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Research Article

The *Fusarium* toxin enniatin B exerts no genotoxic activity, but pronounced cytotoxicity *in vitro*

Claudia Behm, Gisela H. Degen and Wolfram Föllmann

IfADo, Leibniz-Institut für Arbeitsforschung an der TU Dortmund, Dortmund, Germany

Enniatin B, a fungal metabolite produced by various *Fusarium* strains, is a frequent contaminant in cereals used for human foods and animal feeds, but, so far very limited data are available on its toxicity. The aim of this study was to investigate the effects of enniatin B in a battery of short-term tests to evaluate its genotoxic potential. In *Salmonella typhimurium* assays (Ames assay) with the strains TA 98, TA 100, TA 102, and TA 104, both in the presence and absence of an external metabolizing enzyme system (rat liver S9), no mutagenicity was detected up to toxic levels (100 μM) of enniatin B. Likewise, mutagenicity tests in mammalian cells, *i.e.*, the hypoxanthin-guanin-phosphoribosyl-transferase (HPRT) assay with V79 cells performed with and without S9 mix, did not reveal a significant increase in mutant frequency for enniatin B up to 30 μM, a cytotoxic concentration. Additional tests on other types of genotoxicity, *i.e.*, clastogenicity and chromosomal damage, were conducted in V79 cells, applying the alkaline single cell gel electrophoresis (Comet assay with and without FPG, formamidopyrimidine DNA glycosylase, enzyme) and the micronucleus assay. None of these assays revealed a significant genotoxic potential of enniatin B. However, enniatin B exerts pronounced cytotoxic effects in V79 cells as determined by neutral red uptake assay for 48 h exposure: The IC_{20} and IC_{50} values of 1.5 and 4 μM, are higher than those of the more potent *Fusarium* toxin deoxynivalenol (IC_{20} 0.6 μM, IC_{50} of 0.8 μM), but in a similar range as values reported for cytotoxicity of enniatin B in various tumor cell lines. In summary, despite an apparent lack of genotoxic activity, enniatin B can exert biological activity at low micromolar concentrations in mammalian cells.

Keywords: Ames assay / Comet assay / HPRT assay / Mutagenicity testing / Micronucleus assay

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1 Introduction

Fusarium species are prevalent toxin-producing fungi of the northern temperate region [1, 2]. The well known *Fusarium* mycotoxins, *i.e.*, fumonisins, trichothecenes, and zearalenone, can cause a variety of toxic effects [3]. In contrast, other *Fusarium* metabolites such as fusaproliferin, beauvericin, enniatins, and moniliformin have not been well characterized in this respect, although these mycotoxins are now recognized as frequent contaminants of grains [4]. Enniatins, a group of cyclic hexadepsipeptides, have been detected at levels up to several mg/kg in grains from various countries [5 – 8]. Whether an uptake of contaminated grain based products results in adverse effects on human and animal health is as yet unclear, mainly due to very limited data on their toxicity *in vivo* and *in vitro*. But, a potential risk from exposure to enniatins has to be assumed in light of some published data, reviewed recently by Jestoi [4]. Moreover, studies with tritiated enniatin B in rats have shown that this mycotoxin is absorbed upon oral or pulmonary application [9]. Although the bioavailability of enniatins is apparently low upon oral administration, they are stable in the gastrointestinal tract; thus, in addition to systemic also local effects in the GI-tract have to be considered.

Enniatins possess interesting biological activities: They show ionophoric properties, and are known to exert antimicrobial, anthelmintic, and phytotoxic activity as well as cytotoxic effects [4, 10, 11]. The latter effect may be related to their ionophoric properties and their ability to induce

Correspondence: Dr. Wolfram Föllmann, IfADo, Leibniz-Institut für Arbeitsforschung an der TU Dortmund, Ardeystr. 67, 44139 Dortmund, Germany
E-mail: foellmann@ifado.de
Fax: +49-231-1084-456

Abbreviations: DON, deoxynivalenol; **HPRT,** hypoxanthin-guanin-phosphoribosyl-transferase; **MMS,** methylmethane sulfonate; **MN,** micronuclei; **NR,** neutral red

apoptosis in a number of different cell types. Enniatins exert potent cytotoxic activity against cancer cell lines [12, 13], and can act also as potent inhibitors of an ABC membrane transporter related to multidrug-resistance [14]. The latter properties make them interesting candidates for further development as anticancer drug.

