigate the putative involvement of *PPAR* $\gamma$ 2 in the effect of DON on the expression of  
293 estrogen-responsive genes, a siRNA-mediated *PPAR* $\gamma$ 2 silencing approach was conducted in  
294 MCF-7 cell line. Thus, the E2- and ZEA-induced expression of *TFF1/PS2* and *PR* mRNA  
295 were increased in *PPAR* $\gamma$ 2-siRNA-MCF-7 cells compared to control-siRNA-MCF-7 cells  
296 (Fig. 7B-D). These results reflected a lift of inhibition of estrogen signaling, confirming the  
297 efficacy of *PPAR* $\gamma$ 2 silencing. As demonstrated above (section 3.3), DON ( $10^{-5}$  M) in  
298 combination with E2 ( $10^{-10}$  M) or ZEA ( $10^{-7}$  M) induced the expression of *TFF1/PS2* and *PR*  
299 genes to a greater extent than the estrogenic compounds taken separately in control-siRNA-  
300 MCF-7 cells (Fig. 7). However, in these culture conditions, the level of *TFF1/PS2* and *PR*  
301 mRNA did not differ in *PPAR* $\gamma$ 2-siRNA-MCF-7 cells compared to control-siRNA- MCF-7  
302 cells (Fig. 7).

303 **3.8 DON exposure induces ER $\alpha$  phosphorylation at serine 167 in MCF-7 cell line.**

304 In order to explore the ability of DON to activate estrogen signaling in a ligand-independent  
305 manner, the phosphorylation level of ER $\alpha$  at serine 167 (ER $\alpha$  p-Ser<sup>167</sup>) was assessed by  
306 Western blotting in MCF-7 cells. EGF (10 ng/mL), used as a positive control, and DON  
307 ( $10^{-5}$  M) induced an increase in ER $\alpha$  p-Ser<sup>167</sup> staining intensity after culture for 30 min  
308 compared to the control whereas the total ER $\alpha$  staining intensity remained stable in each  
309 condition compared to actin staining intensity (Fig. 8A). Thus, EGF (10 ng/mL) as well as  
310 DON at  $10^{-5}$  M induced an about 2-fold increase in the level of ER $\alpha$  p-Ser<sup>167</sup> compared to the  
311 control (Fig. 8B).

312

#### 313 4. Discussion

314 This work aimed to characterize the effect of DON on estrogen signaling and to investigate  
315 the action mechanisms involved. Our results showed a DON-induced increase in the  
316 expression of estrogen-responsive genes, thus demonstrating for the first time that this  
317 mycotoxin seems to be able to activate the estrogen signaling pathway. Moreover, the  
318 different approaches conducted here enabled us to determine the mechanisms through which  
319 DON exerts its estrogeno-mimetic effects and support a ligand-independent activation of  
320 ER $\alpha$ .

321 It is well known that MCF-7 cells strongly express ER $\alpha$  and depend on E2 to proliferate. As  
322 expected, a 72 h exposure to E2 ( $10^{-10}$  M) resulted in a potent increase in the number of MCF-  
323 7 cells compared to the control. DON did not trigger such stimulatory effect and, on the  
324 contrary, decreased basal and E2-stimulated proliferation of MCF-7 cells at the concentration  
325 of  $10^{-6}$  M. This effect could be explained by the activation of membrane-bound ER $\alpha$  by DON.  
326 Indeed, the stimulation of MCF-7 enriched for membrane ER $\alpha$  with high concentrations of E2  
327 has been reported to inhibit MCF-7 cell proliferation (Zivadinovic et al., 2004). Nonetheless,  
328 the ER $\alpha$ -negative cell line appears to be more sensitive to DON than the MCF-7 cell line,  
329 since concentrations as low as  $10^{-13}$  M of DON reduced the number of MDA-MB-231 cells.  
330 Interestingly, GPER activation has been shown to inhibit the viability of both MCF-7 and  
331 MDA-MB-231 cells (Ariazi et al., 2010; Huang et al., 2020), thus suggesting a putative  
332 activation of GPER in the DON-induced decrease in viability of these cell lines. However,  
333 G36 failed to reverse this effect, thus demonstrating that GPER was not involved in the DON-  
334 induced decrease in the viability of MCF-7 and MDA-MB-231 cells. Hence, these results  
335 obtained after 72 h in culture are thought to underlie the commonly reported cytotoxic effect  
336 of DON rather than an effect on estrogen signaling. Nonetheless, some studies suggest that  
337 shorter exposures may not result in such outcome. Indeed, in a bone marrow-derived cell line,

338 DON ( $25$  and  $50 \times 10^{-6}$  M) decreased cell viability after 24 h exposure but not after 6 h  
339 (Kalagatur et al., 2021). Likewise, in mouse endometrial stromal cells exposed to DON (100  
340 ng/mL), a decrease in cell viability was observed for the longest (24 h and 48 h) but not for  
341 the shortest (6 h and 12 h) periods of exposure (Dai et al., 2017). Similarly, the ATP assay  
342 performed in MCF-7 and MDA-MB-231 cells in our study indicates that exposure to DON  
343 induced a decrease in cell viability after 72 h but not after 6 h. Taken together, our results and  
344 those described above show that the cytotoxic effect of DON is time-dependent *in vitro*. The  
345 exposure time to DON as well as the dose ingested are important parameters which define the  
346 toxicity of DON *in vivo*. However, other factors including the type of species, the  
347 development stage and the tissue considered also play an important role in the toxic effects of  
348 DON *in vivo* (Pinto et al., 2022).

349 Since the strong cytotoxic properties of DON could mask other effects after 72 h of exposure,  
350 the effect of DON on estrogen signaling was investigated under conditions that did not affect  
351 cell viability. Thus, DON has been found to induce the expression of estrogen-responsive  
352 genes *TFF1/PS2*, *PR* and *CTGF* after 6 h of culture. This result shows that, in the range of  
353 micromolar concentrations ( $10^{-5}$  M and  $10^{-4}$  M), DON activates estrogen signaling *in vitro*. To  
354 our knowledge, this is the first report describing that DON alone can induce estrogen  
355 signaling pathway. This effect was also observed when DON was combined with estrogenic  
356 compounds, *i.e.* E2 ( $10^{-10}$  M) and ZEA ( $10^{-7}$  M) with a much larger effect than that measured  
357 for each compound taken separately. Previous studies investigated the possible endocrine  
358 activity of DON in a reporter cell line developed by Willemsen and collaborators by  
359 transfecting MCF-7 cells with the luciferase gene under the control of ERE (MMV-Luc cell  
360 line) (Willemsen et al., 2004). In contrast with our results, these other studies reported no  
361 agonist activity of DON ( $10^{-6}$  M) on ER in the MMV-Luc cell line after 24 h of culture, which  
362 indicates that DON has no effect on estrogen signaling (Demaegdt et al., 2016; Ndossi et al.,

363 2012). We attribute this striking discrepancy, at least in part, to the concentrations used in the  
364 experiments. Indeed, the above-mentioned works used DON at the concentration of  $10^{-6}$  M,  
365 based on previous assays indicating that greater concentrations caused a decrease in cell  
366 viability by more than 10% after 24 h of culture. However, the shortest exposure time (6 h)  
367 performed in our study allowed to overcome any cytotoxic effect of DON. We were then able  
368 to investigate the effect of DON at higher concentrations ( $10^{-5}$  M and  $10^{-4}$  M), *i.e.* those which  
369 appeared to result in the induction of estrogen-responsive genes expression. Furthermore, the  
370 reporter gene in the MMV-Luc cell line is under the control of ERE, so this approach focused  
371 on assessing the ability of DON to mainly activate the direct genomic pathway. However,  
372 estrogen signaling involves different response mechanisms and is made more complex by the  
373 existence of cross-talks between them. The various strategies employed in our study to assess  
374 DON-induced transcriptional activity of estrogen-responsive genes provided an overview of  
375 the mechanisms possibly involved.

