Comparative study of cytotoxicity and oxidative stress induced by deoxynivalenol, zearalenone or fumonisin B1 in human intestinal cell line Caco-2

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Abstract

Fusarium species infestations of cereals crops occur worldwide. Fusarium toxins such as, deoxynivalenol (DON), zearalenone (ZEN) and fumonisin B1 (FB1) have been shown to cause diverse toxic effects in animals and also suspected of disease causation in humans.

From the literature and mechanistic point of view, DON binds to the ribosomal peptidyl-transferase and inhibits protein synthesis specifically and DNA synthesis consequently. ZEN known to be genotoxic, binds to 17β-estradiol receptors, induces lipid peroxidation, cell death and inhibits protein and DNA synthesis. FB1 disrupts sphingolipid metabolism, induces lipid peroxidation altering the cell membrane and causing cell death.

We intended to compare DON, ZEN and FB1 (1–150 μM) cytotoxic effect and the pathways leading to cell death and related to oxidative stress and macromolecules syntheses in a human intestinal cell line in order to tentatively classify them according to their respective potential toxicity.

The comparison reveals that all three mycotoxins bear, at variable degree, the capability of inducing lipid peroxidation (MDA production) and could be classified above 10 μM in decreasing potency order FB1 > DON > ZEN. This effect seems to be related to their common target that is the mitochondria as revealed by MTT test and seemingly not related to sphingoids accumulation concerning FB1.

DON and ZEN also adversely affect lysosomes in contrast to FB1.

The three mycotoxins inhibit protein synthesis with respective IC50 of 5, 8.8 and 19 μM for DON, FB1 and ZEN confirming that protein synthesis is a specific target of DON.

DNA synthesis is inhibited by DON, ZEN and FB1 with respective IC50 of 1.7, 10 and 20 μM. However at higher concentrations DNA synthesis seems to be restored for FB1 and DON suggesting a promoter activity.

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Altogether the potency of the three mycotoxins in macromolecules inhibition is DON > ZEN > FB1 in Caco-2 cells. It appears then that FB1 acts rather through lipid peroxidation while DON affects rather DNA and protein synthesis.

Keywords: Deoxynivalenol (DON); Fumonisin B1 (FB1); Zearalenone (ZEN); Comparative cytotoxicity; Caco-2 cells

1. Introduction

Fusarium species occur widely on plants. They are found in a variety of agricultural products mainly on corn, wheat and other cereal grains for human and animal consumption.

Fusarium toxins namely, deoxynivalenol (DON), zearalenone (ZEN) and fumonisin B1 (FB1) have been shown to cause diverse toxic effects in both experimental animals and livestock and occasionally suspected to cause adverse effects in humans (Mirocha and Christensen, 1974; Kuiper-Goodman et al., 1987; Marasas et al., 1988; Rotter et al., 1996; Gelderblom et al., 1998; Creppy, 2002). These mycotoxins could be found in the same commodities as secondary metabolites of different Fusarium species (Scudamore et al., 1998; Desjardins et al., 2000; Magan et al., 2002; Dawlatana et al., 2002).

Consumption of FB1 is responsible for several animal diseases including pulmonary oedema in swine, equine leukoencephalomacia and nephrotoxicity in different animal species (Marasas et al., 1988; IPCS, 2001).

DON consumption causes acute and chronic toxicity and affects animal feeding behaviour and immune function (Rotter et al., 1996; Lauraitie et al., 1997; Schlatter, 2004; Peskita et al., 2004) and DON and nivalenol (NIV) contamination are reported to contribute to gastrointestinal diseases in exposed humans (Li et al., 1999). DON is known to be clastogenic (Knasmuller et al., 1997).

Human and animal cell lines have been used extensively to study the mechanisms of in vitro toxicity of Fusarium toxins. DON is reported to bind to the ribosomal peptidyl-transferase site and inhibit protein and DNA synthesis, consequently exposure results in decreased cell proliferation (Shifrin and Anderson, 1999).