Both, the possible negative impact as mycotoxin contaminant as well as the interesting pharmacological properties of enniatins call for a better characterization of their toxic potential. However, at present there is no published information on their genotoxicity. Yet, such data are an essential element in a hazard characterization of any chemical. The aim of this study was therefore to investigate the genotoxic potential of enniatin B in a battery of short-term tests: The effects of enniatin B were studied in well established bacterial and mammalian assays that detect mutagenicity, and further tests with V79 cells that detect other types of DNA damage, such as clastogenicity and chromosomal genotoxicity. To choose suitable test concentrations, we also characterized the cytotoxicity of enniatin B in V79 cells and compared it to that of another *Fusarium* mycotoxin, namely deoxynivalenol (DON).

2 Materials and methods

2.1 Materials

Enniatin B was obtained from Axxora Deutschland GmbH (Grünberg, Germany). All other chemicals were purchased in analytical grade from Sigma – Aldrich (Germany), and sterile plastic materials were from Greiner (Frickenhausen) or Corning Costar (Bodenheim, Germany). Cell culture media and supplements were purchased from Seromed (Berlin), and Invitrogen (Karlsruhe, Germany). Ethidium bromide and Hoechst dye 33342 were purchased from Serva (Heidelberg, Germany) and Invitrogen.

2.2 Mutagenicity assay with bacteria (*Salmonella typhimurium*)

The bacterial mutagenicity assay (Ames assay) was performed according to the method of Maron and Ames [15] with *S. typhimurium* strains TA, 98, 100, 102, and 104 (kindly provided by Dr. B.N. Ames of the Department of Biochemistry, University of California, Berkeley, California, USA).

Mutagenicity tests were performed using a preincubation procedure in the presence or in the absence of S9 mix as an external enzymatic metabolizing system as in previous studies with ochratoxin A [16]. Bacteria were preincubated for 20 min at 37°C with the test substances. The test samples consisted of 100 µL bacteria suspension (~10^8 KBE/mL), 100 µL test solution and 500 µL S9 mix or 500 µL phosphate buffer in experiments without external metabolizing system. The test substances were enniatin B in the stated concentrations, sodium azide (NaN_3, 440 µM), 2-nitrofluorene (2-NF, 340 µM), methylglyoxal (MG, 1 µM), 9-aminoacridine (9-ACC, 310 µM) and mitomycin C (MC, 2.1 µM) as positive controls in experiments without S9 mix and 2-aminoanthracene (2-AAN, 37 µM) as positive control in the presence of S9 mix. All substances were dissolved in DMSO and subsequently diluted with phosphate buffer. Controls without test substances contained only phosphate buffer or solvent (DMSO) and S9 mix in experiments in the presence of an external metabolizing enzyme system.

After the preincubation period, 2 mL top agar (0.5% bacto agar in 0.5% sodium chloride in aqua bidest with 0.05 mM biotin/histidine solution) was added and the solution was transferred to a Petri dish (100 mm in diameter) with minimal agar (1.5% bacto agar with 5% glucose and 4% Vogel-Bonner-Minimal-Medium in aqua bidest) and then cultured for 2 days at 37°C. Colony numbers of revertants (spontaneous and induced) were counted with a Biocount 1000 colony counter from Biosys (Karben, Germany).

2.3 Cell culture

V79 cells (purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) were cultured with RPMI 1640 medium supplemented with 10% fetal calf serum, in a humidified incubator at 37°C and 5% CO_2 atmosphere. Cells from stock were seeded in 75 cm^2 flasks in medium with 100 units/mL penicillin and 100 µg/mL streptomycin that was changed twice weekly until cultures reached confluency. For subsequent tests usually cells of passages 20 – 34 were used.