376 The possible interaction between DON and GPER has not yet been studied, although this  
377 receptor has recently emerged as a mediator of endocrine disruptor's action (Périan and  
378 Vanacker, 2020). The potent increase in *CTGF* gene expression observed in both cell lines  
379 after an exposure to DON ( $10^{-5}$  M and  $10^{-4}$  M) for 6 h suggests the involvement of GPER in  
380 DON-mediated effects on estrogen signaling. However, this effect was not reversed by G36,  
381 thus indicating that the DON-induced expression of *CTGF* gene is likely to be GPER-  
382 independent. These results are in correlation with our results on the effect of DON on cellular  
383 viability, which demonstrated no involvement of GPER. Therefore, DON does not seem  
384 capable of activating GPER and the increase in *CTGF* mRNA expression should be attributed  
385 to the activation of other signaling pathways than estrogen signaling, such as the PI3K/Akt  
386 pathway, which was reported to increase the expression of *CTGF* gene (Xu et al., 2015).

387 DON up-regulated *TFF1/PS2* and *PR* gene expression in MCF-7 (ER $\alpha$ -positive) cells without  
388 affecting the expression of *ESR1* and *ESR2* mRNA. Moreover, it had no effect on the  
389 expression of ER $\alpha$ -regulated genes in MDA-MB-231 (ER $\alpha$ -negative) cells. These results  
390 show that the effects of DON on estrogen signaling seem to be specifically mediated by ER $\alpha$   
391 in MCF-7 cell line. On the other hand, DON has been reported to antagonize PPAR $\gamma$ 2  
392 (Demaegdt et al., 2016). Since PPAR $\gamma$ 2 activation is known to inhibit ER $\alpha$ -mediated estrogen  
393 signaling (Qin et al., 2003; Suzuki et al., 2006), the DON-induced disruption of this pathway  
394 described in our study could be the result of the inhibition of PPAR $\gamma$ 2. In order to investigate  
395 this possibility, a siRNA-mediated silencing of *PPAR $\gamma$ 2* mRNA expression was conducted.  
396 The effect of DON on the E2- and ZEA-stimulated expression of *TFF1/PS2* and *PR* genes  
397 remained identical after *PPAR $\gamma$ 2* silencing, thus indicating that the mechanism by which DON  
398 promotes estrogen signaling is independent of the inhibition of PPAR $\gamma$ 2 activity.

399 So, the increase in *TFF1/PS2* and *PR* gene expression following DON exposure suggests the  
400 involvement of either direct or indirect genomic pathway respectively. The latter point is  
401 further supported by the abolition of DON-induced expression of *PR* gene following *ESR1*  
402 silencing in MCF-7 cells. However, 4-OH-TAM does not fully reverse the DON-induced  
403 expression of these genes, indicating that the effect of DON on estrogen signaling is likely to  
404 be mediated by ER $\alpha$  but not through the classical ligand-dependent pathway.

405 The ligand-independent activation of estrogen signaling is based on the phosphorylation of  
406 the receptor on specific residues. Among the numerous phosphorylation sites identified in the  
407 amino-terminal domain of ER $\alpha$ , Ser<sup>167</sup> is of particular interest in MCF-7 cells because its  
408 phosphorylation has been associated to 4-OH-TAM resistance in breast cancer cells  
409 (Campbell et al., 2001; Jiang et al., 2007). The culture in the presence of DON (10<sup>-5</sup> M) for 30  
410 min increased ER $\alpha$  p-Ser<sup>167</sup> to a similar extent than EGF (10 ng/mL), used as a positive  
411 control. This result indicates that DON could modulate estrogen signaling by ligand-

412 independent activation of ER $\alpha$ . In MCF-7 cells, ER $\alpha$  p-Ser<sup>167</sup> could be elicited by various  
413 pathways, including PI3K/Akt or MAPK pathways (Anbalagan and Rowan, 2015; Campbell  
414 et al., 2001; Clark et al., 2001; Lannigan, 2003; Yamnik and Holz, 2010). Interestingly, DON  
415 has been described to be able to activate those signaling pathways. Indeed, *in vivo* studies  
416 demonstrated the activation of PI3K/Akt and MAPK (ERK1/2, JNK, p38, p90-RSK2)  
417 pathways in mouse skin cells following dermal exposure to DON (Mishra et al., 2020, 2014).  
418 Several studies suggested that the activation of these signaling pathways could result from the  
419 “ribotoxic stress response” to DON. This mechanism appears to be common to many  
420 xenobiotics (Laskin et al., 2002) and has been extensively studied in the context of DON  
421 toxicity. In addition to its interaction with the 60S ribosomal subunit leading to the inhibition  
422 of protein synthesis, DON has also been shown to promote the degradation of 28S rRNA,  
423 which triggers the so-called ribotoxic stress response by activating the MAPK pathways  
424 (Iordanov et al., 1997; Moon and Pestka, 2002; Pan et al., 2013; Shifrin and Anderson, 1999).  
425 The hypothesis of the possible involvement of p90-RSK2 in the effects of DON described in  
426 our study is of particular interest. Indeed, p90-RSK2 has been demonstrated to induce ER $\alpha$ -  
427 mediated transcription through either the phosphorylation of Ser<sup>167</sup> or through the binding to  
428 this receptor. These two action mechanisms appear to be independent of each other and  
429 additive. Moreover, although ER $\alpha$ -mediated transcription triggered by p90-RSK2 binding is  
430 inhibited by 4-OH-TAM, a constitutively active p90-RSK2 still induces ER $\alpha$  p-Ser<sup>167</sup> in the  
431 presence of the antagonist, which is in accordance with the effects of DON reported in our  
432 study (Clark et al., 2001).

