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REVIEW

The In Vitro Approach to the Cytotoxicity of a Trichothecene Mycotoxin Nivalenol

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Abstract

Trichothecene mycotoxins are toxic to leukocytes, and one of the leading symptoms of trichothecene toxicosis is leukopenia. In this study, therefore, to elucidate the underlying mechanism of toxicity, we treated promyelocyte (one of the leukocytes) -derived cell line HL60 with a trichothecene mycotoxin nivalenol for 24 h and investigated the toxin's effects. After treatment with 3 or 10 μg/mL nivalenol, morphologic damage was pronounced. The effect of nivalenol on cell proliferation (5-bromo-2-deoxyuridine (BrdU) incorporation) was examined, and the mean 50% inhibitory concentration was 0.16 μg/mL. At 3 and 10 μg/mL, internucleosomal DNA fragmentation, one of the hallmarks of apoptosis, was apparent. Concentrations of nivalenol-caused morphologic damage are in accordance with DNA fragmentation, indicating that nivalenol-caused morphologic change is due to apoptosis. The media of nivalenol-treated cells contained substantial amounts of interleukin (IL)-8, suggesting that IL-8 contributes to the nivalenol-induced phenomena. Conversely, nivalenol decreased monocyte chemotactic protein-1 secretion. We performed BrdU incorporation to assess the effect of 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM), which chelates intracellular calcium ion. BrdU incorporation after concomitant treatment with nivalenol and BAPTA-AM was higher than that after treatment with nivalenol alone. Likewise BAPTA-AM considerably attenuated nivalenol-induced IL-8 secretion. Taking both results together, it appears that nivalenol-caused cytotoxicity depends on intracellular calcium ion.

Discipline: Food
Additional key words: cell proliferation, HL60 cell, interleukin-8, intracellular calcium ion

Introduction

Mycotoxins are secondary metabolites of various fungi. A variety of *Fusarium* fungi produce a number of different mycotoxins of the class of trichothecenes and some other mycotoxins (zearalenone and fumonisins). It is known that there are more than 60 trichothecene mycotoxins, and one of them is nivalenol (Fig. 1). The *Fusarium* fungi are commonly found on cereals grown in the temperate regions of America, Europe and Asia. In Japan, nivalenol contamination of wheat and barley is as prevalent as that of deoxynivalenol, another trichothecene mycotoxin[13,17]. Although the acute toxicity of nivalenol is thought to be equivalent to or more potent than that of deoxynivalenol, the paucity of reports suggests that nivalenol has garnered far less interest than deoxynivalenol. In light of these circumstances, the study of nivalenol toxicity deserves more attention in Japan.

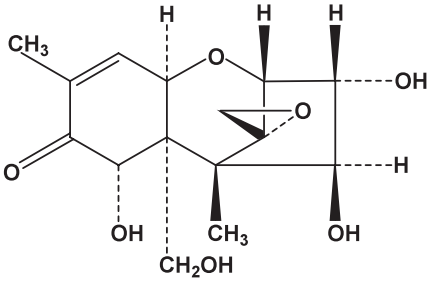

Fig. 1. Chemical structure of nivalenol

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Trichothecene mycotoxins are extremely toxic to rapidly dividing cells, including leukocytes[11], and one of the leading symptoms of trichothecene toxicosis is the leukopenia known as alimentary toxic aleukia[2]. Besides leukopenia, gastrointestinal disturbances such as vomiting and diarrhea are other major adverse effects of these toxins. The International Agency for Research on Cancer (IARC, WHO) assessed carcinogenicity and classified them as "Group 3 (Not classified as to carcinogenicity to humans)"[1]. Our previous result that nivalenol did not exert mutagenicity[9] is consistent with the conclusion of IARC[1]. In our study, to elucidate the mechanism underlying the toxicity of nivalenol, we investigated its cytotoxicity to the human promyelocyte (one of the leukocytes) -derived cell line HL60 after 24 h treatment.

Cytotoxicity

1. Morphology[8]

Change in morphology is the most fundamental adverse effect of toxins; accordingly, we performed a morphological study. At 1 µg/mL (3.2 µM) nivalenol (Fig. 2B), only slight morphologic damage was apparent, and most of the cells looked sound. In contrast, cells treated with 3 or 10 µg/mL nivalenol were clearly damaged (Fig. 2C, D), and more than half of the cells looked dead under these experimental conditions. Using morphologic damage as a criterion, the 50% cytotoxic concentration (CC_{50}) of nivalenol is between 1 and 3 µg/mL.

