Research Article

Enniatins A1, B and B1 from an endophytic strain of *Fusarium tricinctum* induce apoptotic cell death in H4IIE hepatoma cells accompanied by inhibition of ERK phosphorylation

Wim Wätjen[1], Abdessamad Debbab[2, 3], Anke Hohlfeld[1], Yvonni Chovolou[1], Andreas Kampkötter[1], Ru Angelie Edrada[2], Rainer Ebel[4], Abdelhak Hakiki[3], Mahjouba Mosaddak[3], Frank Totzke[5], Michael H. G. Kubbutat[5] and Peter Proksch[2]

[1] Institute of Toxicology, Heinrich-Heine-Universität, Düsseldorf, Germany
[2] Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine-Universität, Düsseldorf, Germany
[3] Laboratoire des Substances Naturelles et Thermolyse Éclair, Faculté des Sciences, Université Mohammed V Agdal, Rabat, Morocco
[4] Department of Chemistry, University of Aberdeen, Old Aberdeen, Scotland, UK
[5] ProQinase GmbH, Freiburg, Germany

Enniatins are mycotoxins which have important impact on human health, *e.g.* as contaminants of cereals, but also are discussed as possible anticancer agents. We investigated toxic effects of enniatins A1, B and B1 isolated from *Fusarium tricinctum* on different cancer cell lines. The enniatins showed moderate activity in HepG2 and C6 cells (EC_{50}-values approximately 10–25 μM), but were highly toxic in H4IIE cells (EC_{50}-values approximately 1–2.5 μM). In H4IIE cells, all enniatins increased caspase 3/7 activity and nuclear fragmentation as markers for apoptotic cell death. Enniatin A1, enniatin B1, and, to a lesser extent, also enniatin B decreased the activation of extracellular regulated protein kinase (ERK) (p44/p42), a mitogen-activated protein kinase which is associated with cell proliferation. Furthermore, enniatins A1 and B1, but not enniatin B were able to inhibit moderately tumor necrosis factor α (TNF-α)-induced NF-κB activation. Screening of 24 additional protein kinases involved in signal transduction pathways (cell proliferation, survival, angiogenesis and metastasis) showed no inhibitory activity of enniatins. We conclude that enniatins A1 and B1 and, to a lesser extent, enniatin B may possess anticarcinogenic properties by induction of apoptosis and disruption of ERK signalling pathway. Further analysis of these substances is necessary to analyse their usefulness for cancer therapy.

Keywords: Apoptosis / Cytotoxicity / Enniatins / ERK / NF-κB

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1 Introduction

Enniatins are fungal metabolites first isolated by Gäumann *et al*. [1] from *Fusarium orthoceras* var. *enniatinium*. They may have an underestimated impact on human health as food contaminants of, *e.g.* cereals, since several reports showed a contamination of human and animal food with these compounds [2–4]. Therefore, further analysis of these compounds becomes increasingly important [5].

We analysed cellular effects of enniatins A1, B and B1 isolated from *F. tricinctum*. The compounds are cyclic hexadepsipeptides, which consist of alternating a-hydroxy-D-isovaleric and *N*-methylamino acid moieties. Enniatin B is a symmetrical compound, it contains three *N*-methyl-L-valine and three α-hydroxy-D-isovaleric molecules, enniatin B1 contains one moiety of *N*-methyl-L-isoleucine, two of *N*-methyl-L-valine and three α-hydroxy-D-isovaleric acid residues. Enniatin A1 is formed by one part *N*-methyl-L-

Correspondence: Dr. Wim Wätjen, Institute of Toxicology, Heinrich-Heine-Universität, P.O. Box 101007, 40001 Düsseldorf, Germany
E-mail: wim.waetjen@uni-duesseldorf.de
Fax: +49-211-81-13013

Abbreviations: CAPE, caffeic acid phenethylester; **ERK,** extracellular regulated protein kinase; **MTT,** 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium-bromide; **SEAP,** secreted embryonic alkaline phosphatase; **TNF-α**, tumor necrosis factor α

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valine, two parts *N*-methyl-L-isoleucine and three parts α-hydroxy-D-isovaleric acid.

Enniatins are known to have antimycobacterial activity as well as insecticidal activity. They inhibit various enzymes, *e.g.* acyl-CoA-cholesterol-acyl transferase [6] and cyclic nucleotide phosphodiesterase. Enniatins have furthermore ionophoric properties [7–9]. Kamyar *et al.* [10] showed that enniatins easily incorporates into the cellular membrane in which it forms cation-selective pores. The enniatins act as ionophores by forming dimeric structures that transport monovalent ions across membranes, particularly mitochondrial membranes, and uncouple oxidative phosphorylation [11].

Beside these effects, also an anticarcinogenic action of enniatins was suggested by Dornetshuber *et al.* [12] showing a selective toxicity of enniatins against cancer cell lines. Cell death was mediated by induction of apoptosis. Here, we analyse toxic effects of enniatins A1, B and B1 isolated from *F. tricinctum* in different cancer cell lines to further elucidate the anticancer effects of these substances. Besides the proapoptotic effects analysed previously by Dornetshuber *et al.* [12], we also evaluated the effect of these compounds on NF-κB and MAPK signal transduction pathway, to get further insights in their cellular action.

2 Materials and methods

2.1 Materials

All chemicals were of analytical grade and were purchased from Sigma (Deisenhofen, Germany). All tissue culture reagents were purchased from PAA (Coelbe, Germany), plastic material for cell cultures was obtained from Falcon (Heidelberg, Germany).