Fumonisins disrupt sphingolipid metabolism, altering the cell membrane and causing cytotoxicity (Riley et al., 1998), while FB1 is reported to induce lipid peroxidation, which may affect DNA integrity leading to DNA oxidized bases (Mohio et al., 2000a, 2003). In addition, recent reports show apoptosis induced by exposure to FB1 (Seefelder et al., 2003) and other Fusarium toxins such as 4-acetyl-12,13-epoxy-9-trichothecene-3,15-diol and fusarenone X (Miura et al., 2002; Oh et al., 2001).

ZEN has strong estrogenic effects due to its competition with 17β-estradiol in the binding to cytosolic oestrogen receptors present in the uterus, mammary gland, hypothalamus and pituitary gland (Kuiper-Goodman et al., 1987). Studies in various species (rodents, pigs and monkeys) have shown that ZEN and its metabolites have estrogenic and anabolic activities (Eisele and Dourmad, 1994).

Subsequently, ZEN was associated with hyperestrogenism and several physiological alterations of the reproductive tract in several laboratory animals such as mice, rats, guinea-pigs, hamsters, rabbits (Creppy, 2002) and domestic animals, (Haschek and Halburton, 1986; Osweiler, 1986). ZEN induces lipid peroxidation, cell death and inhibits protein and DNA syntheses (Abid-Essefi et al., 2004). It has been shown to be genotoxic, and to induce DNA-adduct formation (Pfohl-Leszkowicz et al., 1995; Lioi et al., 2004), DNA fragmentation and micronuclei production (Abid-Essefi et al., 2003; Ouanes et al., 2003).

Evaluation of NIV, DON and FB1 in the human K562 erythroleukemia cell line suggested the following ranking in cytotoxicity, NIV > DON > FB1 (Minervini et al., 2004). It is established that the potential in cytokines induction by DON and NIV is comparable (Ouyang et al., 1995; Hinoshita et al., 1997).

The aim of the present study is to compare cytotoxic effect and the pathway related to oxidative stress in a human intestinal cell line in order to tentatively classify DON, FB1 and ZEN according to their respective potency.
2. Materials and methods

2.1. Chemicals

ZEN, DON and FB1 were obtained from Sigma Chemical Company (St Louis, MO) and were dissolved in ethanol/water (90:10). Dulbecco’s modified eagle medium (DMEM), foetal calf serum (FCS), thiazolyl blue tetrazolium bromide (MTT) and neutral red solution were provided from Sigma-Aldrich. All other chemicals used were of analytical grade.

2.2. Cell culture and treatment

Caco-2 cells, a human colon cancer cell line, originally from Dr. Jing Yu, Tufts School of Medicine (Medford, MA, USA) were from UCL, Bruxelles, Belgium. The cells were grown as monolayer culture in a high glucose concentration (4.5 g/l) DMEM medium supplemented with 10% foetal calf serum (FCS), 8 mM l-glutamine, 1% of mixture penicillin (100 IU/ml) and streptomycin (100 g/ml) incubated at 37 °C in an atmosphere of 5% CO2 –95% air mixture. For cell counting and subculture, the cells were dispersed using 0.05% trypsin and 0.02% EDTA.

2.3. Cell viability assay by MTT

MTT test was used to assess cell viability based on the capacity for viable cells to metabolise a tetrazolium colourless salt to a blue formazan in mitochondria (Loveland et al., 1992). After 72 h of incubation in presence of toxins, DON (1–150 μM), ZEN (1–150 μM) and FB1 (1–150 μM) or the vehicule, 100 μl of 0.5% solution of thiazolyl blue tetrazolium bromide (MTT) were added to each well and 2 h later the medium was eliminated. Subsequently, 100 μl of dimethyl sulfoxide (DMSO) were added to the wells to extract the formazan formed in the viable cells. After 5 min of continuous stirring, the absorbance was determined at 540 nm using a Microplate Reader DYNA TECH MR 4000. The absorbance is proportional to the number of viable cells.

