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Geldanamycin, an Inhibitor of Heat Shock Protein 90, Mitigates Nivalenol-caused Changes in Cytokine Secretion in HL60 Cells

Hitoshi NAGASHIMA*, Hiroyuki NAKAGAWA and Masayo KUSHIRO
Food Safety Division, National Food Research Institute, National Agriculture and Food Research Organization (Tsukuba, Ibaraki 305−8642, Japan)

Abstract
To elucidate the molecular mechanism underlying the toxicity of nivalenol, we investigated the involvement of heat shock protein 90 (Hsp90), a molecular chaperone, in nivalenol-induced cytotoxicity in human promyelocytic leukemia HL60 cells using an Hsp90-specific inhibitor geldanamycin. Cytokine levels and cell proliferation were investigated after 24-h treatment. Nivalenol significantly elicited interleukin-8 (IL-8) secretion, conversely, geldanamycin faintly altered. IL-8 secretion in cells co-treated with these chemicals was much lower than that with nivalenol alone, indicating the importance of Hsp90 for nivalenol-induced IL-8 secretion. Both nivalenol and geldanamycin alone reduced monocyte chemotactic protein-1 (MCP-1) secretion. Regardless of geldanamycin, the values in the nivalenol-treated samples were almost the same. If Hsp90 is indifferent to the nivalenol signal transduction, the effects of these chemicals should be additive. However, statistical analysis shows that these effects are not additive, indicating that geldanamycin mitigates nivalenol's effect on MCP-1 secretion. While nivalenol markedly hindered proliferation, geldanamycin retarded it moderately. The value of cells co-treated with these chemicals was lower than that with nivalenol alone, meaning that geldanamycin does not protect against nivalenol-caused retardation of proliferation. In this study, we showed that Hsp90 is involved in nivalenol-associated changes in cytokine secretion, however, it is unclear whether Hsp90 is involved in the nivalenol-caused retardation of proliferation.

Discipline: Food
Additional key words: interleukin-8, monocyte chemotactic protein-1

Introduction

Mycotoxins are secondary metabolites of various fungi. Mycotoxin contamination of foodstuffs is a problem for many countries, particularly developing countries. A variety of *Fusarium* fungi produces a number of different mycotoxins of the class of trichothecenes and some other mycotoxins (zearalenone and fumonisins). More than 100 trichothecenes are known[5]; nivalenol (Fig. 1) is one such trichothecene. The *Fusarium* fungi are commonly found on cereals grown in the temperate regions of America, Europe, and Asia[1]. Trichothecene mycotoxins are extremely toxic to rapidly dividing cells, including leukocytes, and alimentary toxic aleukia, a type of leucopenia, is one of the leading symptoms of trichothecene toxicosis[6]. We previously reported that nivalenol hinders cell proliferation[11], induces apoptosis[12] and interleukin-8 (IL-8)[11] secretion, and decreases monocyte chemotactic protein-1 (MCP-1)[14] secretion in the human promyelocytic leukemia cell line HL60. Besides, intracellular calcium ion[11, 12], presumably released from the endoplasmic reticulum calcium store by ryano-

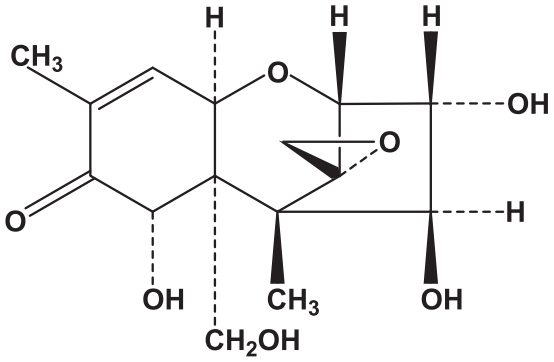

Fig. 1. Chemical structure of nivalenol

*Corresponding author: e-mail nagasima@affrc.go.jp
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dine receptor 1[13], and stress-activated mitogen-activated protein (MAP) kinases[15] occupy crucial positions in the exertion of nivalenol-associated toxicity. Furthermore, specific inhibitors of nuclear factor-κB (NF-κB) alleviate nivalenol-induced toxicity[9].

The 90-kDa heat shock protein (Hsp90), originally identified as a heat- and stress-induced protein, is an evolutionarily conserved molecular chaperone involved in the folding, stabilization, activation, and assembly of its client proteins. Numerous client signaling proteins in a wide range of biological processes have been found to be regulated by Hsp90 under physiological and pathological conditions[17]. In fact, Hsp90 was reported to be indispensable for *Clostridium botulinum* C2 toxin-induced[4] and endotoxin (lipopolysaccharide)-induced[16] toxicities, showing that it potentially also plays a pivotal role in mycotoxin-caused toxicity. In this study, therefore, to elucidate the molecular mechanism underlying the toxicity of nivalenol, we focused on Hsp90 and investigated the effects of geldanamycin, an Hsp90-specific inhibitor[20], on nivalenol-induced cytotoxicity in HL60 cells.