2.2 Plant material and fungal isolation

Fresh healthy rhizomes of *Aristolochia paucinervis* (aristolochiaceae) were collected in January 2006 from the mountain of Beni-Mellal, Morocco. The plant was identified by Professor A. Boulli, Faculty of Sciences and Techniques, Beni-Mellal, Morocco. Voucher specimens have been deposited in the ‚Laboratoire des Substances Naturelles et Thermolyse Éclair', University Mohammed V agdal, Faculty of Sciences, Rabat, Morocco. The rhizomes were rinsed in sterilised distilled water two times. Sterilisation was done by immersing the stems in 70% ethanol for 3 min (two times) followed by rinsing again two times in sterilised distilled water. Then, the rhizomes were cleaved aseptically into small segments (⩾1 cm in length). The material was placed on a petri dish (malt agar medium) containing an antibiotic to suppress bacterial growth (medium composition: 15 g/L malt extract, 15 g/L agar and 0.2 g/L chloramphenicol in distilled water, pH 7.4–7.8) and incubated at room temperature (25°C).

After several days fungal hyphae growing from the plant material were transferred to other plates, incubated again for 10 days, and periodically checked for culture purity.

2.3 Taxonomy of the isolated strain

A piece (0.5 cm^2) of fungal hyphae was removed from the petri dish and lyophilised in a sample tube (2 mL) closed with a hydrophobic membrane (LidBac, Eppendorf, Hamburg, Germany). The lyophilised sample was powdered in a MixerMill MM300 (Retsch, Haan, Germany) after adding a tungsten carbide bead (Qiagen, Hilden, Germany). DNA isolation was performed using the DNeasy plant mini kit (Qiagen) according to the manufacturer's protocol. The procedure includes cell lysis, digestion of RNA by RNase A, removal of precipitates and cell debris, DNA shearing, DNA precipitation and purification. The DNA obtained was dissolved in 50 μL of elution buffer supplied by the manufacturer. PCR was then performed using Hot Star Taq master mix (Qiagen), Taq polymerase and the primer pair ITS1 and ITS4 (Invitrogen, Karlsruhe, Germany) in an iCycler (BioRad, Hercules, CA) thermal cycler according to the following protocol: (i) initial denaturation 95°C, 15 min; (ii) denaturation 95°C, 1 min; (iii) annealing 56°C, 0.5 min; (iv) extension 72°C, 1 min; (v) final extension 72°C, 10 min. Steps 2–4 were repeated 35 times (21 Reference). Each sample consisted of 25 μL of Taq polymerase master mix, 3 μL of primer mix (10 pmol/μL each), 3 μL of template DNA and 19 μL of water. From this, 20 μL was loaded onto an agarose gel (2%). After electrophoresis at 70 V for 60 min, the band due to the PCR product (approximate size 550 bp) was isolated from the gel slice using the PerfectPrep gel cleanup kit (Eppendorf) according to the manufacturer's protocol. The PCR product was then submitted for sequencing (BMFZ, Düsseldorf, Germany) with the primer ITS1.

2.4 Rice culture of isolated fungus

Two Erlenmeyer flasks (1 L each) containing 100 g of rice and 100 mL of distilled water were autoclaved. A small part of the medium from a petri dish containing the purified fungus was transferred under sterile conditions to the rice medium. The fungus strain was grown on solid rice medium at room temperature for 40 days.

2.5 Isolation and identification of enniatins

The culture was extracted with 300 mL ethyl acetate. The obtained ethyl acetate crude extract was dried and partitioned between *n*-hexane and 90% MeOH. The 90% MeOH soluble material (800 mg) was then fractionated by vacuum LC on SiO gel 60 using *n*-hexane: EtOAc/CH_2Cl_2/MeOH gradient elution. Based on TLC using SiO gel F254 plates (Merck, Darmstadt, Germany) and a mixture of EtOAc/

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MeOH/H_2O (77:13:10) as a solvent system, the collected fractions were combined, and next subjected to semi-preparative HPLC (Merck, Hitachi L-7100) on a Eurosphere 100-10 C18 column (300 × 8 mm^2, id) using a H_2O/MeOH gradient. All spectroscopic data obtained by ^1H and ^{13}C NMR (ARX 500 NMR spectrometer), ESI-MS (Finnigan LC Q-Deca mass spectrometer) and optical rotation (Perkin-Elmer-241 MC polarimeter) were in accordance with reference data published for enniatin B [13], enniatin B1 and enniatin A1 [14].

2.6 Cell culture

Tumor cell lines were grown in DMEM medium containing 4.5 g/L glucose and 2 mmol/L L-glutamine, supplemented with foetal bovine serum (FBS): metabolically active H4IIE rat hepatoma cells (10%) and rat C6 glioma cells (5%); HepG2 human hepatoma cells were grown in RPMI medium containing 10% FCS. The cell culture medium contained 100 units/mL penicillin and 100 μg/mL streptomycin and was changed twice *per* week. The cells were maintained in a humidified atmosphere at 37°C with 5% CO_2.

2.7 Determination of cytotoxicity

The effect of isolated compounds on cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium-bromide (MTT) assay [15]. The cells were plated on 96-multiwell plates with 10 000 cells/well. The cells were allowed to attach for 24 h and then treated with different concentrations of the various enniatins for 3 or 24 h. After this treatment the medium was changed and the cells were incubated for 2 h under cell culture conditions with 20 μg/mL MTT. After this incubation time the cells were lysed with 50% ethanol/49% water/1% acetic acid. The concentration of reduced MTT as a marker for cell viability was measured photometrically (560 nm).

2.8 Determination of apoptotic/necrotic cell death

Caspase-3/7-activity was measured using the Apo-ONE homogeneous caspase 3/7 assay (Promega) according to the manufacturers protocol. Briefly, 50 000 cells/well were plated on 96-multiwell plates, allowed to attach for 24 h and treated with enniatins for 24 h. Then, 50 μL of Apo-ONE Caspase-3/7-reagent was added and the increase in fluorescence was measured at 37°C for 3 h (excitation: 485 nm, emission: 535 nm). We further investigated nuclear fragmentation (Hoechst 33342 staining) as an additional feature of apoptotic cell death according to Michels *et al*. [16].