2.4. Cell viability assay by neutral red

Neutral red (NR) test was also performed to assess cytoxicity. Viable cells actively accumulate this dye across cell membrane, therefore after subsequent lysis, absorbance can be used as a measure of cell viability (Balls et al., 1987). The stock solution of NR (3.3 g/l) was diluted to 1/100 in the cell culture medium and the extracting solution consisted of 50% (v/v) ethanol in Milli-Q water with 1% (v/v) acetic acid. After 72 h of incubation in presence of toxins, DON (1–150 μM), ZEN (1–150 μM) and FB1 (1–150 μM) or the vehicule, 150 μl of freshly prepared neutral red solution pre-warmed to 37 °C was added to each well and all plates were incubated at 37 °C for additional 4 h. The cells were washed two times with PBS and 150 μl of the extracting solution were added in each well and plates were shaken for 15 min. The absorbance was determined at 540 nm using a Microplate Reader DYNATECH MR 4000.

2.5. Protein and DNA syntheses

Cells (10^5 cells/ml) were cultured in 24-well multi-dishes (Polylabo, France) for 24 h at 37 °C as described above. Then cultures were incubated in the presence of ZEN (10, 20 and 40 μM), FB1 (5, 10 and 20 μM) and DON (5, 10 and 20 μM) for 24h at 37 °C. A radioactive protein synthesis precursor (1 μCi), [3H]leucine (specific activity, 5 Ci/mmol) or 1 μCi of a radioactive DNA synthesis precursor, [3H]thymidine (specific activity, 85.40 Ci/mmol) was added. After 2 h additional incubation, cells were trypsinised, centrifuged 800 × g/10 min at 4 °C and subsequently, 100 μl of dimethyl sulfoxide (DMSO) were added to the wells to extract the formazan formed in the viable cells. After 5 min of continuous stirring, the absorbance was determined at 540 nm using a Microplate Reader DYNATECH MR 4000. The absorbance is proportional to the number of viable cells.

2.6. Extraction and determination of MDA–thiobarbituric acid (TBA) adduct

After incubation as described above for 24 h at 37 °C cultures were incubated in the presence of DON (5–40 μM), ZEN (5–40 μM) and FB1 (5–40 μM) for 24 h at 37 °C. The absorbance was determined at 540 nm using a Microplate Reader DYNATECH MR 4000. The absorbance is proportional to the number of viable cells.
Abado-Bégoné et al. (1998). The amount of MDA measured is adjusted to the protein content of cellular homogenates determined using the method of Bradford (1976).

2.7. Statistical analyses of data

Results are presented as mean ± S.D. and analysed using a non-parametric statistical test, Mann-Whitney test for significance of differences. Acceptable limit is set at $P < 0.05$.

3. Results

3.1. Effects on cell viability

Caco-2 cells viability was evaluated in the presence of DON, ZEN and FB1 using the MTT and neutral red tests. All toxins diminished cell viability in a concentration-dependent manner (Fig. 1 A).

The effect of DON is already seen with concentration of $1 \mu M$ with both tests. However, this became statistically significant at $10 \mu M$ and the IC50 is $21.5 \mu M$ for Neutral red test and $25 \mu M$ for MTT test.

The IC50 of ZEN in MTT test was $25 \mu M$ and that in neutral red test $15 \mu M$.

FB1 clearly affects cell viability as revealed by MTT test with an IC50 of $21 \mu M$. But the Neutral red test does not show significant cell viability reduction (Fig. 1B), the IC50 being over $150 \mu M$. Cell death caused by FB1 could be evaluated to be in a range of 15–20% in these conditions.

3.2. Effects on proteins and DNA syntheses

The inhibitory effect of all toxins on protein and DNA syntheses in Caco-2 cells is confirmed by the present study. Protein and DNA syntheses were inhibited by DON, ZEN and FB1 in a concentration-dependent manner (Fig. 2A and B). The 50% inhibitory concentrations (IC50) in protein synthesis (Fig. 2B) they were 1.7, 10 and 20 μM for DON, ZEN and FB1, respectively.