2.4 Cytotoxicity assay

Cytotoxicity was assessed by means of the neutral red (NR) uptake assay, and evaluated as described previously [17, 18]. In brief, 5000 V79 cells were seeded in 200 µL RPMI culture medium per well in 96-well culture plates. After 24 h culture medium was withdrawn, and the cells were treated for 24 and 48 h with different concentrations of the test compounds in medium with 5% FCS. DMSO served as a solvent control (0.1%) for enniatin B and ethanol (0.1%) for DON. After that time the medium was withdrawn and substituted by culture medium with neutral red (NR 0.05 mg/mL, preincubated at 5% CO_2 and 37°C for 17 h). After another incubation period of 3 h the medium was withdrawn, and the cells were washed five times with PBS and fixed with 0.2 mL fixative (glacial acetic acid/water/ethanol, 1:49:50) per well which brings the dye NR into solution upon 20 min of shaking (600 rpm) on a plate shaker (MTS2, IKA® Labortechnik, Staufen, Germany). Then absorbance of NR was measured with a plate photometer (340 ATC, SLT, Salzburg, Austria) at 540 nm. NR uptake was expressed in percent relative to the solvent control.

2.5 HPRT assay

The HPRT assay was performed with V79 cells (lung fibroblasts from male Chinese hamster) according to established methods [19, 16]. The cells were incubated for 4 h in serum-free culture medium (RPMI 1640 medium with 100 U/mL penicillin and 100 μg/mL streptomycin) and the stated enniatin B concentrations, either in the presence or in the absence of an external metabolizing enzyme system (S9 mix from rat liver). Prior to and after the treatment period the cells were kept in culture medium supplemented with 10% fetal calf serum. Ethylmethane sulfonate (10 μM EMS, for incubations without metabolic activation) and 7,12-dimethyl benz[*a*]anthracene (60 μM DMBA, for incubations with metabolic activation) served as positive controls.

2.6 Alkaline single cell gel electrophoresis (Comet) assay

The Comet assay was performed based on standard methods [20] with modifications described earlier [21, 22]. Briefly, after enniatin B treatment for 3 or 18 h the cells were washed twice with cold PBS and harvested by trypsin/EDTA treatment. Methylmethane sulfonate (MMS) served as positive control. Suspensions of 1×10^5 V79 cells in 50 μL PBS were added to 500 μL low melting point agarose (0.5% in PBS) and spread onto two agarose-covered microscope slides. When the comet assay was combined with bacterial FPG protein, slides were washed three times (for 5 min each) in enzyme buffer (100 mM NaCl, 20 mM Tris, 1 mM Na_2-EDTA, 0.2 mg/mL bovine serum albumin; pH 7.5) and covered with 200 μL of either buffer or FPG protein in buffer, sealed with a coverslip and incubated for 30 min at 37°C. Slides with and without FPG-post-treatment were processed as follows: After solidification the microgels were placed in 4°C cold lysis buffer (2.5 M NaCl, 0.1 M Na_2-EDTA, 10 mM Tris, 1% v/v *N*-lauroylsarcosine, pH 10; 10% v/v DMSO and 1% w/v Triton X-100 were added 1 h before use). Cells were lysed for a minimum of 2 h at 4°C in the dark. Before placing the microgels in the electrophoresis chamber, they were dipped into distilled water. Alkaline treatment in electrophoresis buffer (300 mM NaOH, 1 mM Na_2-EDTA, pH > 13.5) was carried out for 30 min at 4°C, and then electrophoresis was run for 30 min at 4°C (300 mA, 25 V). After neutralization (0.4 M Tris, pH 7.5) for 15 min and dehydration in ethanol (50, 75, and 100%), the DNA was stained with 50 μL ethidium bromide (20 μg/mL) for immediate analysis. A total of 100 cells from two slides for each condition were analyzed by image analysis using Comet Assay II software (Perceptive Instruments, UK). Observations were made at 400-fold magnification using an epifluorescent microscope DMRB (Leica, Wetzlar, Germany) equipped with an excitation filter of 515 – 535 nm, a 50 W mercury lamp, a barrier filter at 590 nm and a video camera (PE-2020P, Pulnix, Alzenau, Germany). Tail intensity (% DNA in the comet tail) was the parameter used in this study. Median values were calculated for each concentration in each set of experiments.

2.7 *In vitro* micronucleus assay

The micronucleus assay with V79 cells was conducted as described previously [17, 18, 23], with minor adaptations. Initially, 5×10^5 cells were seeded per 25 cm^2 flask in RPMI medium with supplements (see above). On the next day, the culture medium was changed to remove nonattached cells. After 2 days in culture the medium was removed and replaced by fresh culture medium; test compound (enniatin B dissolved in DMSO) or reference chemicals acting as aneugen (VCR = vincristin 10 nM) or as clastogen (MMS, 130 and 220 μM) were added from DMSO stock solutions. Controls were treated with solvent alone (DMSO 0.1% v/v).