2.9 Determination of NF-κB inhibiting activity

Cell transfection: H4IIE were stably transfected with HiFect (Amaxa) transfection reagent according to manufacture's protocol. Briefly, H4IIE cells were seeded at a density of 1.5×10^5 per 35 mm petri dish and incubated overnight. Cells were transfected with 1.6 μg pNF-κB-secreted embryonic alkaline phosphatase (SEAP) and 0.4 μg pTK-Hyg by using 10 μL HiFect transfection reagent in 1 mL serum free DMEM medium. 48 h after transfection, cells were split 1:5 into 100 mm petri dishes and stably transfected cell clones (H4IIE-SEAP) were selected with 400 μg/mL hygromycin.

Reporter gene assay: H4IIE-SEAP cells were seeded at a density of 2×10^5 cells *per* 24-well plates and incubated for 48 h. Cells were preincubated with 1 μM of each enniatin for 1 h and then stimulated with 5 ng/mL tumor necrosis factor α (TNF-α) for 24 h. Activity of the reporter enzyme (SEAP) in the medium was measured using a chemiluminescence-based detection method. In brief, 30 μL conditioned cell culture medium was mixed with 30 μL of 1 × dilution buffer (50 mM Tris, 150 mM NaCl, pH 7.4) and incubated for 30 min at 65°C to heat inactivate endogenous alkaline phosphatase activity. Samples were mixed with 30 μL assay buffer (2 M diethanolamine, 28 mM L-homoarginine) and 30 μL CSPD substrate. After 15 min incubation at dark, SEAP activity was measured in a plate luminometer (Victor 1420, Wallac). In each experiment it was verified that inhibition of SEAP activity was not due to cytotoxic effects (MTT assay).

2.10 Electrophoretic mobility shift assay (EMSA)

Cells (1×10^7 cells/assay) were treated with enniatins, TNF-α or both for 24 h and nuclear extracts were prepared. Double-stranded oligonucleotides containing the sequences corresponding to NF-κB consensus site (5′-AGTTGAGGGGACTTTCCCAGGC-3′,3′-TCAACTCCCCTGAAAGGGTCCG-5′) were 3′-end labelled with biotin. Binding reactions were carried out in a final volume of 15 μL containing 0.8 ng of digoxigenin-labelled double-stranded NF-κB, 5 μg of nuclear extract, 1 μg of poly [d(I-C)] and binding buffer [20 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 2% Tween 20 and 30 mM KCl]. The mixtures were incubated for 15 min at room temperature, followed by another 10 min on ice. Samples were subjected to electrophoresis in 6% nondenaturating polyacrylamide gel in a 0.5 × Tris-borate-EDTA buffer system. The gel was transferred to a nylon membrane (Amersham) by electroblotting. Detection of p65–DNA complexes was performed using the Chemiluminescent Nucleic Acid Detection Module (Pierce Chemical). In brief, after cross-linking, the blot was incubated in blocking buffer for 1 h at 37°C. Streptavidin–peroxidase was added for 15 min in blocking buffer at room temperature, and the blot was then washed six times in wash buffer. The blot was incubated for 5 min in equilibration buffer and developed with the chemiluminescent reagents provided with the kit.

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2.11 Isolation of nuclear proteins

To determine the levels of p65, nuclear extracts were prepared from H4IIE cells. After treatment with enniatins, cells were washed with PBS pH 7.4 and solubilised with buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 10 μg/mL aprotinin, 10 μg mL leupeptin, 1 mM DTT, 1 mM PMSF, 10 mM NaF, 0.6% NP40) and then centrifuged at 10 000 × *g* for 1 min and 4°C. The pellets were resuspended in buffer B (20 mM HEPES, pH 7.9, 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM DTT, 1 mM PMSF, 10 mM NaF, 0.6% NP40), incubated for 25 min at 4°C and then centrifuged at 15 000 × *g* for 5 min and 4°C. The supernatants were used as nuclear protein fraction.

2.12 Western blot analysis

Samples containing equal amounts of protein were resolved by 10 or 12% SDS-PAGE and electrotransferred onto a PVDF membrane (Roche Diagnostics, Mannheim, Germany). Protein loading was assessed by Ponceau S staining of membranes. The membranes were blocked with 3% nonfat dry milk in TBST buffer (20 mM Tris, 500 mM NaCl, 0.01% Tween 20, pH 8.0) and incubated with 1:1000 dilution of antibody against p65 (Cell Signalling). Blots were then probed with the appropriate horseradish peroxidase-conjugated secondary antibody. Bound antibody was visualised using enhanced chemiluminescence reagent (Roche Diagnostics).

2.13 Determination of MAP kinase activation

Cells were seeded in a six-well plate (0.5×10^6/well) for 48 h, then cells were incubated with the enniatins (10 μM) for 2 h. Cells were washed three times with PBS, detached from the plates and harvested by centrifugation (12 000 rpm, 15 min, 4°C). Cell lysis was performed in RIPA buffer including a phosphatase inhibitor cocktail (sodium vanadate, sodium fluoride, sodium molybdate, imidazole, sodium tartrate, okadaic acid) followed by three freeze/thaw cycles. Proteins were collected from supernatant and determined by the Bradford method [17]. After heating, (95°C, 10 min) 8 μg of protein (detection of P-extracellular regulated protein kinase (ERK), P-MEK) and 1 μg of protein (detection of ERK, MEK) were separated on a discontinuous 10 and 4.5% PAGE and then the proteins were transferred to a PVDF membrane at 200 mA for 1 h. The membrane was blocked with 3% nonfat dried milk (Sigma) in TBS buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1 h, and then incubated with the p-ERK antibody sc-7383 (1:1000), p-MEK antibody sc 7995 (1:1000), MEK antibody sc 436 (1:1000) or ERK antibody sc-94 (1:400) (all antibodies: Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. The membrane was washed three times with TBST buffer (TBS + 0.1% Tween20) and incubated with the corresponding HRP-conjugated secondary antibody (1:5000 and 1:2000, respectively) at room temperature for 1 h. The target protein was detected by BM Chemiluminescence Blotting Substrate (Roche, Mannheim) using X-ray film.

