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ABSTRACT

Humans are exposed to different contaminants including mycotoxins. Deoxynivalenol (DON), a potent ribosome inhibitor, is a highly prevalent mycotoxin in the food chain worldwide. Although DON is not genotoxic, we previously showed that it exacerbates the genotoxicity of colibactin, a DNA-crosslinking toxin produced by bacteria in the gut. In the present study, we investigated whether this phenotype can be extended to other genotoxic compounds with different modes of action. Our data showed that, at a dose that can be found in food, DON exacerbated the DNA damage caused by etoposide, cisplatin and phleomycin. In contrast, de-epoxy-deoxynivalenol (DOM-1), a modified form of DON that does not induce ribotoxic stress, did not exacerbate DNA damage. The effect of DON was mimicked with other ribosome inhibitors such as anisomycin and cycloheximide, suggesting that ribotoxicity plays a key role in exacerbating DNA damage. In conclusion, a new effect of DON was identified, this toxin aggravates the DNA damage induced by a broad spectrum of genotoxic agents with different modes of action. These results are of utmost importance as our food can be co-contaminated with DON and DNA-damaging agents.

INTRODUCTION

Humans are exposed to a broad spectrum of food-contaminants, including mycotoxins (Payros *et al.*, 2021a). These toxins produced by fungi are the most common naturally occurring food contaminants and global surveys have estimated that they contaminate up to 70% of world crop production (Streit *et al.*, 2013; Eskola *et al.*, 2020). Mycotoxins can persist during food processing and are thus found in the consumer's meals (Sugita-Konishi *et al.*, 2006).

Deoxynivalenol (DON) is a widespread mycotoxin in food. A recent survey by the European Food Safety Authority (EFSA) reported that almost 50% of cereals are contaminated by this toxin; the highest levels being measured in wheat, maize, and oat grains (Knutsen *et al.*, 2017). Recent assessments using urinary levels as a biomarker, revealed that around 80% of individuals are exposed to DON (Turner *et al.*, 2008; De Santis *et al.*, 2019). Based on its toxicity, a tolerable daily intake (TDI) of 1 µg DON/kg body weight/day has been defined by JEFCA and EFSA (JEFCA, 2011; Knutsen *et al.*, 2017). However, this TDI can be exceeded in some population groups, especially in children (Knutsen *et al.*, 2017; Vin *et al.*, 2020).

Acute exposure to DON is associated with vomiting and bloody diarrhea (Ruan *et al.*, 2020) while chronic exposure decreases food consumption, induces neuro-endocrine changes, and alters immune functions (Maresca, 2013; Pinton *et al.,* 2015; Robert *et al.*, 2017; Terciolo *et al.*, 2018). Upon ingestion of contaminated food, intestinal epithelial cells are the first target of DON (Maresca, 2013; Pinton and Oswald, 2014; Graziani *et al.*, 2015). Its toxicity arises from its capacity to bind and inhibit the peptidyl transferase center in the 60S subunit of the ribosome (Garreau de Loubresse *et al.*, 2014; Pierron *et al.*, 2016a). This results in the inactivation of protein synthesis and a "ribotoxic stress response", which leads to the activation of MAP kinases and their downstream pathways including inflammatory response and oxidative stress (Pestka, 2008; Mishra *et al.*, 2014; Lucioli *et al.*, 2013; Da Silva, Bracarense and Oswald, 2018; Payros *et al.*, 2016). DON alters intestinal epithelium morphology, impairs the barrier function and nutrient absorption (Ghareeb *et al.*, 2014; Pierron *et al*., 2016b; Pinton *et al.*, 2009), modifies intestinal microbiota (Waché *et al.*, 2009), triggers intestinal inflammation (Maresca *et al.*, 2008; García *et al.*, 2018; Pestka, 2010) and increases susceptibility to intestinal inflammatory diseases (Payros *et al.*, 2020, 2021b).

DON is not genotoxic and is not classified as carcinogenic by the International Agency for Research on Cancer (IARC) (International Agency for Research on Cancer, 1993). However, we previously showed that DON exacerbates DNA damage, characterized

by the phosphorylation of the histone H2AX (γH2AX) induced by colibactin, an *Escherichia coli* genotoxin produced mainly in the intestine, and suspected of being involved in colorectal cancer (Nougayrede, 2006; Payros *et al.*, 2017; Pleguezuelos-Manzano *et al.*, 2020; Lopez, Bleich and Arthur, 2021). The aim of the present study was to investigate if realistic doses of DON exacerbate the genotoxicity caused by different DNA damaging agents, and if exacerbation is linked to its ribotoxicity.