433 The activation of numerous signaling cascades may also explain the surprising result of the  
434 highest concentration of DON still leading to a slight increase in *TFF1/PS2* gene expression  
435 in ESR1-siRNA-MCF-7 cells. Even though this effect was sharply reduced compared to that  
436 observed in control-siRNA-MCF-7 cells, it suggests an ER $\alpha$ -independent regulation of

437 *TFF1/PS2* gene expression. Although initially identified in MCF-7 cells and therefore  
438 described as a breast cancer-associated and estrogens-responsive gene, *TFF1/PS2* is a  
439 member of the trefoil factors family, strongly expressed by epithelial cells of the gastro-  
440 intestinal tract which are actually the first target of DON following an oral exposure (Aihara  
441 et al., 2017). Porcine intestinal epithelial cells (IPEC-J2 cells) exposed to DON ( $0,5 \times 10^{-6}$  M)  
442 for 6 h exhibited an increased *TFF1/PS2* mRNA level (Wang et al., 2019). This outcome was  
443 also reported in human goblet cells (HT29-16E cells) when exposed to DON ( $10^{-5}$  M) for 3 h  
444 or 6 h (longer exposures resulting in a strong inhibition of *TFF1/PS2* mRNA expression), and  
445 was associated to the activation of PKR and MAPK (p38 and ERK1/2) pathways (Graziani et  
446 al., 2019). However, this stimulatory effect of DON on expression of *TFF1/PS2* gene in  
447 MCF-7 cell line after knockdown of ER $\alpha$  was not observed in the ER $\alpha$  negative MDA-MB-  
448 231 cell line suggesting that DON-induced signaling pathways in the two cell lines studied  
449 may be different.

450 Therefore, our results clearly indicate that a short-term exposure to DON induces ER $\alpha$  p-  
451 Ser<sup>167</sup> and results in the up-regulation of the expression of estrogen-responsive genes. This  
452 DON-mediated ligand-independent activation of ER $\alpha$  is thought to require the activation of  
453 rapid signaling cascades and/or the transactivation of the receptor by p90-RSK2. Besides, the  
454 activation of rapid signaling pathways could possibly induce the expression of estrogen-  
455 responsive gene in an ER $\alpha$ -independent manner. Thus, the DON-induced disruption of  
456 estrogen signaling pathway seems to result from ER-dependent pathways, while an ER-  
457 independent mechanism could also be involved. Furthermore, the existence of cross-talk  
458 between such ER-dependent and ER-independent pathways could not be excluded (Fig. 9).

459 Our study also demonstrates a combined effect of DON with estrogenic compounds on the  
460 expression of estrogen-responsive genes. Indeed, DON, in combination with either E2  
461 ( $10^{-10}$  M) or ZEA ( $10^{-7}$  M), increased the expression of *TFF1/PS2* and *PR* genes to a greater

462 extent than the estrogenic compound alone. This effect follows an inverted “U-shaped” dose-  
463 response profile: the maximal effect was observed for the intermediate concentration of DON  
464 ( $10^{-5}$  M) while a weaker effect or no effect were observed at low ( $10^{-6}$  M) and high ( $10^{-4}$  M)  
465 concentrations. Studies investigating the effects of endogenous hormones (such as E2) and  
466 endocrine disruptors (such as ZEA) commonly reported such unconventional non-linear dose-  
467 response relationship and several mechanistic hypotheses have been proposed (Lagarde et al.,  
468 2015; Vandenberg et al., 2012). In our study, the non-monotonic dose-response relationship,  
469 characterized by the absence of effect of DON at  $10^{-4}$  M on the E2- or ZEA-induced up-  
470 regulation of estrogen-responsive genes, could be due to the receptor down-regulation.  
471 Indeed, the sustained activation of ER $\alpha$  leads to its degradation by the ubiquitin proteasome  
472 system. This phenomenon is induced by the strong activity of this receptor and also by its  
473 phosphorylation state (Kondakova et al., 2020). In our study, these conditions are fulfilled  
474 during an exposure to ER agonists and in the presence of DON at high concentration.

475 Exposure to cocktails of DON and ZEA has already been studied on various parameters  
476 including intestinal function, nephrotoxicity, sperm viability or steroid hormone production  
477 (Jia et al., 2020; Kolesarova et al., 2017; Liang et al., 2015; Ren et al., 2017; Tassis et al.,  
478 2020). These studies reported either sub-additive, additive, or synergic effects of DON and  
479 ZEA, thus emphasising the difficulty to predict the impact of mycotoxin mixtures by  
480 extrapolating from data obtained with each compound taken separately (Thapa et al., 2021).  
481 Since DON and ZEA are both produced by *Fusarium* fungi (mainly *F. culmorum* and *F.*  
482 *graminearum*), these mycotoxins naturally co-occurred in various regions of the globe (Ma et  
483 al., 2018; Palumbo et al., 2020; van der Fels-Klerx et al., 2021), making our result relevant  
484 regarding human and animal exposure, as demonstrated by many other reports. The  
485 assessment of the effects of DON in combination with its metabolites may also be relevant,

486 especially since two of them, namely 3-acetylDON and 15-acetylDON, have been shown to  
487 exhibit agonist effects on the ER activity (Demaegdt et al., 2016).

488

489

490 **5. Conclusion**

491 **Short-term exposure to DON results in estrogen signaling activation *in vitro*, which is**  
492 **evidenced by the up-regulation of estrogen-responsive gene expression, demonstrating a**  
493 **transient disruption of this signaling pathway induced by this mycotoxin. This effect**  
494 **does not appear to involve neither direct binding to ER $\alpha$  or GPER nor cross-talk with**  
495 **PPAR $\gamma$ 2 signaling, but is rather thought to be the consequence of the DON-induced**  
496 **ligand-independent activation of ER $\alpha$  by phosphorylation. Further studies are needed to**  
497 **investigate the upstream mechanisms involved. Moreover, this work also supports the**  
498 **significance of considering the exposure window to characterize the effects of endocrine**  
499 **disruptors, since many adverse effects could occur after a short exposure time. Finally,**  
500 **our study highlights a combined effect of DON and ZEA on estrogen signaling**  
501 **disruption. Considering the natural co-occurrence of these mycotoxins, this result is**  
502 **highly relevant with regard to animal and human exposure and supports the need to**  
503 **assess the effects of mycotoxins in cocktail in addition to those of each compound taken**  
504 **separately.**