2.14 Protein kinase activity assay

All biochemical protein kinase activity assays were performed in 96-well FlashPlates™ from Perkin Elmer/NEN (Boston, MA, USA) in a 50 μL reaction volume. The reaction cocktail contained 20 μL of assay buffer, 5 μL of ATP solution (in H_2O), 5 μL of test compound (in 10% DMSO), 10 μL of substrate and 10 μL of purified recombinant protein kinase. Final concentration of ATP was 1 μM. The assay for all enzymes contained 60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 μM Na-orthovanadate, 1.2 mM DTT, 50 μg/mL PEG 20000, 1 μM [γ-^{33}P]-ATP (approx. 5×10^5 cpm *per* well). Amount of protein kinases used *per* 50 μL assay were: AKT1, ARK5, CDK2/CycA, VEGF-R3, FLT3, MET, PDGF-Rb, 0.1 μg; Aurora A, Aurora B, CDK4/CycD1, VGF-R2, 0.05 μg, B-RafVE, IGF1-R, 0.02 μg; COT, 0.4 μg; EGF-R, Ins-R, 0.025 μg; EPHB4, SRC, 0.01 μg; ERBB2, FAK, PLK1, SAK, TIE2, CK2-a1, 0.2 μg. The following substrates were used at the specified concentrations: GSK3(14–27), 1 μg/50 μL: AKT1; tetra(LRRWSLG), 0.25–0.5 μg/50 μL: Aurora A, B; MEK1 KM, 0.25 μg/50 μL: B-Raf; Histone H1, 0.125 μg/50 μL: CDK2/CycA; Rb-CTF, 0.5 μg/50 μL: CDK4/CycD1; Poly(Glu,Tyr)4:1, 0.125 μg/50 μL: EGF-R, EPHB4, ERBB2, FAK, IGF1-R, SRC, VEGF-R2, VEGF-R3, Tie2; Poly(Ala,Glu,Lys,Tyr), 0.125 μg/50 μL: FLT3, INS-R, Met, PDGF-Rb; Casein, 0.2–1.0 μg/50 μL: PLK1, CK2a1. Autophosphorylation was measured for ARK5, COT and SAK. Compounds were tested at a final concentration of 1 μg/mL (this corresponds to 1.51 μM enniatin A1, 1.55 μM enniatin B1 and 1.59 μM enniatin B2).

2.15 Statistics

Data are given as mean ± SEM of at least three independent experiments. The significance of changes in the test responses was assessed using a one-way ANOVA followed by LSD posthoc test (Analyse-it, Leeds, UK), differences were considered to be significant at $p < 0.05$.

3 Results

3.1 Isolation of enniatins

Enniatins A1, B1 and B (Fig. 1) were isolated from *F. tricinctum* obtained from rhizomes of *A. paucinervis*. All enniatins were obtained from fraction four following VLC,

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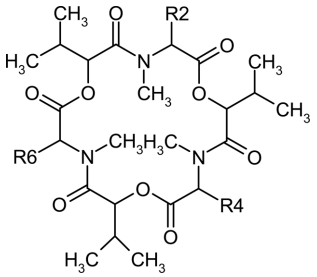

Figure 1. Basic enniatin structure. Enniatin A1:$R_2 = R_4 = -CH(CH_3)CH_2CH_3$, $R_6 = -CH(CH_3)_2$, enniatin B1: $R_2 = R_4 = -CH(CH_3)_2$, $R_6 = -CH(CH_3)CH_2CH_3$, enniatin B: $R_2 = R_4 = R_6 = -CH(CH_3)_2$.

identification was performed by UV, ^1H and ^{13}C NMR spectroscopy, including COSY and DEPT spectra, MS and measurement of optical rotation $[\alpha]_D$. Spectral data were in accordance with published data [13, 14].

3.2 Toxicity of enniatins in different tumor cell lines

The toxic potential of the isolated enniatins was analysed using H4IIE rat hepatoma, HepG2 human hepatoma and C6 rat glioma cells employing the MTT reduction assay (Fig. 2): The enniatins showed a moderate toxicity in C6 glioma and HepG2 hepatoma cells [EC_{50}-values (24 h): 2.5–10 μM and 10 μM, respectively], whereas they exerted a prominent toxicity in H4IIE cells with EC_{50}-values in the low micromolar range (1 and 1.5 μM, respectively). Experiments with a shorter incubation time (3 h) revealed also a significant toxicity for all enniatins (Fig. 2B): EC_{50}-values for enniatin A1 and B1 were approximately 5 μM, while enniatin B showed a lower toxicity (EC_{50}-value approximately 10 μM).

3.3 Induction of apoptotic cell death by enniatins

We further focused on H4IIE cells and showed that enniatins-induced apoptotic cell death in H4IIE cells detected via increased caspase 3/7 activity (Apo-ONE assay): A significant increase in enzyme activity was found for all enniatins at 1 μM, but not at 0.5 μM (Fig. 3A). We further investigated the induction of apoptotic cell death by analysing the amounts of fragmented nuclei. All enniatins increased the amounts of fragmented nuclei at concentrations of 1 and 2.5 μM (Fig. 3B, shown for enniatin B1).

3.4 Modulation of NF-κB pathway by enniatins

The transcription factor NF-κB regulates not only a variety of physiologic processes including cell growth, oxidative stress response, but also apoptosis. For this reasons, we analysed whether enniatins interfered with the activation of NF-κB causal by the cytokine TNF-α (H4IIE cells stably transfected with pNF-κB-SEAP reporter plasmid). Incubation with TNF-α (5 ng/mL, 24 h) increased SEAP activity 3.3-fold. Preincubation with 1 μM (1 h) of the enniatins A1 and B1, but not enniatin B significantly lowered NF-κB-activity after stimulation by TNF-α to 73 ± 7.7% and 72 ± 5.3% of TNF-α stimulated value, respectively. On the other hand, basal NF-κB activity was not modulated by the enniatins (Fig. 4A). As positive control the known NF-κB inhibitor CAPE (caffeic acid phenethylester) was used: incubation of H4IIE cells with 40 μM CAPE (1 h) inhibited basal NF-κB-dependent transcriptional activity Furthermore, it completely blocked TNF-α-induced SEAP activity to control levels.