METHODS

Toxins and reagents. DON, etoposide, cisplatin, anisomycin and cycloheximide were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France), and phleomycin (closely related to bleomycin) from Invivogen (Toulouse, France). DOM-1, a kind gift from G. Schatzmayr and D. Moll, was obtained by transforming crystalline DON (Romer Labs, Tulln, Austria) as previously described (Pierron *et al.*, 2016a). Stock solutions were stored at -20 °C; etoposide (5 mM), phleomycin (13.78 mM), DON (5 mM), DOM-1 (5 mM) and anisomycin (75 µM) were dissolved in DMSO; cisplatin (1.5 mM) and cycloheximide (18 mM) were dissolved in water.

Cell treatments. Non-transformed rat intestinal epithelial cells (IEC-6, ATCC CRL-1592) were cultured in complete DMEM GlutaMAX™ medium (Fisher Scientific) supplemented with 10% fetal calf serum, 1% non-essential amino acids (Fisher Scientific) and 0.1 U/mL bovine insulin (Sigma-Aldrich), at 37 °C with 5% CO_2. Human colon adenocarcinoma cells (HT-29) were cultured in complete McCoy's 5a Modified medium (Fisher Scientific) supplemented with 10% fetal calf serum and 1% non-essential amino acids (Fisher Scientific), at 37 °C with 5% CO_2. Cells were seeded in black 96-well plates (Greiner bio-one, Les Ulis, France) or Labtech (Fisher Scientific) and grown for 24-48 h to reach ~ 80% confluence before treatment. Cells were washed three times with warm HBSS before treatment. Cells were incubated for 4 h at 37 °C with 5% CO_2 in DMEM Hepes medium (Fisher Scientific) containing different concentrations of genotoxins (1 to 5 µM etoposide, 15 to 25 µM cisplatin, or 1 to 5 µM for phleomycin) and ribotoxins. Control cells were treated with DMSO vehicle (Sigma-Aldrich). After treatment, cells were washed three times with cold PBS and fixed with 4% formaldehyde (Fisher Scientific) for 20 min at room temperature before In-Cell Western or immunofluorescence assays.

Immunofluorescence staining. After fixation, cells were permeabilized for 15 min with PBS 0.25% Triton X-100 and blocked for one hour in blocking solution (PBS 5% normal goat serum 0.01% Tween 20). Cells were incubated with monoclonal primary antibody anti γH2AX diluted 1:500 (mouse monoclonal clone JBW301, Millipore, Burlington, USA) and anti S33-pRPA32 diluted 1:500 (rabbit polyclonal, Bethyl, Montgomery, USA) in blocking solution, for 3.5 h at room temperature. Following washing in PBS 0.05% Triton X-100, cells were incubated for 2 h in the dark at room temperature with anti-mouse AlexaFluor 488 and anti-rabbit AlexaFluor 568 (Invitrogen, Whaltham) diluted 1:1000. After three washes with PBS, Labtech were mounted using Fluoroshield containing DAPI (Sigma-Aldrich) and examined with a Zeiss LSM 710 confocal microscope.

Quantification of DNA damage by In-Cell Western analysis. Quantification of γH2AX by In-Cell Western analysis was performed as previously described (Martin *et al.*, 2013; Theumer *et al.*, 2018). Briefly, the fixed cells were permeabilized with 0.2% Triton X-100 and incubated in Maxblock (Active Motif) before immuno-staining with rabbit monoclonal anti-γH2AX diluted 1:200 (20E3; Cell Signaling, Saint-Quentin en Yvelines, France) followed by near-infrared-fluorescent secondary antibody diluted 1:500 (IRDye 800CW; Rockland) and staining of DNA with RedDot2 diluted 1:1000 (Biotium, Interchim, Montluçon, France). The DNA and γH2AX signals were measured at 680 and 800 nm with an Odyssey infrared imaging scanner (LI-COR Science Tec, Les Ulis, France). The genotoxic index was calculated by dividing the γH2AX fluorescence by the corresponding DNA fluorescence and normalized with the average fluorescence in untreated control cells (Tronnet and Oswald, 2018). All the data from three biological replicates are presented.