Materials and methods

1. Chemicals and cells

Nivalenol was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). Geldanamycin[2] was purchased from Merck KGaA (Darmstadt, Germany) and dissolved in DMSO. Cell proliferation ELISA, BrdU (Colorimetric) was purchased from Roche Diagnostics GmbH (Penzberg, Germany). The human promyelocytic leukemia cell line HL 60 was purchased from RIKEN Cell Bank (Tsukuba, Japan) and cultured in RPMI (Roswell Park Memorial Institute) 1640 medium (Invitrogen Corp., Carlsbad, CA USA) containing 10% fetal calf serum (JRH Biosciences Inc., Lenexa, KS, USA).

2. Determination of cytokine levels

HL60 cells (1×10^5 cells) in 0.5 mL of RPMI 1640 medium containing chemical(s) were cultured in each well of a 24-well culture plate for 24 h, whereupon the media were collected. To achieve the maximum IL-8 concentration, HL60 cells were treated with 1 μg/mL (3.2 μmol/L) of nivalenol, because IL-8 secretion peaked at this concentration in HL60 cells[11]. The morphology of the cells[11] and the extent of DNA laddering[12] suggested that the cells were still viable in 1 μg/mL of nivalenol. The collected media were centrifuged at 5,000 × g for 5 min to obtain the supernatants and remove cells and cell debris. Further, the supernatants were analyzed to determine the levels of IL-8 and MCP-1 using Quantikine Human IL-8 Immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA) and Quantikine Human CCL2/MCP-1 Immunoassay kit (R&D Systems, Inc.), respectively, according to the manufacturer's recommended procedures. The determination of each cytokine level was performed three times.

3. Cell proliferation

Cell proliferation was investigated by measuring 5-bromo-2-deoxyuridine (BrdU) incorporation during DNA synthesis as described by a previous study[8]. HL60 cells (1.5×10^4 cells) in 100 μL of RPMI 1640 medium containing chemical(s) were placed in each well of a 96-well microtiter plate for BrdU incorporation, and cell proliferation was assessed after 24 h of culture. Cell proliferation was examined five times.

4. Statistics

Data were expressed in terms of mean ± standard deviation. Intergroup differences were analyzed by Tukey's test. The interaction was evaluated using two-way analysis of variance (ANOVA) with replication. If there was significant interaction, we judged that the effects of two chemicals are not additive. A P value of less than 0.05 was considered statistically significant.

Results and discussion

To our knowledge, no studies report the role of Hsp90 in nivalenol toxicity. Here, therefore, we examined the importance of Hsp90 in the exertion of nivalenol toxicity in HL60 cells.

We investigated the effects of the Hsp90-specific inhibitor geldanamycin[20] on cytokine secretion. We chose the lowest effective concentration of geldanamycin - 20 nmol/L - as the final concentration. This working concentration was determined in preliminary experiments. Nivalenol significantly elicited IL-8 secretion in HL60 cells (283.1% of the value in the vehicle-treated samples), conversely, geldanamycin alone faintly altered IL-8 secretion (93.5%; Table 1). The secretion of IL-8 in cells concomitantly treated with nivalenol and geldanamycin was much lower (195.5%) than IL-8 secretion in cells treated with nivalenol alone (283.1%; Table 1). ANOVA with replication shows a statistically significant ($P < 0.05$) interaction between the effects of nivalenol and geldanamycin, meaning that Hsp90 is likely to be important for nivalenol-induced IL-8 secretion in HL60 cells. Almost equivalent results were obtained by 50 nmol/L geldanamycin experiments (unpublished data). Malhotra et al.[7], Teruya et al.[19], and Yeo et

Table 1. Effect of geldanamycin on nivalenol-induced interleukin-8 secretion

	Nivalenol (1 μg/mL)			
	–		+	
None	100	± 9.7*	283.1	± 24.4*, ‡
Geldanamycin (20 nmol/L)	93.5	± 7.0†	195.5	± 9.1†, ‡

HL60 cells were treated with the chemicals, as indicated, for 24 h. The results are expressed in terms of mean ± standard deviation (n = 4). Interleukin-8 (IL-8) secretion in the vehicle-treated samples is defined as 100% (82.4 pg/mL). Intergroup differences were analyzed by Tukey's test. Intergroup differences between values labeled with the same superscript symbols (*,†, and‡) are statistically significant ($P < 0.05$).