2. Cell Viability Test[8]

(1) 5-Bromo-2-Deoxyuridine (BrdU) Incorporation

We performed three commonly used cell viability tests, which are applied to assess cytotoxicity. Because cell proliferation is the most essential biological phenomenon of living creatures, we measured BrdU incorporation during DNA synthesis to determine the rate of cell proliferation. The mean value of 50% inhibitory concentration (IC_{50}) of nivalenol was 0.16 µg/mL (0.51 µM) (Table 1). Our result is consistent with that of Minervini et al.[7], who reported a value of 0.6 µM. In contrast, IC_{50} concentrations reported by Thuvander et al.[14] (0.24 to 0.36 µM) and Johannisson et al.[3] (approximately 0.2 µM) were lower than ours. Both of these other groups tested human lymphocytes, which might be more vulnerable to nivalenol than other cell types would be.

(2) Water-Soluble Tetrazolium (WST)-8 Assay

Results of the WST-8 assay which measures mitochondrial succinic dehydrogenase activity indicated that the mean IC_{50} of nivalenol was 0.40 µg/mL (1.28 µM) (Table 1). The effect of nivalenol on mitochondrial succinic dehydrogenase activity has been examined using the

Table 1. Cell viability of nivalenol-treated HL60 cells

	BrdU (IC_{50})	WST-8 (IC_{50})	LDH (CC_{50})
Nivalenol (µg/mL)	0.16 ± 0.03	0.40 ± 0.03	> 10

Results are presented as mean ± standard deviation of 4 (BrdU) or 3 (WST-8 and LDH) experiments.

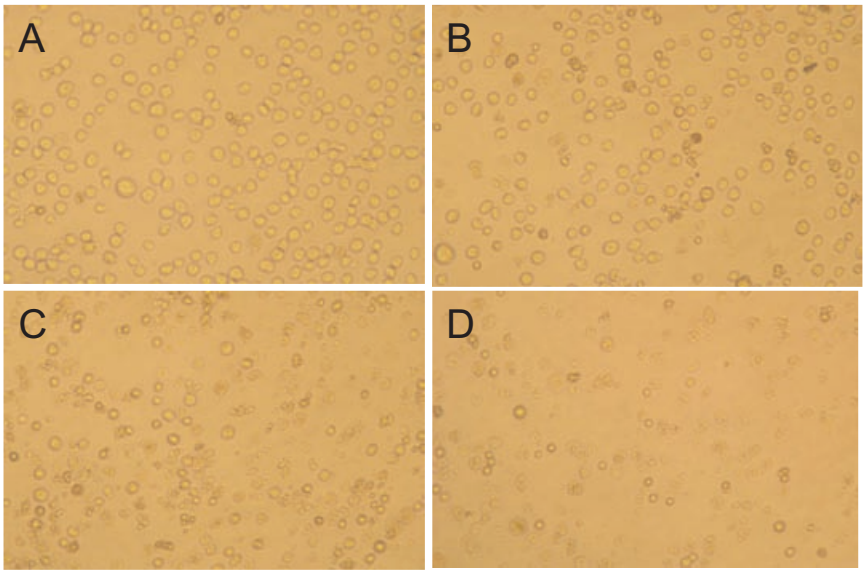

Fig. 2. Morphological study of nivalenol-treated HL60 cells
(A) Vehicle control. Cells treated with (B) 1 µg/mL, (C) 3 µg/mL or (D) 10 µg/mL nivalenol for 24 h. (Magnification, 100×).

MTT assay[7,12,16], which determines enzyme activities by the same principle as the WST-8 assay, but the results of these previous studies vary. Minervini et al. reported that the IC_{50} of nivalenol in K562 cells was 0.5 μM[7]. However, Yang et al. did not observe any effect of nivalenol on RAW 364.7 and U937 cells at concentrations of 10 μg/mL or lower[16], whereas according to Sugita-Konishi and Pestka, the IC_{50} of nivalenol in U937 cells was approximately 1 μg/mL (3.2 μM)[12]. It is likely that these discrepancies are due to differences in experimental conditions, including cell type.