Statistical analyses. P-values were calculated using one-way analysis of variance (ANOVA) followed by multiple comparisons with Bonferroni's multiple-comparison correction using GraphPad Prism 7.0. For In-Cell Western analyses, the data are expressed as mean ± SEM.

RESULTS

DON exacerbates the genotoxicity caused by etoposide, cisplatin and phleomycin

To assess whether DON modifies the toxicity of a variety of genotoxins, cultured cells were treated with DON combined with one of the three DNA-damaging compounds with different modes of action: etoposide (ETP), a topoisomerase inhibitor, which causes DNA

double strand breaks (Hande, 1998); cisplatin (CPT), which causes DNA adducts and crosslinks (Dasari and Tchounwou, 2014); phleomycin (PHM), which causes oxidation of bases and single strand breaks (Chen and Stubbe, 2005). More precisely, non-transformed rat intestinal epithelial IEC-6 cells were treated for 4 h with 3 µM ETP, 20 µM CPT or 3 µM PHM, alone or combined with 10 µM DON or 10 µM DMSO used as control (vehicle). DNA damage was visualized using immunofluorescence confocal microscopy of phosphorylated H2AX (called γH2AX), a robust and quantitative DNA damage marker (Rogakou *et al.*, 1998). Control cells and cells treated only with DON exhibited low basal levels of γH2AX in nuclei (Figure 1). Treatment with the different genotoxins led to an increase in γH2AX staining whereas cells treated with both DON and the genotoxins exhibited exacerbated γH2AX signals (Figure 1). Increased γH2AX staining was also observed in human colon cancer HT-29 cells treated with DON and the genotoxins (Figure S1).

To confirm that treatment with DON increased DNA damage caused by the genotoxins, a second DNA damage marker, phosphorylated RPA32 (pRPA32), which is phosphorylated in response to genotoxic stress (Dueva and Iliakis, 2020), was examined. Cotreatment with DON and the genotoxins also increased the levels of pRPA32 compared to the genotoxins alone (Figure S2). In conclusion, DON exacerbates the DNA damage caused by a variety of DNA-damaging compounds with different modes of action.

DON induces dose-dependent exacerbation of the DNA damage caused by a variety of genotoxins

To quantify the exacerbation of DNA damage caused by the mycotoxin, IEC-6 cells were treated with varying doses of the three genotoxins together with 10 µM DON, then γH2AX levels in the cell population were measured by In-Cell Western assay. DNA damage increased when the dose of etoposide was increased from 1 µM to 5 µM. Additionally, for each dose of etoposide, cotreatment with 10 µM DON markedly exacerbated DNA damage. Similarly, DNA damage caused by cisplatin and phleomycin increased with an increase in the dose of genotoxin and were significantly exacerbated at a dose of 10 µM DON (Figure 2).

To assess whether DON at realistic doses could exacerbate the DNA damage caused by the different genotoxins, the cells were treated with single doses of DNA-damaging compounds combined with DON at doses ranging from 0.3 µM to 10 µM. DNA damage exacerbation increased with an increase in the dose of DON from 1 µM DON for cisplatin and phleomycin, and from 3 µM DON for etoposide (Figure 3, Figure S3). DON exacerbation of DNA damage was not associated with cell death assessed by DNA staining and quantification

(Table S1), consistent with the normal morphology of DAPI-stained nuclei in the cells treated with both DON and the genotoxins (Figure 1). Taken together, these results show that DON exacerbates DNA damage caused by different genotoxins at a dose as low as 3 µM.

De-epoxy-deoxynivalenol (DOM-1) does not exacerbate the DNA damage caused by the different genotoxins

To probe the mechanism by which DON exacerbates the DNA damage, we investigated if ribosome inhibition was implicated. Cells were treated with de-epoxy-deoxynivalenol (DOM-1), a detoxified form of DON that binds to but does not inhibit the ribosome (Pierron *et al.*, 2016a). In contrast to cells treated with the genotoxins and DON, γH2AX levels were similar in cells treated with the genotoxins alone or combined with 10 µM DOM-1 (Figure 4). DOM-1 and/or the genotoxins did not result in cell death (Table S1). Thus, non-ribotoxic DOM-1 does not exacerbate the DNA damage caused by different genotoxic compounds.