We further analysed the effect of enniatin A1 and B1 on NF-κB activation by EMSA experiments and detection of nuclear NF-κB by Western blot analysis of nuclear proteins: preincubation with enniatin A1 and B1 prior to stimulation with TNF-α decreases the binding of NF-κB to the oligonucleotides containing the NF-κB binding site by a factor of 0.6 and 0.8, respectively, as seen by EMSA analysis. The enniatins itself resulted not in an increased DNA binding of NF-κB (Fig. 4B). Analysing the protein amount of NF-κB in the nucleus we see no difference in protein amount after preincubation with enniatins (Fig. 4C).

3.5 Effects of enniatins on protein kinases

Since, in many tumors a constitutive activation of the mitogen-activated (MAP kinase) signalling pathway occurs, we analysed the effect of enniatins on MAP kinase signalling pathways in our cellular model. Incubation of H4IIE cells with enniatins (10 μM, 2 h) strongly diminished phosphorylation (=activation) of the ERK-MAP kinase (p44/p42). This enzyme is constitutively active in our model system, since the cells were maintained in 10% FBS medium containing a variety of growth factors which are strong activators of this pathway. In analogy to the results of NF-κB activity, enniatins A1 and B1 were more active in inhibition of ERK phosphorylation than enniatin B (Fig. 5A) causing a reduction of 74 or 70% of control value in contrast to the 42% reduction of ERK phosphorylation caused by enniatin B. The decrease in ERK phosphorylation by enniatin A1 occurred after incubation times of 1 h and at concentrations as low as 2.5 μM (Fig. 5B). We furthermore see a similar reduction of the phosphorylation of the upstream kinase MEK: a reduction to 47, 68 and 56% of MEK phosphorylation (compared to the control) was seen for enniatins A1, B and B1, respectively (Fig. 5C).

We further analysed the effect of the enniatins on different protein kinases involved in different signal transduction pathways including protein kinases associated with cell proliferation (ARK5, Aurora A, Aurora B, B-Raf, CDK2, CDK4, COT, EGF-R, ErbB2, FLT3, PDGF-Rb, PLK1, SAK), survival (Akt1, CK2, IGF1-R), angiogenesis (EphB4, Tie2, VEGF-R2, VEGF-R3) and metastasis (FAK,

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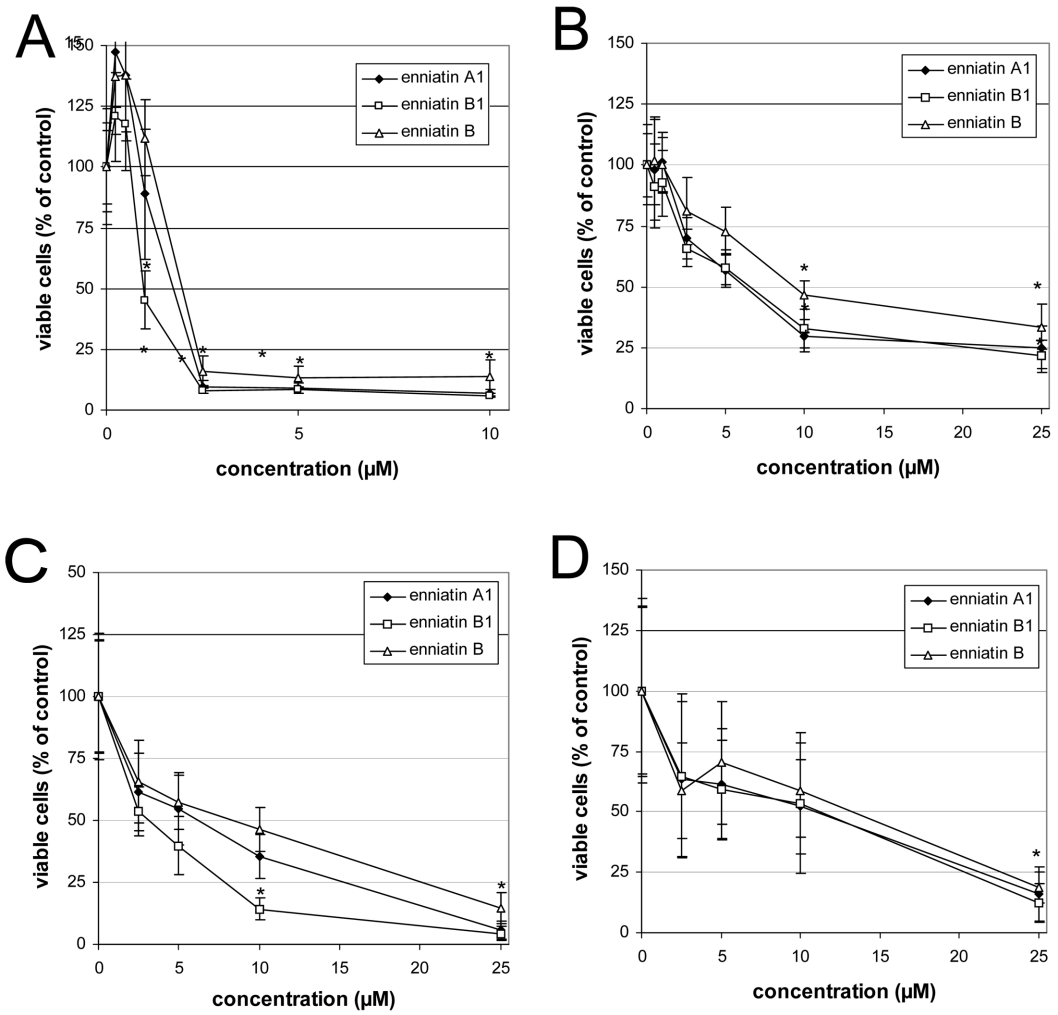

Figure 2. Cytotoxicity of enniatins in different cell lines. H4IIE hepatoma (A), C6 glioma (C) and HepG2 hepatoma (D) cells were incubated with enniatins for 24 h or 3 h (B, H4IIE cells), then MTT reduction as a marker of cell viability was measured (absorbance at 560 nm). Results are expressed as viable cells in percent of control (absorbance values of DMSO control (only shown for enniatin A1): 0.288 ± 0.044 (H4IIE, 24 h), 0.174 ± 0.028 (H4IIE, 3 h), 0.532 ± 0.135 (C6, 24 h), 0.301 ± 0.106 (HepG2, 24 h). Data are means ± S.E.M ($n = 3$), *: $p < 0.05$ *versus* corresponding control (DMSO).