After an incubation period of about 1.5 cell cycles (18 h for V79 cells), to allow expression of micronuclei, the medium was removed, and cells were detached by disaggregation with trypsin/EDTA (0.05% trypsin/0.02% EDTA). Cells were then subjected to hypotonic conditions with 0.4% KCl. After centrifugation ($130 \times g$, 10 min) the cell pellets were resuspended in 5 mL ice-cold methanol/acetic acid (3:1) as fixative for 15 min at RT. This fixation step was done three times, and the cell pellet resuspended in 0.5 mL fixative. Some drops of the suspension were transferred onto glass slides, and cells were fixed by heat treatment. The slides were stained with acridine orange solution (0.05 mg/mL) and examined using a DM LB fluorescence microscope (Leica, excitation filter 440 – 490 nm, barrier filter I3) at 400 × magnification, For each concentration, at least 2000 cells with well preserved cytoplasm were examined and analyzed for micronuclei (MN). Scoring for MN followed published criteria [refs. in 17, 18]: Only cells with intact cytoplasm, where the micronucleus size was maximally 1/3 of the main nucleus and showed a similar color and shape, were counted. The number of MN scored in at least 2000 cells per group was recorded, and calculated as micronucleus rate (number of MN per 1000 cells) for each condition. The effect was assessed in two independent experiments.

2.8 Staining of cells for detection of apoptosis

To investigate nuclear fragmentation as a feature of apoptotic cell death, cells were grown on glass slides for 24 h and then incubated with the solvent control DMSO or alternatively with 1 μM Enniatin B for another 24 h. Then, without medium change, nuclei were stained according to Michels *et al*. [24] with 100 μM Hoechst dye 33342 for 15 min and sealed with a glass coverslip. Cells with fragmented nuclei were counted and photographed at 400-fold

Table 1. *Salmonella* mutagenicity (Ames) assay with Enniatin B in different *S. typhimurium* strains in the presence or absence of an external metabolizing enzyme system (rat liver S9-mix)

Strain	TA 98		TA 100		TA 102		TA 104	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Number of revertants [mean ± SD]								
Buffer control	46 ± 4	50 ± 5	198 ± 5	193 ± 4	362 ± 6	455 ± 5	440 ± 4	455 ± 5
Solvent control (DMSO)	45 ± 2	51 ± 2	166 ± 3	156 ± 5	327 ± 6	432 ± 7	426 ± 6	439 ± 4
100 nM Enniatin B	43 ± 5	50 ± 7	164 ± 6	157 ± 2	365 ± 4	439 ± 8	451 ± 7	449 ± 9
1 μM Enniatin B	45 ± 4	45 ± 3	146 ± 9	141 ± 5	345 ± 3	422 ± 9	464 ± 13	435 ± 7
10 μM Enniatin B	40 ± 4	42 ± 2	134 ± 6	140 ± 4	338 ± 7	423 ± 8	478 ± 3	435 ± 4
30 μM Enniatin B	42 ± 3	37 ± 7	138 ± 5	135 ± 9	320 ± 16	400 ± 4	473 ± 3	425 ± 8
100 μM Enniatin B	34 ± 3	26 ± 1	101 ± 7	78 ± 22	242 ± 7	295 ± 20	474 ± 8	376 ± 13
Positive controls								
2-NF	1994 ± 26	–	–	–	–	–	–	
NaN_3	–	–	2403 ± 14	–	–	–	–	–
MC	–	–	–	–	1413 ± 31	–	–	–
MG	–	–	–	–	–	–	2196 ± 54	
2-AAN	–	2141 ± 49	–	2470 ± 61	–	1241 ± 42	–	1341 ± 40

DMSO, dimethylsulfoxide (3.7%); positive controls and final concentrations in brackets: 2-NF, 2-nitrofluorene (340 μM); NaN_3, sodium azide (440 μM); MC, mitomycin C (2.1 μM); MG, methylglyoxal (1 μM); 2-AAN, 2-aminoanthracene (37 μM).

magnification using a DMLB fluorescence microscope (Leica, excitation: 365 nm, beamsplitter: 395 nm, emission: 420 nm barrier).