The ribosome inhibitors anisomycin and cycloheximide exacerbate the DNA damage caused by the different genotoxins

To confirm the role of ribosome inhibition in exacerbating DNA damage, the capacity of two ribosome inhibitors with distinct targets to exacerbate DNA damage was tested. Cells were treated with anisomycin, which, like DON, inhibits the A site of the ribosome, or with cycloheximide, which binds to the E site and interferes in the translocation step of protein synthesis (Schneider-Poetsch *et al.*, 2010). Both inhibitors significantly increased the DNA damage in the cells cotreated with etoposide, cisplatin or phleomycin, in a similar way to DON without causing cell death (Figure 5, Table S1). Altogether, these results indicate that the ribotoxicity induced by DON does exacerbate DNA damage.

DISCUSSION

Given its intrinsic toxicity and prevalence, DON is a major concern for food safety (Knutsen *et al.*, 2017; Payros *et al.*, 2016). The toxicity of DON is well documented, but little is known about its interactions with other toxins (Alassane-Kpembi *et al.*, 2017; Luo *et al.*, 2019). We recently observed that DON increases genotoxicity induced by colibactin, a bacterial toxin that causes peculiar DNA-interstrand crosslink lesions (Payros *et al.*, 2017; Bossuet-Greif *et al.*, 2018; Xue *et al.*, 2019). The aim of the present study was to determine whether this phenotype extends to other genotoxins with other modes of action, and, if so, to investigate the mechanism involved. We observed that DON also exacerbates the genotoxicity

of three well-known drugs: etoposide, phleomycin and cisplatin, that respectively induce DNA double strand breaks, single strand breaks, and adducts (Smart *et al.*, 2008; Povirk, 1996; Siddik, 2003). Thus, although DON is not inherently genotoxic, it exacerbates DNA damage caused by a broad spectrum of genotoxic agents.

The exacerbation of DNA damage caused by DON has been linked to its ribotoxicity. Indeed, the non-ribotoxic DON derivative DOM-1 (Pierron *et al.*, 2016a) did not exacerbate genotoxicity. Conversely, ribotoxins with modes of action similar to or distinct from that of DON (anisomycin and cycloheximide respectively) reproduced the DNA damage exacerbating phenotype, suggesting that the ribosome inhibitor mode of action is not critical for DNA damage exacerbation. The mechanism by which DON and other ribotoxins aggravate the genotoxicity of various genotoxins remains to be identified but several hypotheses can be proposed. First, ribosomes play an important role in genome preservation through ribosomal proteins that have a direct role in DNA repair (Mao-De and Jing, 2006). In addition, upon genotoxic stress, the cell reprograms mRNA translation to quickly synthesize proteins involved in the stress response (Kabilan *et al.*, 2020; Spriggs, Bushell and Willis, 2010). Thus, DON interference with ribosome function could increase DNA damage by disturbing DNA damage response. Second, ribotoxins such as DON and anisomycin trigger the "ribotoxic stress response" with the recruitment of its main mediator, the protein kinase R (PKR) (Zhou *et al.*, 2014). Activated PKR has been reported to interact functionally with DNA repair proteins, to repress the repair response, and to sensitize the cells to DNA damaging agents (Bennett *et al.*, 2006; Zhang *et al.*, 2004). Third, DON and ribotoxins have been reported to cause upregulation of inflammatory cytokines such as interleukins or IFNγ, and expression of transcription factors such as NF-κB (Pestka, 2010; Cano *et al.*, 2013; Luo *et al.*, 2021). It is known that inflammation negatively regulates the DNA repair machinery (Jaiswal *et al.*, 2000). For example, some studies reported that over-expression of NF-κB triggers the shutdown of tumor suppressor p53 activity, which plays an important role in DNA repair systems (Hudson *et al.*, 1999; Gudkov, Gurova and Komarova, 2011). Thus, ribotoxin-induced inflammatory response could sensitize the cells to DNA damage by influencing cell response to DNA damage. Additional studies are needed to explore these hypotheses.