Met, SRC). None of the enniatins showed significant inhibitory effects to any of these kinases at a concentration of 1 µg/mL (this corresponds to 1.51 µM enniatin A1, 1.55 µM enniatin B1 and 1.59 µM enniatin B).

4 Discussion

Enniatins are known as contaminants of human food; the impact of these contaminants on human health is still under discussion. Several studies showed a toxic potential at micromolar concentrations [18], hence these substances may have an underestimated toxic potential. Contamination of food with enniatins is a relatively widespread phenomenon: analysing 38 Finnish grain samples, Jestoi *et al*. [3] detected enniatins B and B1 in all samples, and enniatin A1 in 95% of the samples. The same group analysed contamination levels of different *Fusarium* mycotoxins from randomly selected grain-based products purchased from Finnish and Italian markets [4]: enniatins B and B1 were also the most predominant mycotoxins in the commercially available samples (present in 97% of the samples). Investigating enniatin content in maize from south-western Nigeria, Adejumo *et al*. [19] reported that enniatins A1, B and B1 were present in 3, 7 and 3% of the samples, respectively.

Logrieco *et al*. [2] analysed the content of enniatins in 13 wheat samples affected by head blight. *F. avenaceum* was the dominant species (91%) isolated from all samples, but isolates of *F. tricinctum* (3%) were also recovered. Enniatin B (up to 4.8 µg/g) was detected in 12 samples, enniatin B1 (up to 1.9 µg/g) was detected in eight samples and enniatin A1 (up to 6.9 µg/g) was detected in ten samples. The same

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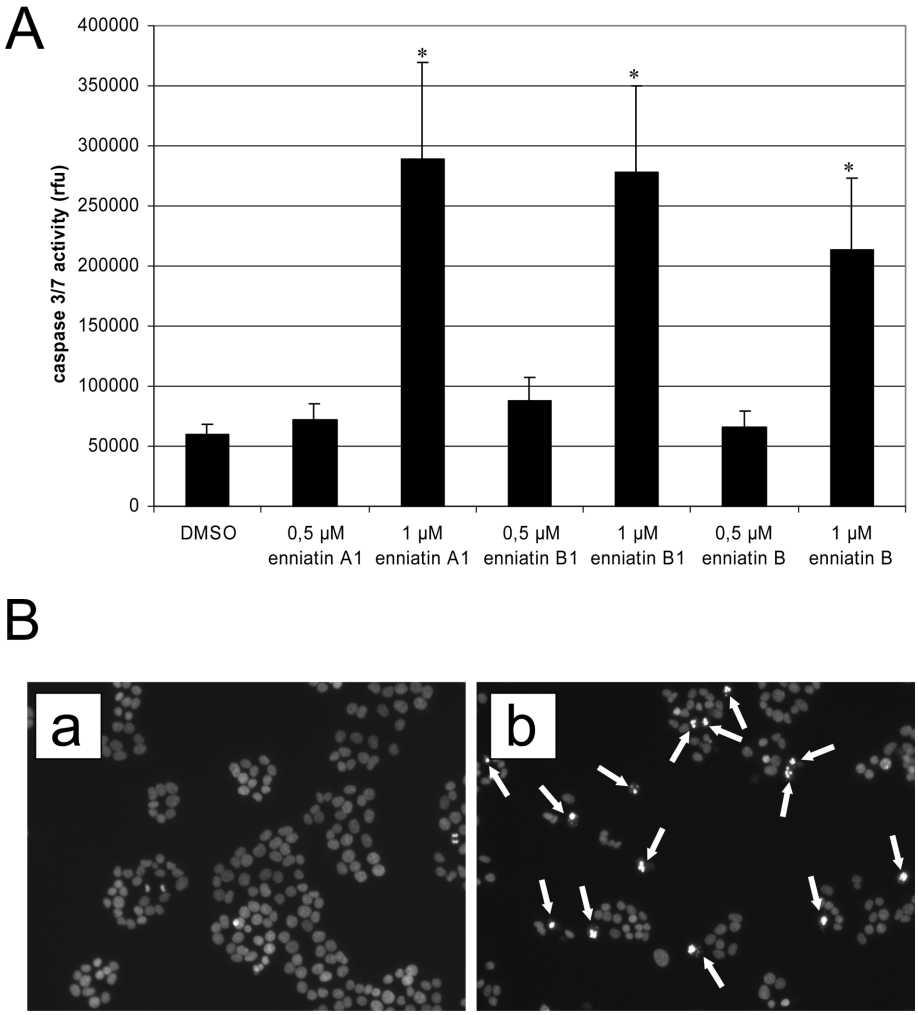

Figure 3. Induction of apoptosis in H4IIE cells. H4IIE cells were incubated with enniatins for 24 h, then caspase 3/7 activity was measured using homogeneous Apo-ONE assay (Promega). Results are expressed as increase in relative fluorescence units (rfu) for 3 h ± SD ($n = 3$), *: $p < 0.05$ *versus* control (DMSO). (B) Apoptosis is further confirmed by nuclear fragmentation (Hoechst staining): (a) nuclei of control cells, (b) incubation with 1 µM enniatin B1 (24 h), apoptotic cells are marked with an arrow.

group investigated the mycotoxin content of 96 fungal isolates belonging to 28 species in the *Gibberella fujikuroi* complex. Enniatins were produced by 4 species, enniatin production ranged from 2 to 131 µg/g [20]. From these studies we may conclude that wheat contamination with enniatins is probably underestimated, since enniatins seem to be widespread wheat head blight agents and are produced in relatively high levels by *Fusarium* species, *e.g. F. tricinctum*.