3 Results

3.1 Assessment of mutagenicity in the Ames assay

In the Ames assay four different *S. typhimurium* strains were used: Strain TA 98 detects frame-shift mutations whereas TA 100 detects base-pair substitutions. The strains TA 102 and TA 104 detect mutagenicity induced by oxidative mutagens and cross-linking agents. In all four strains enniatin B did not induce an increase in mutation rates (Table 1), neither in the absence or the presence of an external metabolizing enzyme system (S9 mix from rat liver). When *S. typhimurium* were treated with 100 μM enniatin B, lower density of the bacterial background lawn revealed toxicity which caused also a decrease in revertant rates. The spontaneous mutation rates for each strain were in the range of historical controls, and the respective positive controls indicated a successful performance of the assay.

3.2 Cytotoxicity of enniatin B

The cytotoxic effects of enniatin B and deoxynivalenol (DON) for comparison were measured with the neutral red uptake assay in V79 cells treated for 24 h and for 48 h. Figure 1 depicts a typical result: A decrease of neutral red uptake was observed in cells treated for 48 h with enniatin B levels above 1 μM whilst concentrations ≤1 μM were not cytotoxic compared to solvent controls. DON was more cytotoxic in this test condition: Concentrations above 0.3 μM decreased neutral red uptake by 20%. Levels resulting in an inhibition of 20, 50, and 80% (IC_{20}, IC_{50}, and IC_{80}) of neutral red uptake were deduced from concentration-effect curves of multiple tests. For enniatin B an IC_{20} of 1.5 μM and an IC_{50} of 4 μM were determined (Table 2). For DON the IC_{20} and IC_{50} values were about 0.6 and 0.8 μM for the 48 h treatment. As expected, cytotoxicity of the mycotoxins is less pronounced with shorter treatment periods (24 h).

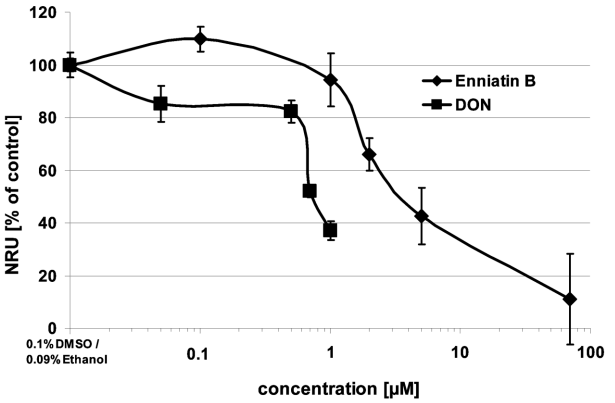

Figure 1. Cytotoxicity of Enniatin B and deoxynivalenol (DON) in V79 cells determined after 48 h incubation with the neutral red uptake assay. Neutral red uptake is given as percent of uptake into untreated cells (solvent control).

Table 2. Cytotoxicity of enniatin B and deoxynivalenol in V79 cells determined with the neutral red uptake assay. IC-values are given as median of at least 3 – 6 independent experiments

	Inhibitory concentration [μM]		
	IC_{20}	IC_{50}	IC_{80}
24 h treatment			
Enniatin B	2.1	36.0	70.5
Deoxynivalenol	0.6	1.1	18.0
48 h treatment			
Enniatin B	1.5	4.0	54.0
Deoxynivalenol	0.6	0.8	2.5

Table 3. HPRT assay with Enniatin B in V79 cells in the presence or absence of an external metabolizing enzyme system (rat liver S9-mix)

	– S9	+S9
Buffer control	29 ± 18	23 ± 17
Solvent control (DMSO)	20 ± 13	15 ± 11
0.1 μM Enniatin B	18 ± 2	n.d.
0.3 μM Enniatin B	18 ± 13	42 ± 25
0.5 μM Enniatin B	32 ± 9	n.d.
1 μM Enniatin B	29 ± 32	30 ± 18
3 μM Enniatin B	30 ± 32	17 ± 1
10 μM Enniatin B	29 ± 13	29 ± 18
20 μM Enniatin B	39 ± 66	32 ± 15
EMS	1278 ± 202	–
DMBA	–	133 ± 62

DMSO, dimethylsulfoxide (0.1%), positive controls and final concentrations in brackets: EMS, ethylmethane sulfonate (10 M), DMBA, 7,12-dimethyl benz[*a*]anthracene (60 μM), n.d., not determined.