505

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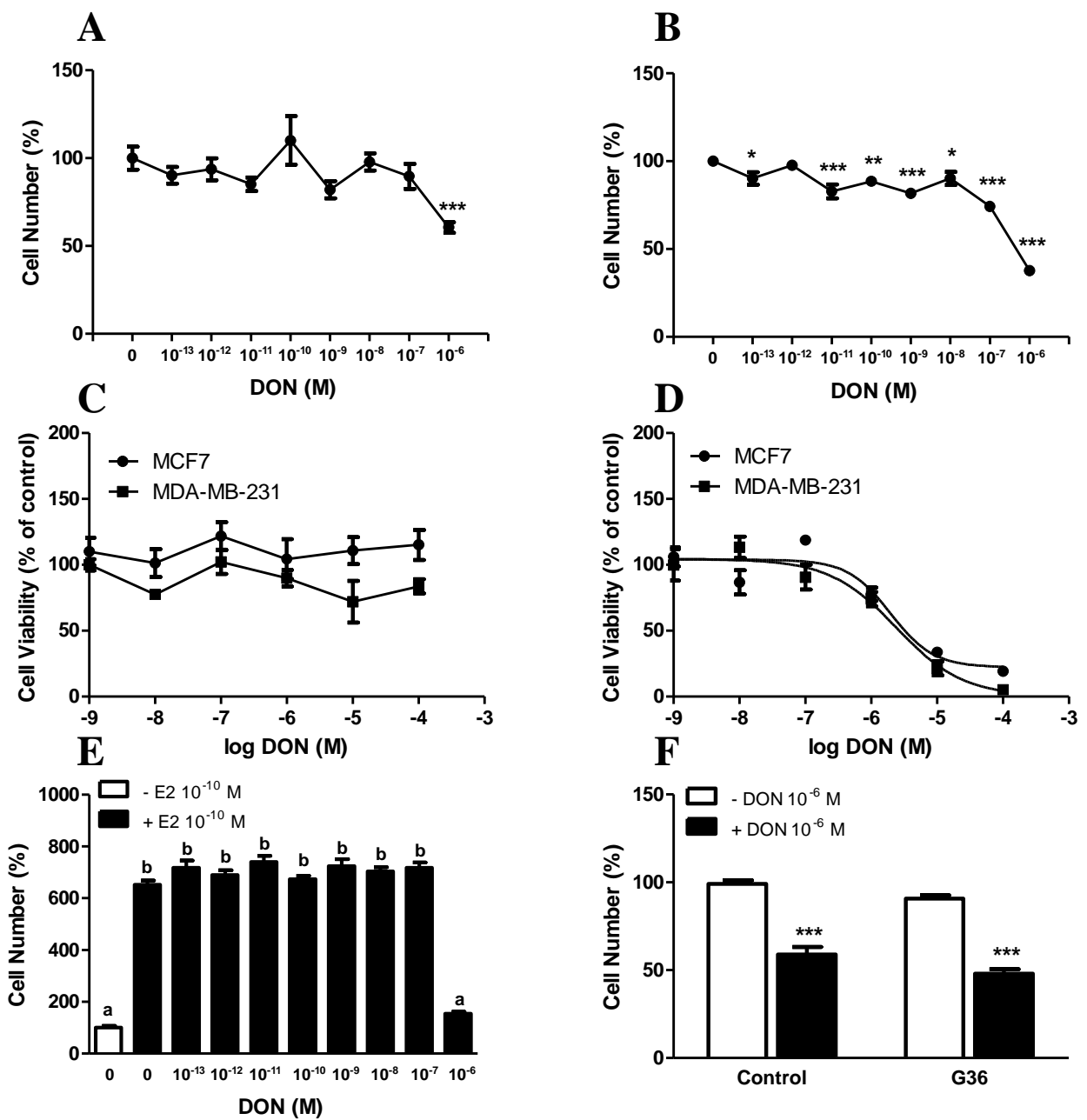
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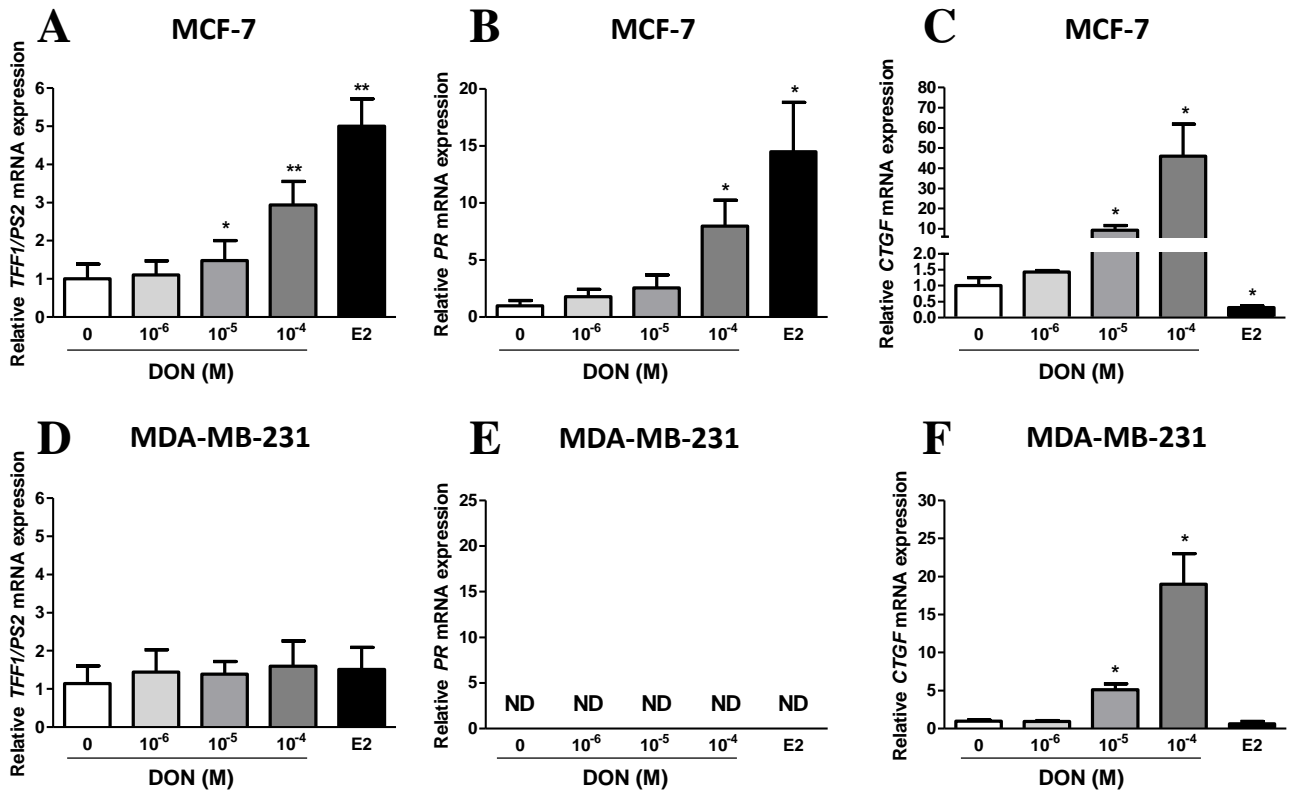
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**Table 1.** Primers used in real time PCR.