Exacerbation of genotoxicity was observed from a dose as low as 1 µM of DON. This result is biologically pertinent given the concentrations of DON to which consumers are exposed. Indeed, DON concentrations of 0.16-2 µg/mL (0.5-7 µM) can be considered as realistic in the human gut (Sergent *et al.*, 2006; Maresca, 2013). The lower concentration corresponds to prolonged daily intake by consumers and the higher one corresponds to the

level that can be reached after consumption of heavily contaminated food (Knutsen *et al.*, 2017; Vin *et al.*, 2020; Alassane-Kpembi *et al.*, 2013). Consequently, the level of DON to which humans are exposed could potentiate the genotoxicity of foodborne genotoxins. Humans are exposed to many naturally occurring dietary genotoxins, or that are produced during food processing (Sakita *et al.*, 2017; Goldman and Shields, 2003). These genotoxins can cause different forms of DNA damage such as single or double strand breaks and adducts (Barnes *et al.*, 2018). For example, a daily intake of 26 ng/kg body weight of heterocyclic amines, which are formed during cooking and cause DNA strand breaks and adducts, has been described in the US population (Layton *et al.*, 1995). Similarly, European are exposed to 2 µg/kg body weight of the DNA strand break-inducing heavy metal cadmium (European Food Safety Authority, 2012).

Given the high prevalence of DON in human food, one can assume that intestinal epithelial cells are co-exposed to this mycotoxin together with dietary genotoxins. The present study demonstrates that realistic doses of DON exacerbate DNA damage induced by various type of genotoxic drugs (etoposide, cisplatin and phleomycin) and preliminary results in our laboratory indicate that DON aggravated DNA damage in intestinal cells exposed to dietary genotoxins such as pesticide or alcohol-derived compounds. DNA damage is pivotal in cancer, because it can lead to gene mutations, chromosomal instability and ultimately, cell transformation and neoplasia (Jackson and Bartek, 2009). We therefore suggest that DON could enhance the carcinogenic potential of intestinal mutagens. In 1993, IARC concluded that DON cannot be classified with respect to its carcinogenicity for humans (International Agency for Research on Cancer, 1993; Claeys *et al.*, 2020). However, a preliminary report on a large-scale epidemiological study including half a million participants from 10 European countries followed for 15 years points to an association between the risk of proximal colon cancer and long-term exposure to DON (Huybrechts *et al.*, 2019). Further studies are needed to examine whether exposure to DON (and other ribosome inhibitors) could promote cancer by exacerbating the genotoxicity of endogenous and dietary mutagens.

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FIGURE LEGENDS:

Figure 1: DON exacerbates phosphorylation of H2AX caused by etoposide, cisplatin and phleomycin. Non-transformed rat intestinal epithelial IEC-6 cells were treated for 4 hours with 10 μM DON or 10 μM DMSO vehicle combined with 3 μM ETP, 20 μM CPT or 3 μM PHM, and γH2AX was examined by immunofluorescence. Representative photos. Scale bar = 20 μm.

Figure 2: Dose-dependent genotoxicity of etoposide, cisplatin and phleomycin and its exacerbation by DON. Non-transformed rat intestinal epithelial IEC-6 cells were treated for 4
hours with 10 μM DON or 10 μM DMSO vehicle combined with different doses of ETP (red), CPT (blue) or PHM (green), then H2AX phosphorylation levels were quantified by In-Cell Western. All the data are expressed as mean ± SEM (3 independent experiments). Statistical analysis was performed using one-way ANOVA with Bonferroni's multiple-comparison correction. Values that differ significantly from vehicle are indicated by black asterisks, and values that differ significantly from the genotoxin alone are indicated by red asterisks. n.s.: not significant, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

Figure 3: DON exacerbates genotoxicity caused by etoposide, cisplatin and phleomycin in a dose-dependent manner. Non-transformed rat intestinal epithelial IEC-6 cells were treated for 4 hours with the doses of DON shown in the figure, combined with 5 μM ETP (red), 25 μM CPT (blue) or 5 μM PHM (green), then H2AX phosphorylation levels measured in three independent experiments were quantified by In-Cell Western analysis. All the data are expressed as mean ± SEM (3 independent experiments). All P-values are calculated using one-way ANOVA with Bonferroni's multiple-comparison correction. Values that differ significantly from the vehicle are indicated by black asterisks, and values that differ significantly from the genotoxin alone are indicated by red asterisks. ***: $p < 0.001$, ****: $p < 0.0001$, n.s: not significant.