3.3 Assessment of mutagenicity in the HPRT assay

The HPRT assay is a widely used test to study mutagenicity in mammalian cells and allows detection of point mutations and small deletions. Enniatin B was tested over a wide range of concentrations, up to levels that are cytotoxic for V79 cells. In contrast to the positive controls (DMBA and EMS), the frequency of mutations was not significantly increased by treatment with enniatin B in tests with and without addition of S9 mix (Table 3).

3.4 DNA damage assessed in the Comet assay

The induction of DNA strand breaks was determined in V79 cells treated for 3 h and for 18 h with graded concentrations of enniatin B. The Comet assay was performed in the standard version at pH > 13 which detects alkali labile sites (DNA strand breaks) and with addition of formamido pyrimidine glycosylase (FPG) protein, an endonuclease which enables detection of oxidative DNA base lesions and of AP sites induced by alkylating agents such as MMS [21]. An increase in tail intensity was chosen as parameter for DNA damage, and detected in cells treated with the positive control agent (MMS), both in the standard Comet assay and the FPG-modified version. Cells exposed to enniatin B (0.1 up to 100 μM) did not show similar increases in DNA damage (Table 4). Although some values of treated samples were above the solvent control values, no consistent concentration-dependent increase in tail intensity was observed.

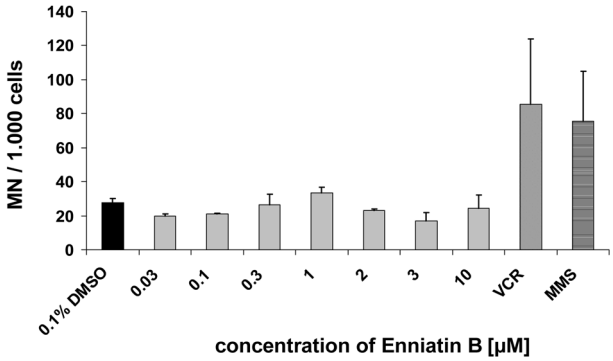

Figure 2. Determination of micronuclei (MN) in V79 cells. Cells were treated for 18 h with enniatin B (0.03 – 10 μM) or solvent and positive control agents (MMS = methylmethane sulfonate, VCR = vincristin). The mean value ± SD of two independent experiments is shown where at least 2000 cells per group were scored for micronuclei.

3.5 Induction of micronuclei

The micronucleus (MN) assay is now widely used to detect chromosomal damage induced by agents with clastogenic or aneugenic activity [25]. Cells treated with positive control compounds (MMS and VCR) for 18 h (about 1.5 cell cycles for V79 cells to allow expression of micronuclei), showed a clear increase in micronuclei frequency, whilst treatment with enniatin B up to cytotoxic levels (10 μM) did not result in MN rates different from those of the solvent control (Fig. 2).

3.6 Detection of nuclear fragmentation

Upon microscopic analysis of V79 cells only few nuclei with signs for nuclear fragmentation were detected in untreated cultures stained with Hoechst 33342 dye (Fig. 3a control) whilst nuclei showing clear nuclear fragmentation were frequently observed in cells treated with 1 μM enniatin B for 24 h: Figure 3b depicts a cluster of cells with a higher portion of apoptotic cells, typical but not representative for the entire sample evaluated in more microscopic fields. Overall, about 10% of all cells showed nuclear fragmentation under the given exposure to enniatin B.