To estimate the adverse potential of the enniatins, we investigated toxic effects of enniatins A1, B and B1 on H4IIE, HepG2 and C6 cells. While the enniatins showed only a moderate toxicity in HepG2 and C6 cells (EC_{50}-values approximately 10 – 25 µM), they showed toxic effects in H4IIE cells with EC_{50}-values of approximately 1 – 2.5 µM. Toxicity in the low micromolar range was also reported for a mixture of enniatins (enniatin A, A1, B and B1) in different cell lines [12], however after a longer incubation time (72 h, MTT assay). High toxicity was also reported for the enniatins A, A1, B, B1, B2 and B3 in MRC-5 cells [18]: EC_{50}-values (BrdU assay) were reported as 1.4 µM (enniatin A1), 1.4 µM (enniatin B1) and 3.6 µM (enniatin B). When the Alamar Blue assay was used, EC_{50}-values were reported as 6.9 µM (enniatin A1), 4.7 µM (enniatin B1) and 9.8 µM (enniatin B). Similar to our results, Ivanova *et al*. [18] found lower toxicity for the enniatins in HepG2 cells: EC_{50}-values were 18.1 µM (enniatin A1), 36 µM (enniatin B1) and 435 µM (enniatin B) as measured in the Alamar Blue assay.

Recently, Dornetshuber *et al*. [12] reported that a mixture of enniatins exerted profound p53-dependent cytostatic and p53-independent cytotoxic activities especially against human cancer cells, suggesting a potential quality of enniatin as an anticancer drug. We further investigated if enniatins may be useful as anticancer pharmacons: Dornetshuber *et al*. [12] investigated different aspects of enniatin-induced apoptosis and cell cycle distribution in KB-3-1 cells (analysis of cyclin proteins, Bcl-proteins, caspases, PARP, p53, p21). We focussed our investigations on H4IIE cells due to the strong toxic effects observed in this cell line. The ennia-

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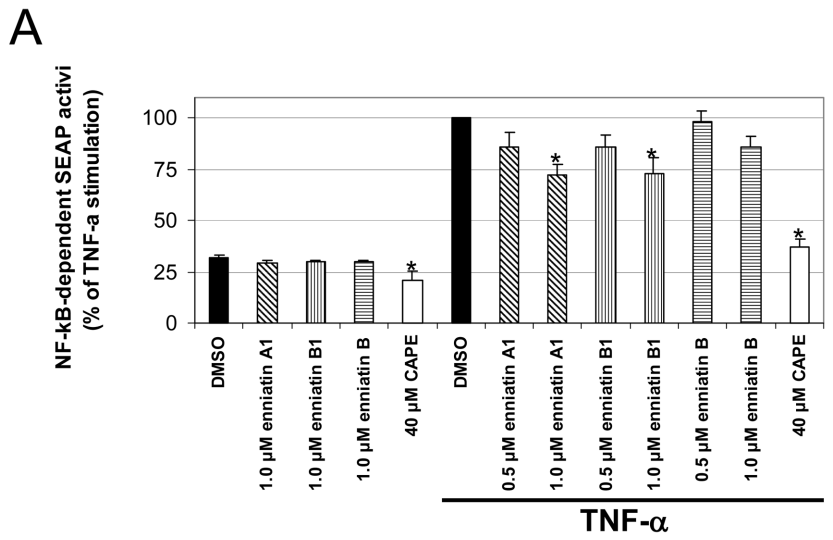
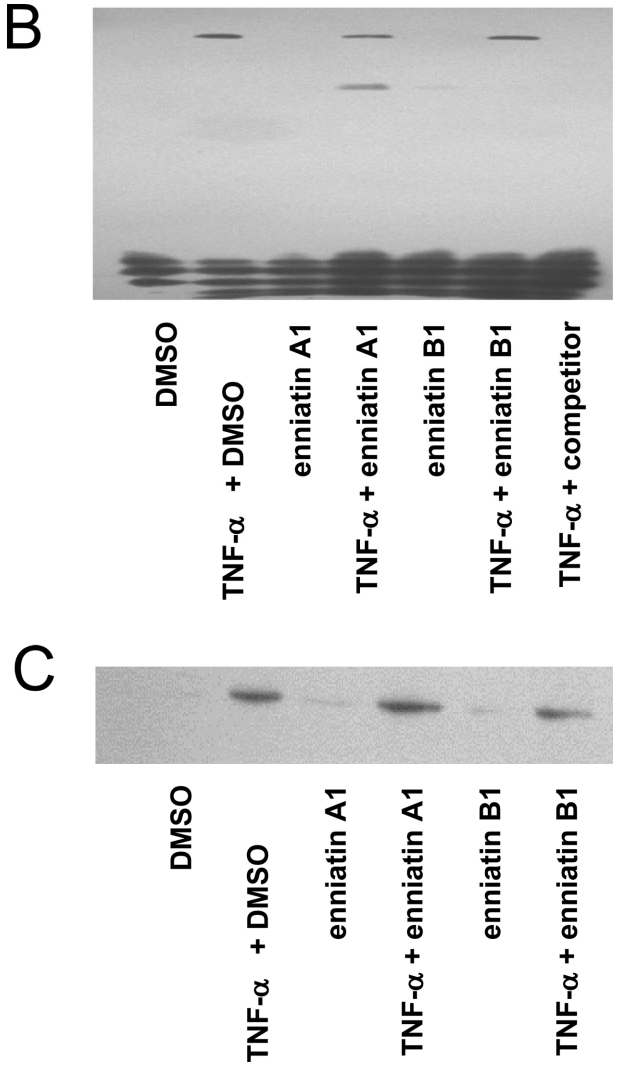


Figure 4. Effects of enniatins on NFκ-B activation. (A) H4IIE cells stably transfected with pNF-κB-SEAP reporter plasmid were preincubated with Enniatins (1 μM, 1 h) or 40 μM (1 h) of the NF-κB inhibitor CAPE and then stimulated with 5 ng/mL TNF-α for 24 h. Cell culture supernatants were assayed for SEAP activity, results are expressed as fold activity of the TNF-α stimulated cells (means ± SEM, $n = 4$). (B) Cells were preincubated with 1 μM enniatin A1 or B1 and then stimulated with 5 ng/mL TNF-α for 24 h. Nuclear proteins were isolated and analysed for binding to oligonucleotides containing the NF-κB-consensus sequence (EMSA, $n = 2$ with essentially the same results); (C) Cells were preincubated with 1 μM enniatin A1 or B1 and then stimulated with 4 ng/mL TNF- for 24 h. Protein amounts of nuclear NF-κB were analysed (Western blot, $n = 2$ with essentially the same results).



tins A1-, B1- and B-induced apoptosis in hepatoma cells as detected by caspase 3/7 activation and nuclear fragmentation in low micromolar concentrations after 24 h.