Figure 4: DOM-1 does not exacerbate genotoxicity caused by etoposide, cisplatin and phleomycin. Non-transformed rat intestinal epithelial IEC-6 cells were treated for 4 hours

with 10 μM DON or DOM-1 and 5 μM ETP, (red), 20 μM CPT (blue), or 5 μM PHM (green), then H2AX phosphorylation levels measured in three independent experiments were quantified by In-Cell Western analysis. All the data are expressed as mean ± SEM (3 independent experiments). All Pvalues are calculated using one-way ANOVA with Bonferroni's multiple-comparison correction. Values that differ significantly from the vehicle are indicated by black asterisks, and values that differ significantly from the genotoxin alone are indicated by red asterisks. ***: $p < 0.001$, ****: $p< 0.0001$, n.s: not significant.

Figure 5: Cycloheximide and anisomycin exacerbate genotoxicity caused by etoposide, cisplatin and phleomycin. Non-transformed rat intestinal epithelial IEC-6 cells were treated for 4 hours with 1 μM CHX or 100 pM ANC combined with 5 μM ETP (red), 20 μM CPT (blue) or 5 μM PHM (green), then H2AX phosphorylation measured in three independent experiments was quantified by In-Cell Western analysis. All the data are expressed as mean ± SEM (3 independent experiments). All P-values are calculated using one-way ANOVA with Bonferroni's multiplecomparison correction. Values that differ significantly from the vehicle are indicated by black asterisks, and values that differ significantly from the genotoxin alone are indicated by red asterisks. **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, n.s: not significant.