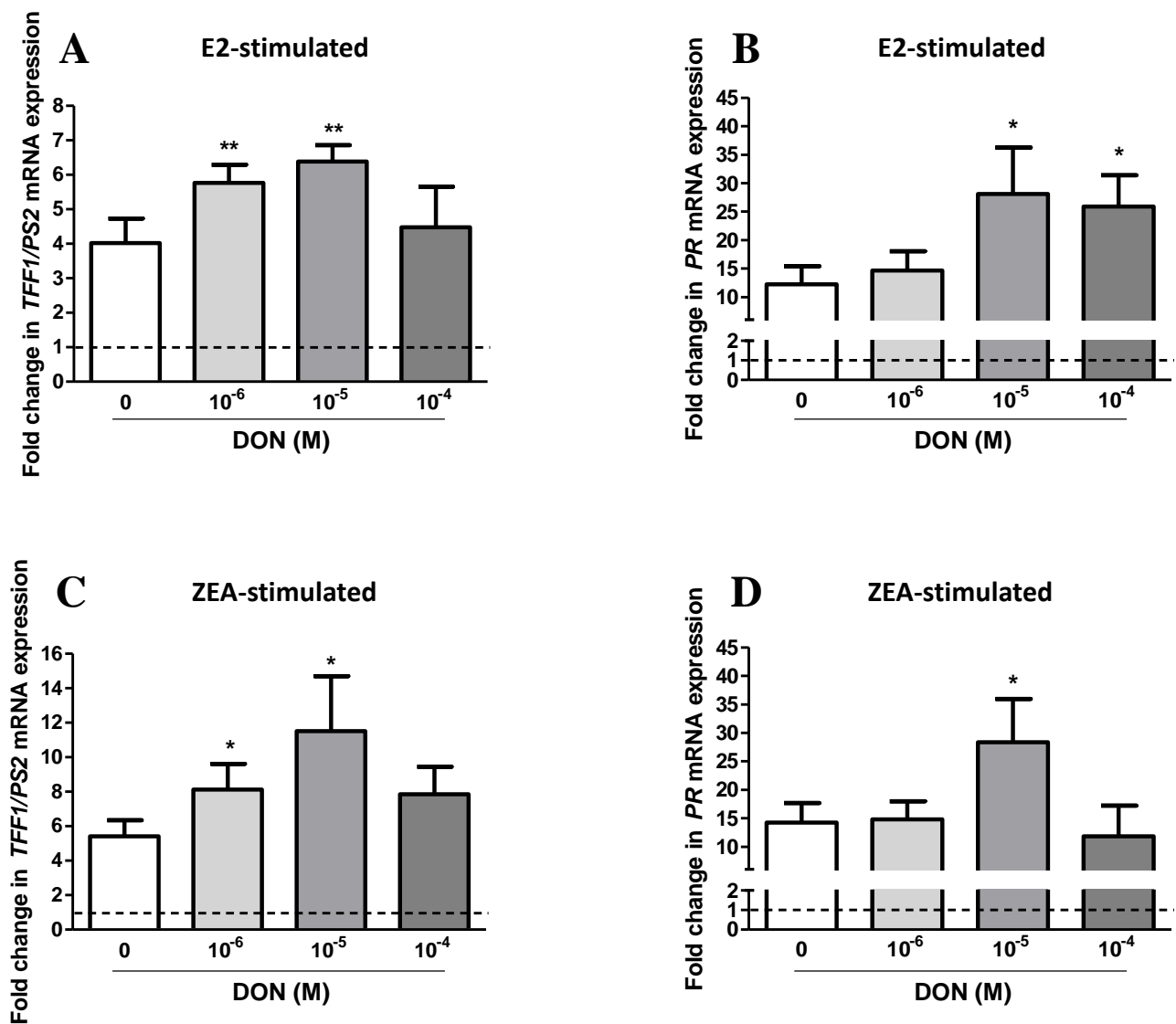
| Gene            | Forward primer (5' → 3') | Reverse primer (5' → 3') | Size (pb) |
|-----------------|--------------------------|--------------------------|-----------|
| <i>GAPDH</i>    | AGCCGCATCTTCTTTTGC       | AATGAAGGGGTCATTGATGG     | 162       |
| <i>TFF1/PS2</i> | CACCATCGAGAACAAGGTGA     | AGCCCTTATTTGCACACTGG     | 161       |
| <i>PR</i>       | TCGAGCTCACAGCGTTTCTA     | CACCATCCCTGCCAATATCT     | 183       |
| <i>CTGF</i>     | TACCAATGACAACGCCTCCT     | CCGTCGGTACATACTCCAGA     | 207       |
| <i>ESR1</i>     | GACAGGGAGCTGGTTCACAT     | CAGGATCTCTAGCCAGGCAC     | 111       |
| <i>ESR2</i>     | TGCGCCTGGCTAACCTCCTGA    | CCGCGAAGCACGTGGGCATT     | 148       |



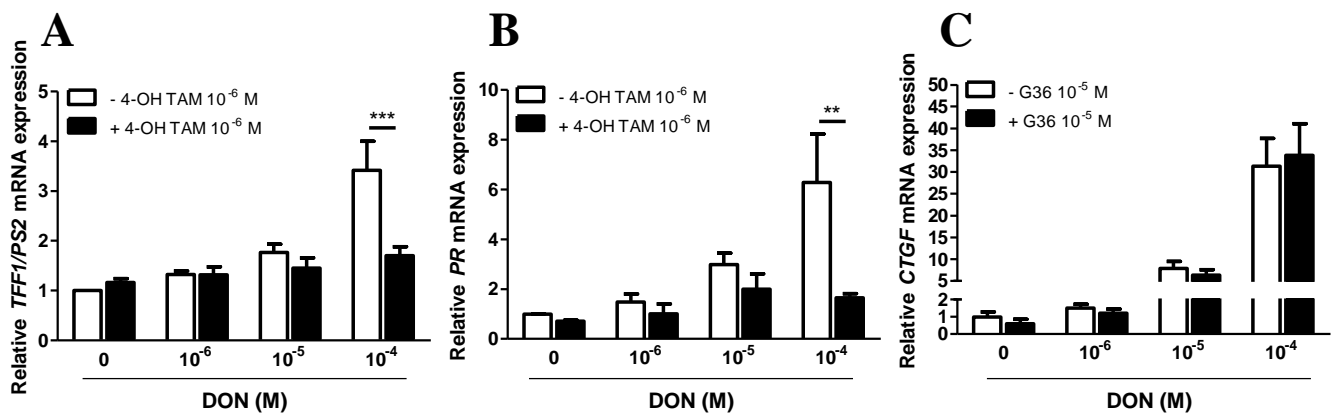
**Figure 1. Effect of DON on viability of MCF-7 and MDA-MB-231 cell lines.** MCF-7 (A) or MDA-MB-231 (B) cells were cultured for 72h with increasing concentrations of DON then the cell number was determined using a crystal violet cell proliferation assay. The effect of DON on cell viability was characterized using an ATP luminescent cell viability assay after culture of MCF-7 or MDA-MB-231 cell lines in the presence of increasing concentrations of DON for 6 (C) or 72 h (D). The effect of DON was studied on the E2-induced proliferation of MCF-7 cell line (E). Cells were cultured for 72 h with E2 at  $10^{-10}$  M and in the presence of increasing concentrations of DON. The cell number was then determined using a crystal violet cell proliferation assay. The involvement of GPER in the DON-induced decrease in the number of MCF-7 cells was investigated (F). Cells were cultured for 45 min with or without G36 ( $10^{-5}$  M) and for further 72 h in the presence or absence of DON ( $10^{-6}$  M). The cell number was determined using a crystal violet cell proliferation assay. All results are expressed as percentage of cells number relative to the control condition. Data are shown as mean  $\pm$  SEM of 3 independent experiments. Statistical significance was analysed by a paired t-test (A, B and F) or an ANOVA followed by a Bonferroni post-hoc test (E). Different lowercase letters indicate significant differences ( $p < 0.05$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  indicate significant difference compared to (A and B) the control condition, (F) the corresponding condition without DON.