For concentrations above 5 μM of DON, DNA synthesis inhibition seemed to be reversed so that the percent inhibition is of 45% only for a concentration of 20 μM. It seemed that a plateau of inhibition is reached at 80–90% for ZEN and 60–65% for FB1.

3.3. Effects on oxidative stress

DON, ZEN and FB1 increased significantly the production of MDA, a biomarker of lipid peroxidation in Caco-2 cells, (Fig. 3) from the concentration of $10 \mu M$. At higher concentrations the most effective in oxidative stress is FB1 whereas the less effective is ZEN.

4. Discussion

An attempt has been made to study the effects of Fusarium toxins (DON, ZEN and FB1) on cell viability using MTT and Neutral red assays on the same cell line, as well as on the inhibition of DNA and protein synthesis, using radio-labelled precursors. In addition to these effects, oxidation of cell membrane lipids was measured by MDA formation. Experiments were carried out using the same experimental conditions for all toxins, required for direct comparison. It appears evident that Caco-2 cells are sensitive to DON, ZEN and FB1. The choice of toxin concentrations to assess cellular macromolecules syntheses inhibition was made on the basis of concentrations that reduced cells viability as determined by neutral red uptake and/or by the reduction of tetrazolium salt (MTT test) by mitochondria. Literature data on inhibitory concentrations in other cell lines have also taken into account (Mobio et al., 2000a; Ouanes et al., 2003; Seefelder et al., 2003; Sundstol Eriksen et al., 2004; Minervini et al., 2004).

Incubation of Caco-2 cells with toxins resulted in the decrease of neutral red uptake by DON and ZEN in a concentration-dependent manner, whereas FB1 did not affect significantly this uptake, indicating that apparently cell membrane permeability was not modified by FB1, as already reported (Babich and Borenfreund, 1987; Creppy et al., 2004).

ZEN is the toxin that the most adversely affects Caco-2 membrane integrity as showed by IC50 values in neutral red test, $15 \mu M$ as compared to $21 \mu M$ for DON.

In contrast to neutral red uptake the MTT assay showed that all toxins affect the mitochondrial metabolism through succinate dehydrogenase activity.
These results clearly point at mitochondria as a target of the three toxins.

FB1 is the most cytotoxic in MTT test while this toxicity cannot be demonstrated by neutral red test. This situation deserves attention for being instructive from several points of view. The neutral red is a weak cationic dye that readily penetrates cell membranes by non-ionic diffusion, accumulating intracellularly in lysosomes, where it binds with anionic sites in the lysosomal matrix. Alterations of the cell surface or of the sensitive lysosomal membrane lead to lysosomal fragility and other changes that gradually become irreversible (Babich and Borenfreund, 1987). The main advantage of neutral red assay is that it detects only viable cells (Babich and Borenfreund, 1987). Neutral red is preferentially up taken into the lysosomes/endosomes of
the cells. Combination with MTT test tells us that FB1 is fairly toxic to Caco-2 cells by altering mitochondrial dehydrogenases as reported also by Minervini et al. (2004). In this context one may assume that DON, ZEN and FB1 damage cells by a mitochondrial pathway. But one may also assume that FB1 moreover does not target lysosomes in contrast to ZEN and DON.

This lysosome destabilization detected by neutral red, coupled to mitochondrial damages revealed by MTT test might be strongly involved in the apoptosis known to be induced in several cell lines by ZEN and DON (Sun et al., 2002; Maresca et al., 2002; Ouanes et al., 2003; Abd-Essef et al., 2004). Interestingly such a mechanism has been fairly demonstrated by Yuan et al. (2002) for other toxicants.