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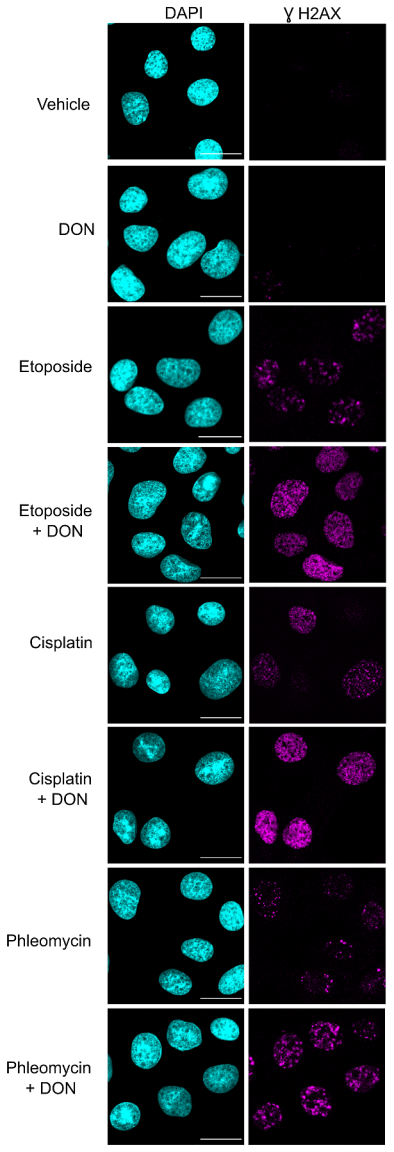
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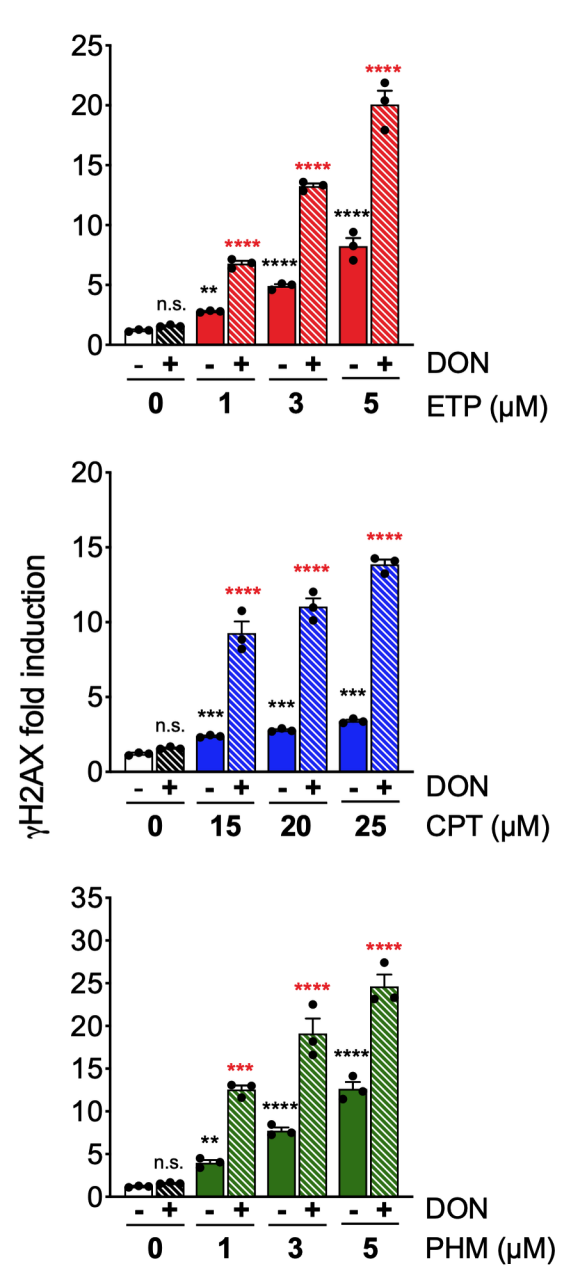
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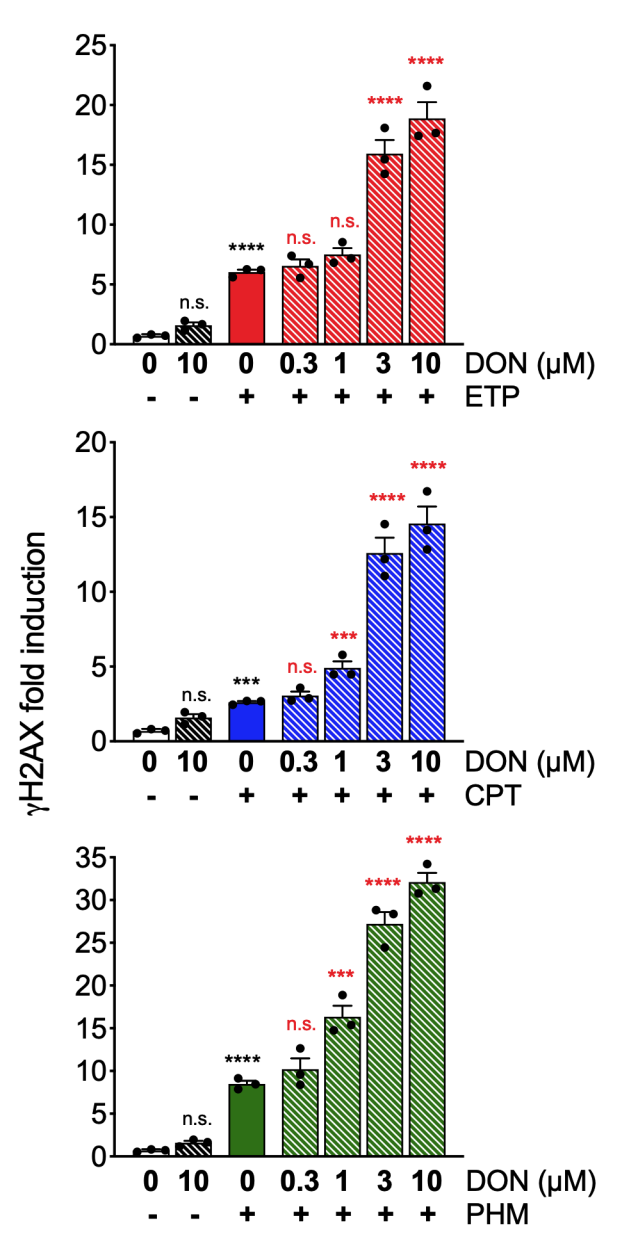