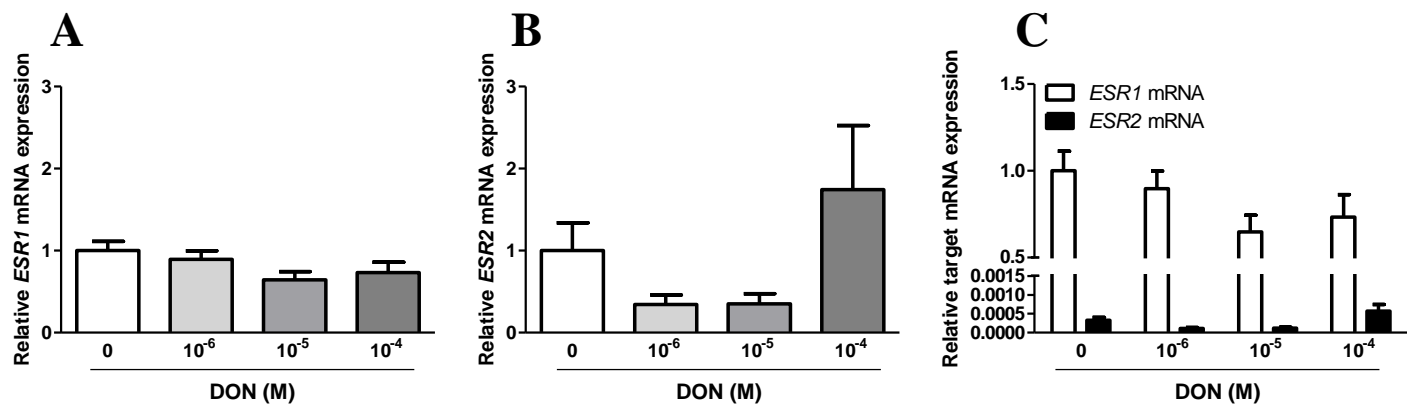
**Figure 2. Effect of DON on the expression of estrogen-responsive genes in MCF-7 (A-C) and MDA-MB-231 cell lines (D-F).** Cells were incubated for 6 h with increasing concentrations of DON or E2 ( $10^{-10}$  M). After extraction of total RNA, the expression of *TFF1/PS2* (A and D), *PR* (B and E) and *CTGF* (C and F) mRNA was quantified by RT-qPCR, mRNA levels of the targeted genes were normalized against *GAPDH* mRNA level. ND, not detected. Data are shown as mean  $\pm$  SEM of 3 (E-F), 4 (A, B) or 5 (D) independent experiments. Statistical significance was analysed by a paired t-test. \* $p < 0.05$ , \*\* $p < 0.01$  indicate significant difference compared to control condition.



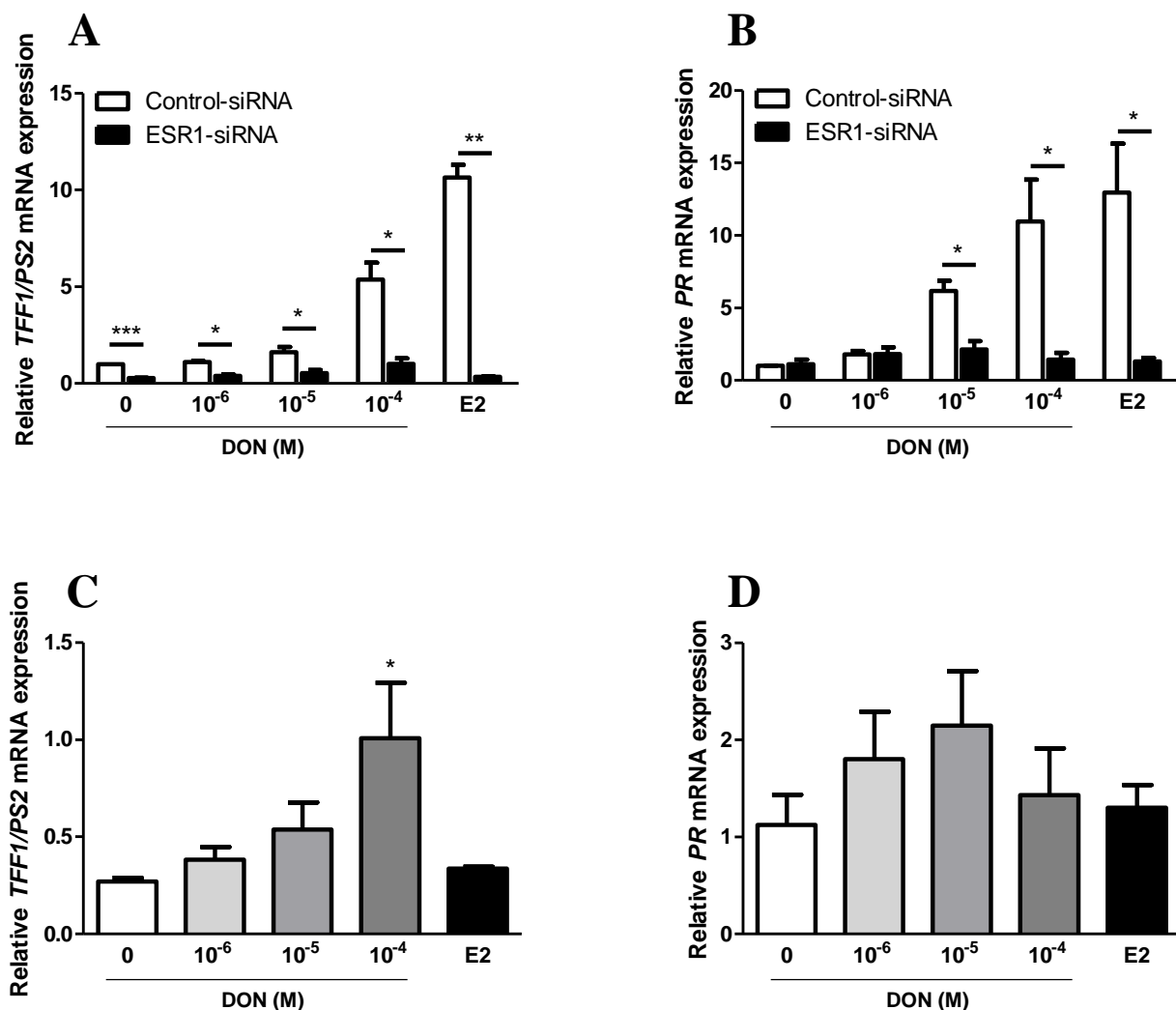
**Figure 3. Effect of DON on the expression of estrogen-responsive genes induced by E2 (A, B) or ZEA (C, D) in MCF-7 cell line.** Cells were incubated for 6 h with E2 ( $10^{-10}$  M) and ZEA ( $10^{-7}$  M) alone or in combination with increasing concentrations of DON. After extraction of total RNA, the expression of *TFF1/PS2* (A and C) and *PR* (B and D) mRNA was quantified by RT-qPCR, mRNA levels of the targeted genes were normalized against *GAPDH* mRNA level. Data are shown as mean  $\pm$  SEM of 3 independent experiments. Statistical significance was analysed by a paired t-test. \* $p < 0.05$ , \*\* $p < 0.01$  indicate significant difference compared to control with E2 or ZEA.