Table 4. Analysis of DNA damage by enniatin B in V79 cells with the Comet assay conducted in the presence or absence of fpg enzyme

Tail intensity	3 h Treatment		18 h Treatment	
	−fpg	+fpg	−fpg	+fpg
Experiment 1				
Medium control	1.4	6.8	1.9	12.4
Solvent control (DMSO)	1.8	5.2	2.1	20.2
50 μM MMS	3.3	41.4	3.8	46.0
0.1 μM Enniatin B	2.1	3.7	5.3	10.5
0.3 μM Enniatin B	2.7	6.5	2.6	12.5
1 μM Enniatin B	3.9	6.3	2.9	9.8
3 μM Enniatin B	4.1	10.0	2.3	6.1
5 μM Enniatin B	2.9	7.0	4.8	6.7
10 μM Enniatin B	10.3	6.5	2.4	10.4
30 μM Enniatin B	2.8	3.9	2.1	6.5
50 μM Enniatin B	n.d.	n.d.	2.9	5.4
100 μM Enniatin B	n.d.	n.d.	2.2	6.9
Experiment 2				
Solvent control (DMSO)	1.7	19.2	2.9	7.0
50 μM MMS	4.4	49.8	15.3	33.4
0.1 μM Enniatin B	1.0	6.6	2.4	9.1
0.3 μM Enniatin B	1.6	11.5	2.2	6.5
1 μM Enniatin B	1.3	9.3	1.9	5.1
3 μM Enniatin B	1.4	4.9	2.0	4.3
5 μM Enniatin B	1.1	6.8	8.1	5.3
10 μM Enniatin B	1.1	5.1	4.3	7.9
30 μM Enniatin B	1.4	6.5	3.9	3.7
50 μM Enniatin B	2.0	5.5	n.d.	n.d.
100 μM Enniatin B	2.9	11.8	n.d.	n.d.

DMSO, dimethylsulfoxide (0.1%); MMS, methylmethane sulfonate; n.d., not determined.

4 Discussion

Enniatins belong to a group of emerging *Fusarium* mycotoxins [4], recently described as frequent contaminants in grain samples used for human food and animal feed in Italy and in Scandinavian countries [5–8]. By means of a multi-mycotoxin method [26] enniatin B was now also detected in wheat samples from Northrhine-Westphalia in Germany, in levels similar to those of DON (Dehne K.H., Spiteller M. *et al*., unpublished data). But, in contrast to DON and some other mycotoxins, there is only very limited data on the toxicity of enniatin B and related cyclic hexadepsipeptides [4] despite their interesting biological properties [10–12; and Section 1].

To the best of our knowledge this is the first investigation on the genotoxic/mutagenic potential of enniatins. We studied enniatin B with a battery of short-term *in vitro* assays using bacteria and mammalian cells that cover all relevant endpoints for mutagenicity and other genotoxic effects [25, 27]. Our results with two mutagenicity assays (Ames test, Table 1 and HPRT test in V79 hamster lung fibroblasts, Table 3) argue clearly against such a potential of enniatin B or metabolites generated by rat liver enzymes. With regard to chromosomal genotoxicity, enniatin B failed to induce an increase in micronuclei (Fig. 2), and thus lacks clastogenic and aneugenic properties. Also, the standard alkaline Comet assay and the FPG-modified Comet assay (Table 4) revealed no genotoxic activity for enniatin B. Whilst a few values in treated samples were above those of solvent controls, there was no consistent concentration-related increase in DNA damage (tail intensity). As apoptosis is known to complicate assessments of clastogenic damage [28, 29], this process is likely to explain isolated increases in tail intensity. In line with recent data on enniatins and induction of apoptosis in other cell types [12, 13] we observed also clear signs of nuclear fragmentation in V79 cells (Fig. 3) treated at and below cytotoxic levels of enniatin B.

Despite the apparent lack of genotoxicity, enniatin B exerts pronounced cytotoxicity in V79 fibroblasts although it is less potent than DON (Table 2). Other than DON which is degraded in the gastrointestinal tract [30], enniatin B is apparently stable [9] and may thus exert prolonged effects upon ingestion. But, the potential impact of an exposure cannot be evaluated at present due to very limited data on their toxicity *in vivo*. Yet, a number of *in vitro* studies have assessed the cytotoxicity in cell culture [reviewed in ref. 4]. For instance, Ivanova *et al*. [31] performed a study in cell lines of human origin (HepG2 and MRC-5 cells) and found enniatins A, A1, B, B1, B2, and B2 to be cytotoxic at IC_{50}-concentrations of 2–36 μM in the Alamar blue assay. In the