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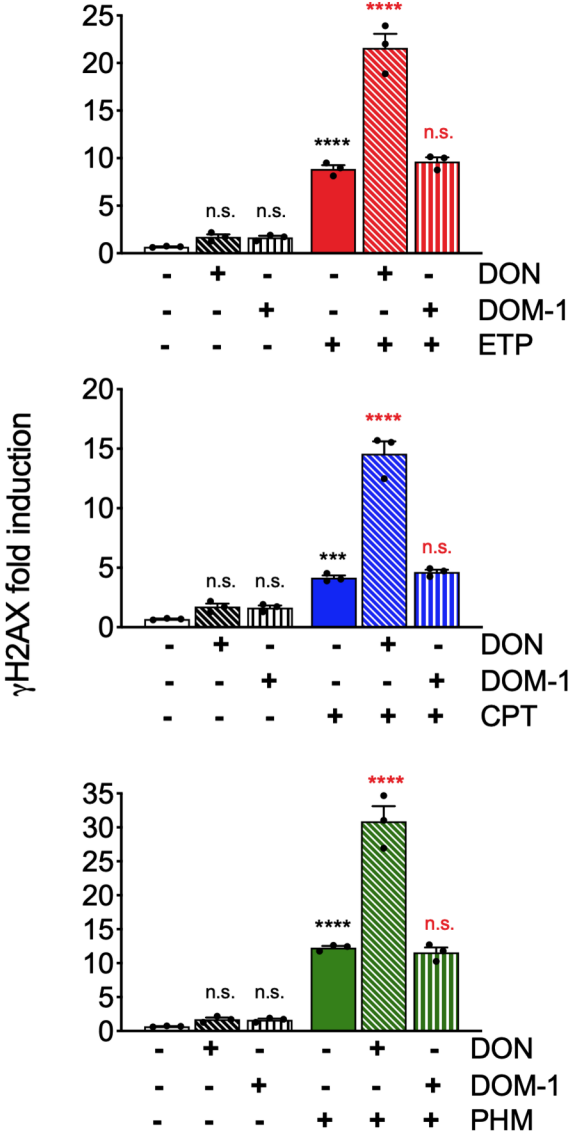
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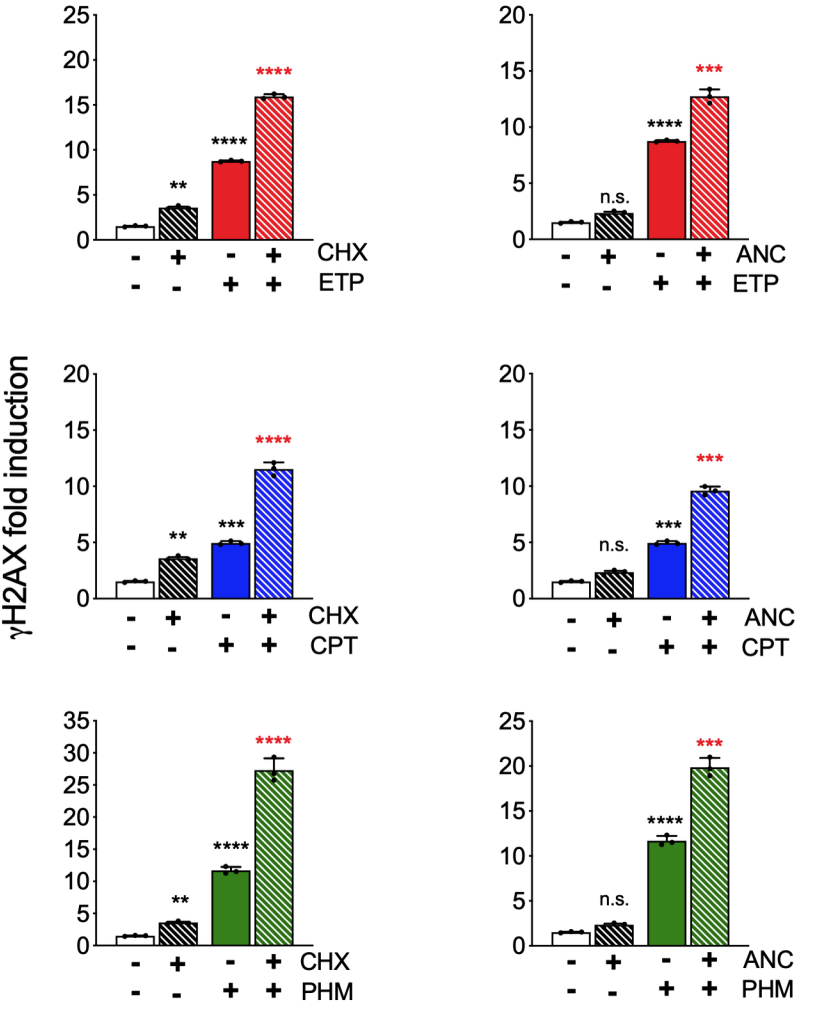
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GRAPHICAL ABSTRACT