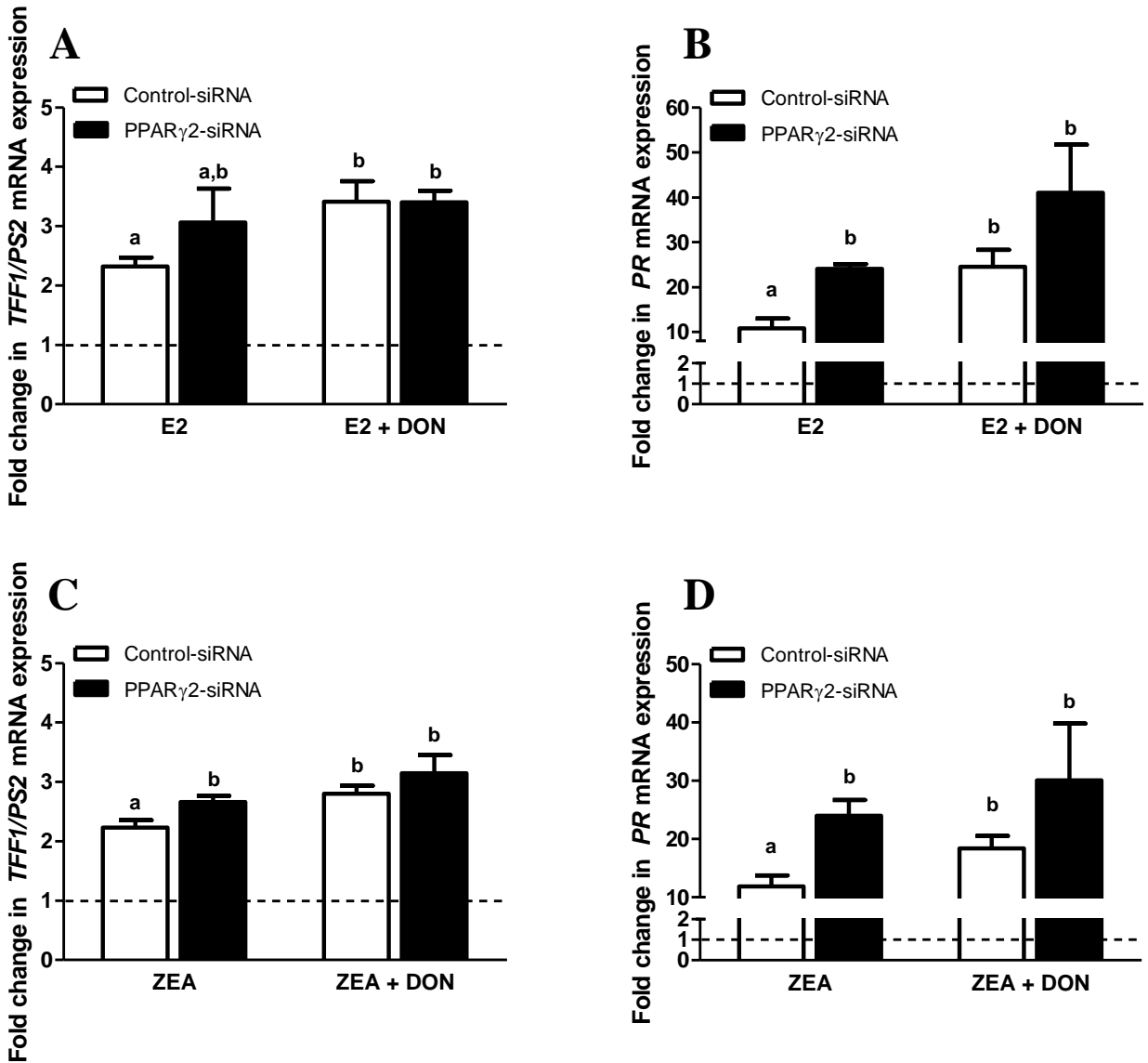
**Figure 4. Effect of ERs antagonists on the expression of DON-induced estrogen-responsive genes in MCF-7 cell line.** Cells were pre-incubated for 45 min with (A and B) 4-OH-TAM ( $10^{-6}$  M) or (C) G36 ( $10^{-5}$  M) and then incubated for 6 h with increasing concentrations of DON. After extraction of total RNA, the expression of *TFF1/PS2* (A), *PR* (B) and *CTGF* (C) mRNA was quantified by RT-qPCR, mRNA levels of the targeted genes were normalized against *GAPDH* mRNA level. Data are shown as mean  $\pm$  SEM of 3 (B), 4 (C) or 5 (A) independent experiments. Statistical significance was analysed by a paired t-test. \*\* $p < 0.01$ , \*\*\* $p < 0.001$  indicate significant difference compared to the corresponding condition without antagonist.



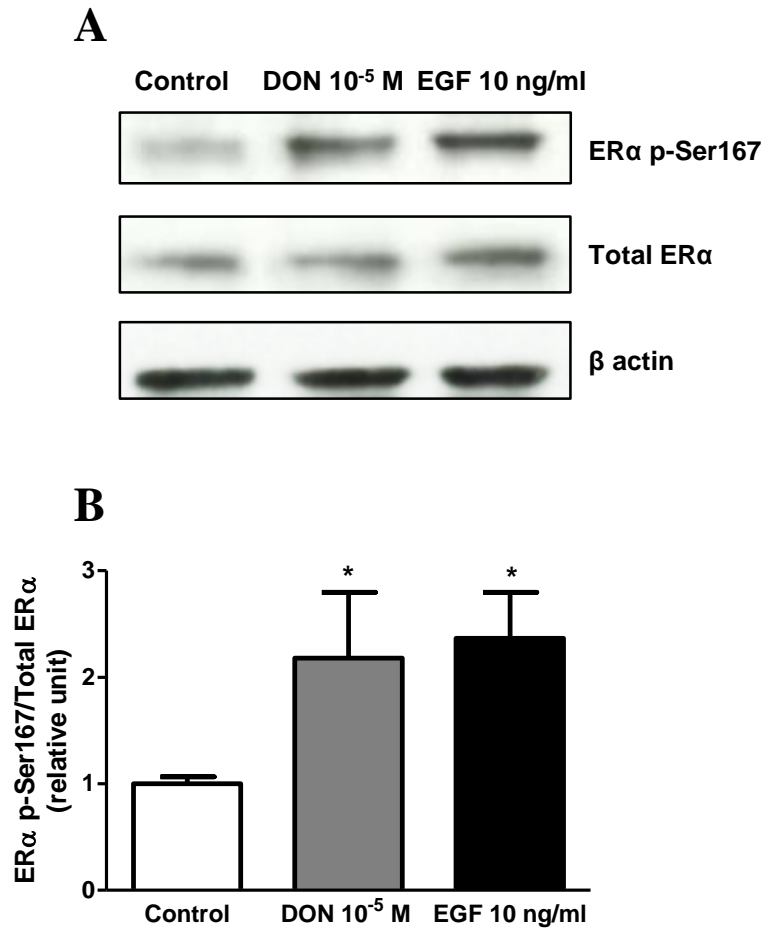
**Figure 5. Effect of DON on the expression of nuclear estrogen receptor in MCF-7 cell line.** MCF-7 cells were incubated for 6 h with increasing concentrations of DON. After extraction of total RNA, the expression of *ESR1* (A and C) and *ESR2* (B and C) mRNA were quantified by RT-qPCR, mRNA levels of the targeted genes were normalized against *GAPDH* mRNA level. Data are shown as mean  $\pm$  SEM of 3 independent experiments. Statistical significance was analysed by a paired t-test.