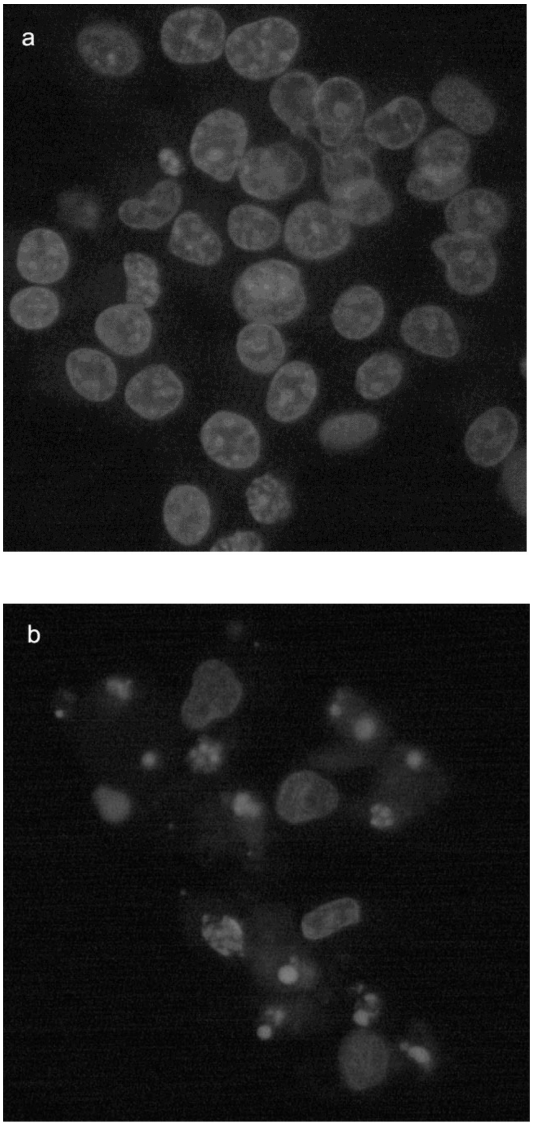

Figure 3. V79 cells stained with Hoechst 33342 to investigate nuclear fragmentation. (a) Shows a control culture and (b) depicts a cluster of cells from a culture treated with 1 μM enniatin B for 24 h.

BrdU assay, which was more sensitive in this case, IC_{50} values varied between 0.6 and 14.4 μM. With the MTT assay, Dornetshuber *et al*. [12] determined cytotoxicity of a mixture of enniatins in 25 different human tumor cell lines and in human fibroblasts and human endothelial cells: They found several tumor cells to be more susceptible to enniatins (IC_{50} 1.61 – 4.88 μM) compared to nontumor cells (IC_{50} 7.9 to >10 μM), and they concluded that cytotoxicity was due to induction of apoptosis via alteration of mitochondrial membranes. As p53-dependent cytostatic and p53-independent cytotoxic effects were more pronounced in human cancer cells, Dornetshuber *et al*. [12] suggested the use of enniatins as potential anticancer drugs. What triggers apoptosis and p53 activation by enniatins is not known so far; but their view [12] that no direct induction of DNA strand breaks or a production of oxygen species occur in enniatin treated cells is supported by our negative results obtained with the Comet assay in this study.

Another recent study by Wätjen *et al*. [13] assessed the cytotoxicity of the enniatins A1, B and B1 in HepG2, C6 and H4IIE cells: Cytotoxicity was moderate (IC_{50} values 10 – 25 μM) in HepG2 human hepatoma cells and C6 rat glioma cells, but stronger (IC_{50} values 1 – 2.5 μM) in H4IIE rat hepatoma cells. Cytotoxicity in the latter cells was accompanied by an increase in caspase 3/7 activity and nuclear fragmentation. Moreover, the activation of ERK kinase and activity of NF-κB was decreased by enniatins. As discussed by the authors [13], these are interesting aspects in the context of a use of enniatins as potential anticancer drugs.

In conclusion, further investigations are needed to elucidate the mode of action and determine the toxicity of enniatin in tumor and nontumor cells, also in light of our finding that cytotoxicity of enniatin B in V79 cells (Table 2) was in the same range of concentrations as those reported for this and closely related enniatins in various tumor cells [12, 13, 31]. Also studies on the toxicity of enniatins in combination with other mycotoxins are indicated considering the occurrence of such mixtures in *Fusarium* contaminated grains.

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