We further analysed, whether intracellular signal transduction pathways were modulated by the enniatins: enniatin A1, enniatin B1, and, to a lesser extent, enniatin B decreased activation of ERK kinase (p44/p42), a mitogen-activated protein kinase which is associated with cell proliferation. The decrease in ERK phosphorylation occurred after incubation times of 1 h and at concentrations as low as 2.5 μM enniatin A1 Inhibition of this pathway may be interesting for further tumor therapy, since this kinase is permanently active in several tumors. A similar inhibition was detected for the upstream kinase MEK by enniatins A1 and B1. The activity of 24 further protein kinases involved in cell proliferation, survival, angiogenesis and metastasis was likewise analysed, but no inhibition was detected up to a maximal concentration of 1 μg/mL. Therefore, we conclude that the enniatins exert their cellular actions at the ERK and/or MEK or upstream of these kinases but downstream of the EGF-receptor and B-raf. Furthermore, it was found that enniatin A1 and B1, but not enniatin B modulate the activity of NF-κB, a transcription factor that is involved in many critical physiological responses. Furthermore, unregulated NF-κB activity also contributes to many human diseases and many cancers. Enniatins did not inhibit the basal activity of this transcription factor, but the amount of activation after stimulation was decreased. Since, NF-κB is commonly discussed as a transcription factor responsible for cell survival, this inhibition may also contribute to the toxic effects of the enniatins. Since, we detect a similar decrease in NF-κB DNA binding like analysed in the reporter gene assay, but we find no decrease in nuclear NF-κB protein after stimulation with TNF-α, we suggest

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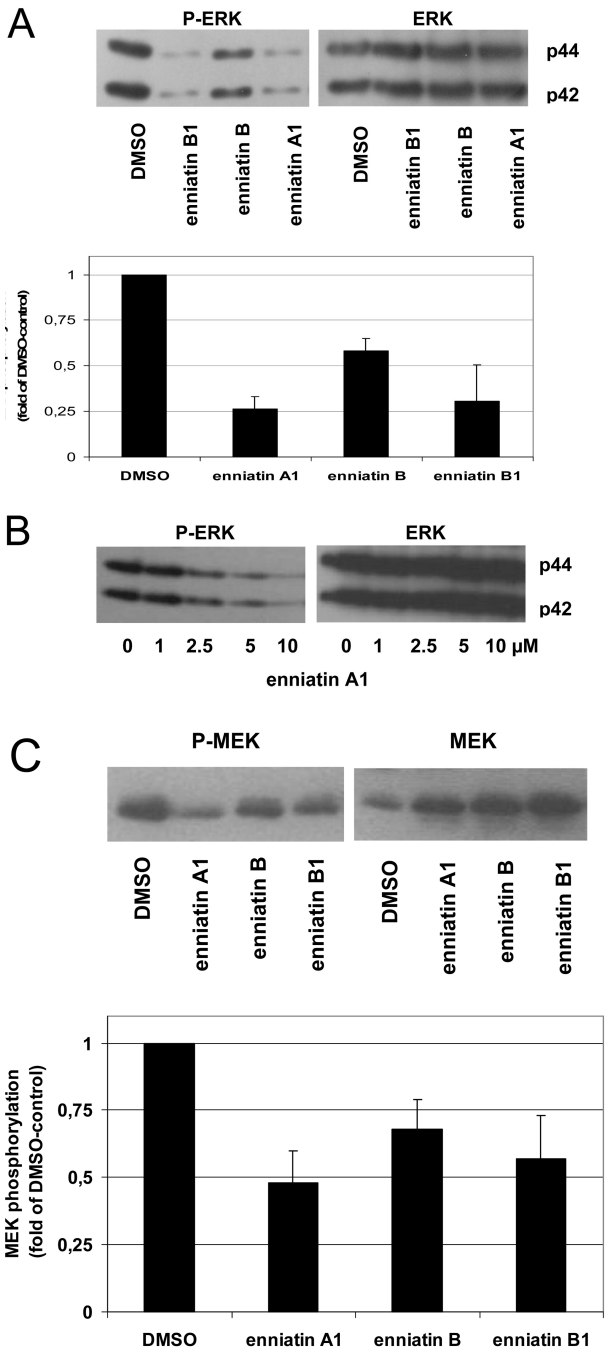

Figure 5. Effects of enniatins on MAP kinase activation. (A) Effect of enniatins (10 µM, 2 h) on ERK phosphorylation ($n = 3$ with essentially the same results), (B) reduction of ERK phosphorylation by enniatin A1 (var. concentrations, incubation time: 1 h) ($n = 2$ with essentially the same results), (C) Effect of enniatins (10 µM, 2 h) on MEK phosphorylation ($n = 3$ with essentially the same results).

that enniatins do not inhibit the translocation of NF-κB from the cytosol to the nucleus, but the DNA binding affinity of this transcription factor thereby inhibiting its activity.

We conclude that enniatins A1, B1 and B may be useful for the development of new chemical compounds in cancer therapy due to (i) relatively high toxicity, (ii) induction of apoptotic cell death, (iii) inhibition of NF-κB-signalling and (iv) disruption of the ERK signalling pathway. Further analysis of these substances is necessary to analyse their potential significance in cancer therapy.

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