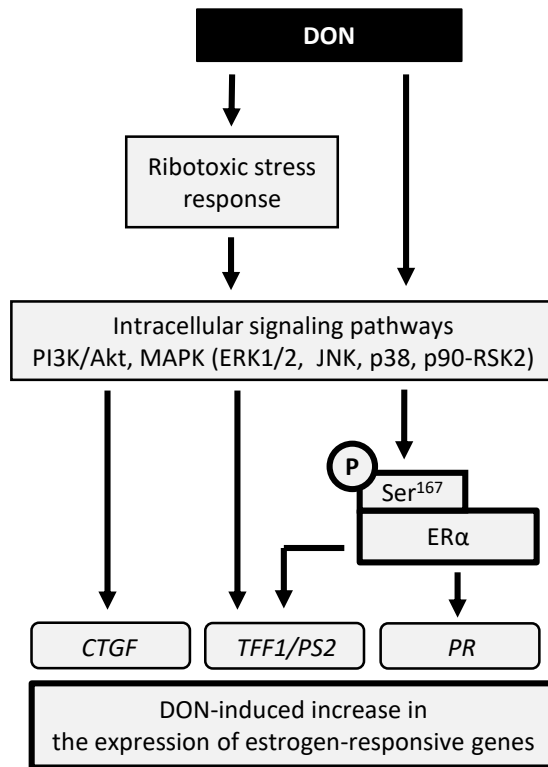
**Figure 6. Effect of *ESR1* silencing on the expression of DON-induced estrogen-responsive genes in MCF-7 cell line.** MCF-7 cells were transfected with siRNA targeting *ESR1* mRNA (ESR1-siRNA) or an off-target siRNA (control-siRNA) used as a negative control. 48 h post-transfection, cells were incubated for 6 h with increasing concentrations of DON or E2 (10<sup>-10</sup> M). After extraction of total RNA, the expression of *TFF1/PS2* (A and C) and *PR* (B and D) mRNA was quantified by RT-qPCR, mRNA levels of the targeted genes were normalized against *GAPDH* mRNA level. A and B, results obtained for cells transfected with control-siRNA and ESR1-siRNA. C and D, focus on the results obtained for cells transfected with ESR1-siRNA only. Data are shown as mean ± SEM of 3 independent experiments. Statistical significance was analysed by a paired t-test. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001 indicate significant difference compared to the corresponding condition in MCF-7 transfected with control-siRNA (A and B) or the control condition in MCF-7 transfected with ESR1-siRNA (C and D).



**Figure 7. Effect of *PPAR* $\gamma$ 2 silencing on the effects of DON on the expression of estrogen-responsive genes induced by E2 (A, B) or ZEA (C, D) in MCF-7 cell line.** MCF-7 cells were transfected with siRNA targeting *PPAR* $\gamma$ 2 mRNA (*PPAR* $\gamma$ 2-siRNA) or an off-target siRNA (control-siRNA) used as a negative control. 24 h post-transfection, cells were incubated for 6 h with E2 ( $10^{-10}$  M) or ZEA ( $10^{-7}$  M) alone or in combination with DON ( $10^{-5}$  M). After extraction of total RNA, the expression of *TFF1/PS2* (A and C) and *PR* (B and D) mRNA was quantified by RT-qPCR, mRNA levels of the targeted genes were normalized against *GAPDH* mRNA level. Data are shown as mean  $\pm$  SEM of 3 independent experiments. Statistical significance was analysed by an ANOVA followed by a Bonferroni post-hoc test. Different lowercase letters indicate significant differences ( $p < 0.05$ ).



**Figure 8. Effect of DON on the level of ERα phosphorylation at serine 167 in MCF-7 cell line.** MCF-7 cells were incubated for 30 min with DON (10<sup>-5</sup> M) or EGF (10 ng/mL), used as a positive control. ERα phosphorylation at serine 167 site (ERα p-Ser<sup>167</sup>) was analysed by Western Blot from whole cell lysates. **A**, representative Western Blot of ERα p-Ser<sup>167</sup>, total ERα and β-actin served as loading control. **B**, diagram of ERα p-Ser<sup>167</sup> staining quantification obtained using the Image J software and normalized against the corresponding total ERα value. Data are shown as mean ± SEM of 3 independent experiments. Statistical significance was analysed by a paired t-test. \*p<0.05 indicate significant difference compared to control condition.



**Figure 9. Proposed scheme illustrating the mechanisms of DON effects on the induction of estrogen signaling pathway in MCF-7 cell line.** Our results demonstrate that the incubation of MCF-7 cell line with DON for 6 h induced the expression of estrogen-responsive genes. This scheme summarizes the different mechanisms investigated in our study to explain this effect (boxed in bold), as well as some mechanisms not investigated in our study but suggested by our results in the light of literature data. We show that the expression of DON-induced estrogen-responsive genes resulted neither from direct binding of DON to GPER and to ER $\alpha$ , nor from its antagonistic activity on PPAR $\gamma$ 2. Rather, we hypothesize that DON could trigger the ribotoxic stress response or other mechanisms, leading to the activation of intracellular signaling pathways which in turn could induce the ligand-independent activation of ER $\alpha$  through its phosphorylation at Ser<sup>167</sup>. Moreover, these DON-induced signaling pathways could induce the expression of *TFF1/PS2* and *CTGF* genes in an ER-independent manner.

**Table 1.** Primers used in real time PCR.

| Gene            | Forward primer (5' → 3') | Reverse primer (5' → 3') | Size (pb) |
|-----------------|--------------------------|--------------------------|-----------|
| <i>GAPDH</i>    | AGCCGCATCTTCTTTTGC       | AATGAAGGGGTCATTGATGG     | 162       |
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| <i>PR</i>       | TCGAGCTCACAGCGTTTCTA     | CACCATCCCTGCCAATATCT     | 183       |
| <i>CTGF</i>     | TACCAATGACAACGCCTCCT     | CCGTCGGTACATACTCCAGA     | 207       |
| <i>ESR1</i>     | GACAGGGAGCTGGTTCACAT     | CAGGATCTCTAGCCAGGCAC     | 111       |
| <i>ESR2</i>     | TGCGCCTGGCTAACCTCCTGA    | CCGCGAAGCACGTGGGCATT     | 148       |