(3) Lactate Dehydrogenase (LDH) activity in culture medium

LDH is leaked to the culture medium after cell death and subsequent rupture of the cell membrane. Cells in medium containing either nivalenol or 0.1% Tween 20 were cultured, and then media were subjected to LDH assay. We assumed the activities of the Tween 20-treated controls to be 100%. At 10 μg/mL nivalenol or lower, supernatant LDH activity was less than 50% that in Tween 20-treated samples (CC_{50}; Table 1) under our experimental conditions. Although HL60 cells treated with 10 μg/mL nivalenol showed profound morphologic change (Fig. 2D), presumably the cells did not burst. Minervini et al. used trypan blue exclusion, another test that is based on breakage of cell membrane[7], to monitor cell viability. They found that 80% of cells were viable even at 25.0 μg/mL nivalenol[7]—a finding that is consistent with our result regarding cytoplasmic LDH activity.

3. DNA Ladder[9]

Apoptosis is a kind of cell death. When cells are exposed to certain chemicals, they commit suicide by activating an intracellular death program. Internucleosomal DNA fragmentation (DNA ladder) is one of the most common hallmarks of apoptosis. We investigated whether nivalenol induces DNA fragmentation in HL60 cells (Fig. 3). At 3 and 10 μg/mL nivalenol, an apparent DNA ladder was observed (Fig. 3; lanes 5, 6), showing that nivalenol induces apoptosis in HL60 cells. A faint DNA ladder was observed at 1 μg/mL nivalenol (Fig. 3; lane 4). Concentrations of nivalenol-caused morphologic damage (Fig. 2; significant at 3 and 10 μg/mL, slight at 1 μg/mL nivalenol) are in accordance with DNA fragmentation (Fig. 3), indicating that marked nivalenol-caused morphologic change is due to apoptosis. Ueno et al. reported that DNA fragmentation was observed at 0.01 μg/mL nivalenol in HL60 cells[15], however, because of some differences in experimental conditions, we were not able to detect DNA fragmentation even at 0.3 μg/mL (Fig. 3; lane 3).

Cytokine Secretion

1. Interleukin (IL) -8[8]

The proinflammatory cytokines are indispensable to the development of diverse pathologic phenomena; accordingly, we investigated whether nivalenol induces IL-8, one of the proinflammatory cytokines, secretion from HL60 cells. Cells were cultured in the media containing various concentrations of nivalenol, and then the levels of IL-8 in the culture media were quantified. Nivalenol increased IL-8 secretion from HL60 cells (Table 2). The values from samples treated with 3 or 10 μg/mL nivalenol were smaller than that from cells exposed to 1 μg/mL nivalenol, probably because of damage to the cells (Fig. 2C, D). Sugita-Konishi and Pestka also found that nivalenol induced IL-8 secretion[12], consistent with the possibility that IL-8 is responsible for nivalenol-induced pathologic phenomena.

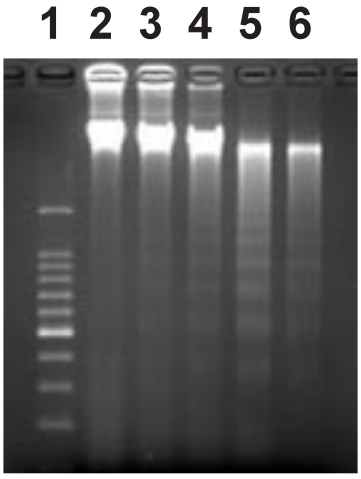

Fig. 3. Internucleosomal DNA fragmentation from nivalenol-treated HL60 cells

Lane 1 contains molecular weight marker (100 bp DNA ladder). Lane 2 corresponds to negative control (HL60 cells treated with vehicle only). Lanes 3-6 correspond to HL60 cells treated with 0.3, 1, 3, and 10 μg/mL nivalenol for 24 h, respectively.

Table 2. Nivalenol induced IL-8 secretion from HL60 cells

Nivalenol (μg/mL)	IL-8 (pg/mL)
0	33.2 ± 6.1
0.3	61.2 ± 4.4[a]
1	314.1 ± 12.9[a]
3	130.1 ± 18.5[a]
10	42.1 ± 2.7

Results are presented as mean ± standard deviation (n = 4).
a: $P < 0.05$ (Dunnett's test) versus value for vehicle-only control.