Table 2. Effect of geldanamycin on nivalenol-induced decrease in monocyte chemotactic protein-1 secretion

	Nivalenol (1 μg/mL)			
	–		+	
None	100	± 2.1*, ‡	69.9	± 0.8*
Geldanamycin (20 nmol/L)	76.1	± 0.9†, ‡	70.2	± 1.8†

HL60 cells were treated with the chemicals, as indicated, for 24 h. The results are expressed in terms of mean ± standard deviation (n = 4). Monocyte chemotactic protein-1 (MCP-1) secretion in the vehicle-treated samples is defined as 100% (191.1 pg/mL). Intergroup differences were analyzed by Tukey's test. Intergroup differences between values labeled with the same superscript symbols (*, †, and‡) are statistically significant ($P < 0.05$).

Table 3. Effect of geldanamycin on cell proliferation

	Nivalenol (0.3 μg/mL)			
	–		+	
None	100	± 12.8*, ‡	14.9	± 3.5*
Geldanamycin (20 nmol/L)	83.7	± 9.0†, ‡	8.6	± 2.9†

HL60 cells were treated with the chemicals, as indicated, for 24 h. The results are expressed in terms of mean ± standard deviation (n = 6). Cell proliferation in the vehicle-treated samples is defined as 100%. Intergroup differences were analyzed by Tukey's test. Intergroup differences between values labeled with the same superscript symbols (*, †, and‡) are statistically significant ($P < 0.05$).

al.[22] reported that geldanamycin abated tumor necrosis factor-α[7], *Legionella pneumophila*[19], and *Helicobacter pylori*[22] -induced IL-8 secretion, respectively. In these reports, the authors indicated that the inhibition of IL-8 secretion was achieved via Hsp90-mediated NF-κB inactivation[7, 19, 22]. Since NF-κB specific inhibitors decrease nivalenol-induced IL-8 secretion[9], geldanamycin may exert its effect through NF-κB inactivation in our experiments.

Nivalenol reduced MCP-1 secretion (69.9% of the value in the vehicle-treated samples; Table 2), which was reported in our previous study[14]. Similarly, geldanamycin reduced MCP-1 secretion (76.1%; Table 2). We found that, regardless of the presence of geldanamycin, the values in nivalenol-treated samples were almost the same (69.9% versus 70.2%; Table 2). Provided that Hsp 90 is indifferent to the nivalenol signal transduction pathway(s), the effects of nivalenol and geldanamycin are expected to be additive. However, ANOVA with replication shows a statistically significant ($P < 0.05$) interaction between the effects of nivalenol and geldanamycin, meaning that the effects of these chemicals on MCP-1 secretion are not additive, and geldanamycin mitigates the effect of nivalenol on HL60 cells. Again, almost equivalent results were obtained by 50 nmol/L geldanamycin experiments (unpublished data). To our knowledge, the relationship between Hsp90 and MCP-1 secretion has not been previously reported, hence, this is the first study that reports this association.

Next, the effect of geldanamycin on cell proliferation was documented. In our hands, cell proliferation was the most sensitive measure of cell viability[11]. The 50% inhibitory concentration of nivalenol was about 0.16 μg/mL (0.51 μmol/L) in HL60 cells[11]; therefore, we performed our experiments with 0.3 μg/mL (0.96 μmol/L) of nivalenol. As we reported previously[11], nivalenol alone markedly hindered cell proliferation (14.9% of vehicle-treated control value; Table 3). On the other hand, treatment with geldanamycin alone led to a moderate deceleration of cell proliferation (83.7%; Table 3). Because geldanamycin was originally isolated in the process of screening for substances inhibiting the proliferation of protozoa[2], there are numerous studies concerning the inhibition of proliferation, including mammalian cultured cells[3, 18, 21]. Our result shows for the first time that proliferation of HL60 cell is also impeded by geldanamycin. The value of cells concomitantly treated with nivalenol and geldanamycin was lower (8.6%) than that of cells treated with nivalenol alone (14.9%; Table 3). With regard to cell proliferation, since no protective effect of geldanamycin was observed, it is unclear whether Hsp90 is concerned with the nivalenol-caused retardation of cell proliferation. Nivalenol-caused retar-

dation of cell proliferation was antagonized by an NF-κB inhibitor[9]. As we mentioned above, since geldanamycin impairs NF-κB activity[7, 19, 22], geldanamycin was anticipated to alleviate nivalenol-caused retardation of cell proliferation, but did not (Table 3). Other than NF-κB, geldanamycin down-regulates cell proliferation-promoting cellular factors such as c-Myc[21], c-Src[10, 20], and c-Raf-1[18] mediated by Hsp90 inactivation. Our results indicate that with respect to the nivalenol-caused retardation of cell proliferation, the effects of inactivation of these crucial factors would be more potent than the protective effect of NF-κB inhibition in HL60 cells.

We showed that Hsp90 plays a role in nivalenol-associated changes in cytokine secretion in HL60 cells. Because copious client proteins are known to be regulated by Hsp90[17], further studies are required to identify which Hsp90 client protein(s) contribute to nivalenol-caused changes in cytokine secretion in order to elucidate the detailed mechanism of nivalenol-induced cytotoxicity in HL60 cells.

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