Since FB1 induces apoptosis in several cell lines our data suggest that this could be explained by a pathway involving mitochondria mainly. Seefelder et al. (2003), found that the apoptosis induced by 10 μM of FB1 is not triggered by the ceramide synthase inhibition as previously hypothesised by many groups, (Riley et al., 1998; Peska et al., 2004). Several other mechanisms independent from accumulation of sphingoids in cells exposed to FB1 have been invoked by some authors. These sphingoids-independent mechanisms are, effect of tumour necrosis factor and/or protein kinase C activation and phosphoprotein phosphatase inhibition, (Yeung et al., 1996; Fukada et al., 1996; He et al., 2001; Jones et al., 2001). It is striking to note that all these mechanisms are connected.
Fig. 3. Malondialdehyde (MDA) production induced by 24h incubation with increasing concentrations (5–40 \mu M) of toxins DON, ZEN and FB1 in Caco-2 cells. Results are expressed as the mean ± S.D. in nmoles of MDA/mg of protein measured by the Bradford method. Significantly different from the control, *P ≤ 0.05, **P ≤ 0.001.

and could be related to oxygen reactive species that are induced by FB1. Altogether these data confirm those of Mobio et al. (2000a,b) and Mobio et al. (2003) and strongly suggest that epigenetic mechanisms might prevail in FB1 cellular effects (Mobio et al., 2000a; Creppy, 2002).

All the tested toxins induce lipid peroxidation as measured by MDA production in Caco-2 cells. Above a concentration of 10 \mu M of toxins they could be classified in a decreasing order according to their oxidative potential, FB1 > DON > ZEN. It is remarkable that this classification superimposes on the hydrogen bond acceptor count, respectively 16, 6, and 5. As compared to DON and ZEN, FB1 is the most active in inducing oxygen reactive species, that at their turn induce several cytokines such as tumour necrosis factor-alpha, interleukins, nuclear factors with mitogenic activity that may underlay tumour promoter activity (Hwang et al., 2004).

All toxins tested inhibit cellular macromolecules synthesis, DON being the most effective in both protein and DNA synthesis inhibition. DON is indeed known to specifically inhibit protein synthesis by impairing peptidyl-transferase (Cannon et al., 1976; Shifrin and Anderson, 1999). Other two toxins have no specific target known in the protein synthesis machinery. Our data raise the question of whether or not and how protein and DNA synthesis inhibition are related in the case of the mycotoxins studied. The relationship between protein synthesis inhibition and DNA synthesis inhibition could be at a regulatory level since proteins are needed for DNA synthesis, replication and even degradation.

Concerning DNA synthesis inhibition the present data have revealed some relevant situations that need to be discussed. It has been shown previously that FB1 does not inhibit completely DNA synthesis even with high concentrations (Mobio et al., 2000a; Tajima et al., 2002). The present data on FB1 appear paradoxical since MTT test indicates unexpectedly high cytotoxicity while neutral red and DNA synthesis indicate low inhibition. Our hypothesis is still that FB1 bears pro mutagenic properties, i.e. in case of mutations; cells will continue to grow in the presence of FB1. This is largely supported by the findings of Gelderblom et al. (1998) and it could be also related to stress proteins transcription through mitogenic cytokins network (Roberts and Kimber, 1999; Evans et al., 2003; Hwang et al., 2004). In some aspects DON seems to have similar features in DNA synthesis in which a strong inhibition is observed with lower concentrations that is not reflected by the neutral red assay and protein
synthesis inhibition. At 5 μM of DON, DNA synthesis is already inhibited by 85–90% whereas protein synthesis is inhibited by only 40–45%. Afterwards, DNA synthesis seemed to be somehow restored at higher concentrations of DON.

Finally the comparison reveals that all the tested mycotoxins bear the capability of inducing lipid peroxidation (MDA production) and could be classified on that basis, FB1 > ZEN > DON. They target mitochondria and/or lysosomes, and they inhibit protein synthesis and DNA synthesis, DON > ZEN > FB1, by different mechanisms however. Consequently each one reduces cellular viability. Because these mycotoxins target the same cellular organelles could one hypothesise that combinations of these toxins will lead to additive or synergistic effects?

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