2. Monocyte Chemotactic Protein (MCP) -1

Next, we addressed MCP-1 secretion, because MCP-1 is also known as one of the proinflammatory cytokines. Cells were treated exactly the same as IL-8 quantification[8], and then the levels of MCP-1 in the culture media were quantified using the Quantikine Human CCL2/MCP-1 Immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA). Nivalenol clearly decreased MCP-1 secretion from HL60 cells (Table 3) in a dose-dependent manner. Since the values of IL-8 secretion from samples treated with nivalenol were greater than that from cells exposed to vehicle (Table 2), reduction of MCP-1 secretion is not accounted for by the damage to the cells. Kinser et al. reported that deoxynivalenol upregulated the MCP-1 mRNA in mouse spleen cells[4], however, they did not document the secretion of MCP-1.

Intracellular Calcium Ion

1. Cell Proliferation[8]

Because many extracellular signals are known to increase intracellular calcium ion concentration, we investigated the effects of the intracellular calcium ion chelator 1,2-bis(2-aminophenoxy)ethane-*N*, *N*, *N′*, *N′*-tetraacetic acid tetraacetoxymethyl ester (BAPTA-AM) on nivalenol toxicity in HL60 cells. We chose BrdU incorporation for this purpose because in our hands, this test was the most sensitive measure of cell viability (Fig. 2, Table 1). Treatment with BAPTA-AM alone slightly hindered cell proliferation (78.5% that of vehicle-treated control samples; Table 4). Cell proliferation after concomitant treatment with nivalenol and BAPTA-AM (17.1%) was greater than that after treatment with nivalenol alone (10.3%), and the difference between these treatments was statistically significant ($P < 0.05$; Table 4). That BAPTA-AM moderately impaired the cyotoxicity of nivalenol indicates that this effect is partially dependent on intracellular calcium ion.

2. IL-8 Secretion[9]

BAPTA-AM lessened nivalenol-caused retardation of cell proliferation in HL60 cells (Table 4), therefore, we investigated the chelator's effect on nivalenol-induced IL-8 secretion. While exposure to BAPTA-AM alone moderately induced IL-8 secretion, the chelator clearly reduced nivalenol-induced secretion (59.1%) (Table 5). That BAPTA-AM evidently impaired the effect of nivalenol indicates that IL-8 secretion is dependent on intracellular calcium ion. Nozawa et al.[10], Marino et al.[5] and Matsubara et al.[6] reported that BAPTA-AM decreased *Helicobactor pylori*, endozepine triakontatetraneuropeptide and histamine-induced IL-8 secretion, indicating that our case is not an exceptional phenomenon.

Conclusions

In the present study, we showed that nivalenol retards cell proliferation and induces apoptosis and corresponding morphological change and IL-8 secretion in HL60 cells. In addition, it appears that intracellular calcium ion plays a role in nivalenol-caused cytotoxicity in these cells. However, further studies are needed to elucidate the detailed mechanism of cytotoxicity evoked by nivalenol.

Table 3. Nivalenol lessened MCP-1 secretion from HL60 cells

Nivalenol (μg/mL)	MCP-1 (pg/mL)
0	112.6 ± 0.7
0.3	84.3 ± 1.8[a]
1	78.6 ± 0.6[a]
3	73.0 ± 0.3[a]
10	72.0 ± 2.1[a]

Results are presented as mean ± standard deviation (n = 4).
a: $P < 0.05$ (Dunnett's test) versus value for vehicle-only control.

Table 4. BAPTA-AM restored on nivalenol-caused retardation of HL60 cell proliferation

	Nivalenol (0.3 μg/mL)	
	–	+
None	100 ± 20.6[a]	10.3 ± 4.1[ac]
BAPTA-AM (0.5 μM)	78.5 ± 21.3[b]	17.1 ± 3.0[bc]

Results are given as mean ± standard deviation (n = 6). The value for vehicle-treated samples was defined as 100%.
a, b, c: $P < 0.05$ (Tukey's test) between values labeled with the same letter.

Table 5. BAPTA-AM attenuated nivalenol-induced IL-8 secretion in HL60 cells

	Nivalenol (1 μg/mL)	
	–	+
None	100 ± 4.1[ad]	594.5 ± 88.5[ac]
BAPTA-AM (1.5 μM)	154.4 ± 6.9[bd]	351.6 ± 29.0[bc]

Results are given as mean ± standard deviation (n = 4). The value for vehicle-treated samples was defined as 100%.
a, b, c, d: $P < 0.05$ (Tukey's test) between values labeled with the same letter.

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