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Research Article

Interactions between ABC-transport proteins and the secondary *Fusarium* metabolites enniatin and beauvericin

Rita Dornetshuber[1, 2], Petra Heffeter[2], Michael Sulyok[3], Rainer Schumacher[3], Peter Chiba[4], Stephan Kopp[4], Gunda Koellensperger[5], Michael Micksche[2], Rosa Lemmens-Gruber[1] and Walter Berger[2]

[1] Department of Pharmacology and Toxicology, University of Vienna, Vienna, Austria
[2] Institute of Cancer Research, Department of Medicine I, Medical University of Vienna, Vienna, Austria
[3] Christian Doppler Laboratory for Mycotoxin Research, Department IFA-Tulln, University of Natural Resources and Applied Life Sciences, Vienna, Austria
[4] Institute of Medical Chemistry, Medical University of Vienna, Vienna, Austria
[5] Department of Chemistry, University of Natural Resources and Applied Life Sciences, Vienna, Austria

Enniatins (ENN) and beauvericin (BEA) exert cytotoxic properties. Here, we observed that their impact on Ca^{2+}-homeostasis can be reversed by exogenous ATP. Thus, we investigated whether membrane-located ATP-binding cassette (ABC) transporters influence ENNs- and BEA-induced cytotoxicity. In short-term exposure assays breast cancer resistance protein (ABCG2)-overexpression weakly but significantly reduced the cytotoxic activity of BEA but not ENNs. In contrast, multidrug resistance-associated protein-1 (ABCC1)- and *P*-glycoprotein (ABCB1)-overexpression was not protective under identical conditions. ABCG2-mediated resistance against BEA was reversible by ABCG2 modulators. In long-term exposure assays, ABCG2 and ABCB1 significantly protected against ENNs- and to a lesser extent BEA-induced cytotoxicity. Moreover, both fusariotoxins potently inhibited the ABCG2- and ABCB1-mediated efflux of specific fluorescent substrates, with BEA being more effective. Additionally, ATPase and photoaffinity-labelling assays proofed interaction of both substances with ABCG2 and ABCB1. Remarkably, 2 years selection of KB-3-1 cells against both fusariotoxins resulted only in two-fold ENNs but negligible BEA resistance. Interestingly, the selected sublines displayed upregulation of multidrug resistance proteins and crossresistance to other chemotherapeutics. Summarizing, ABCG2 and ABCB1 slightly but significantly protect human cells against ENNs- and BEA-induced cytotoxicity. However, both mycotoxins potently interact with ABCB1 and ABCG2 transport functions suggesting influences on bioavailability of xenobiotics and pharmaceuticals.

Keywords: ABC-transport proteins / Beauvericin / Enniatin / Multidrug resistance / Mycotoxins

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1 Introduction

Mycotoxins are worldwide distributed fungal secondary metabolites and their presence in food and feed, as the result of fungal diseases in crops, can present a danger to animal and human health [1]. Under certain environmental conditions *Fusarium* spp. produce several mycotoxins such as the cyclic hexadepsipeptides enniatins (ENNs) and beauvericin (BEA) [2]. These secondary metabolites belong to the most frequent cereal contaminants. For example, pearl millet

Correspondence: Professor Walter Berger, Institute of Cancer Research, Department of Medicine I, Medical University Vienna, Borschkegasse 8a, 1090 Vienna, Austria
E-mail: walter.berger@meduniwien.ac.at
Fax: +43-1-4277-65169

Abbreviations: ABC, ATP-binding cassette; **ABCB1,** *P*-glycoprotein; **ABCC1,** multidrug resistance-associated protein-1; **ABCG2,** breast cancer resistance protein; **BEA,** beauvericin; **BSO,** buthionine sulphoximine; **CSA,** cyclosporine A; **ENN,** enniatin; **ENN_{mix},** enniatin mixture of 3% A, 20% A_1, 19% B and 54% B_1 homologues; **FACS,** fluorescence-activated cell sorting; **FCS,** fetal calf serum, **GSH,** glutathione; **IAAP,** iodoarylazidoprazosin; **LC/ESI-MS/MS,** ESI triple quadrupole MS; **MDR,** multidrug resistance; **MTT,** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **PhA,** pheophorbide A; **Rh123,** rhodamine 123

which is widely consumed in regions of Africa and Asia as well as maize and corn is regularly contaminated with BEA [3]. Beside deoxynivalenol, ENNs were found as the most predominant mycotoxins in conventional and organic grain-based products purchased from Italian and Finnish markets [2]. However, data on the impact of BEA and ENNs on human and animal health are limited. Both substances, which are well-known inhibitors of cholesterol acyltransferase [4], possess antimicrobial, insecticidal and ionophoric properties [5, 6]. Moreover, BEA increases ion permeability in biological membranes by forming a complex with essential cations (Ca^{2+}, Na^{+}, K^{+}) consequently affecting the ionic homeostasis. Also the ionophoric properties of ENNs are based on the ability to incorporate into cell membranes forming cation selective pores with high affinity for K^{+}, Mg^{2+}, Ca^{2+} and Na^{+} [6, 7]. Moreover, several studies concerning BEA suggested that this fusariotoxin also exerts substantial cytotoxic effect on invertebrate and in mammalian cells including human cell lines of myeloid origin [8, 9]. For several human cancer cell lines, the cytotoxicity of BEA has been described to be based on apoptosis induction *via* the mitochondrial pathway [10]. Furthermore, BEA was found to exert antiangiogenic activity and migration inhibitory effects at sublethal concentrations in human cancer cell lines [11]. Recently, we have shown that also at higher concentrations (low μM) ENNs exert profound cytotoxic activity against human cancer cell lines based on apoptosis induction. In contrast, short-term ENNs-treatment with low, subtoxic concentrations (nM) significantly increased tumour cell proliferation, suggesting that frequent exposure to low ENNs concentrations by grain product consumption might have tumour promoting functions [12].

Based on these data, it might be hypothesized that both, ENNs and BEA, could cause yet unknown effects to the health of domestic animals and humans when entering the food chain. However, the human body is known to be highly protected against xenobiotics especially in the epithelial intestinal barrier. For example, the *Fusarium* mycotoxin nivalenol, a world wide natural contaminant of natural and processed grain, is a known substrate for the ATP-binding cassette (ABC) transporters *P*-glycoprotein (ABCB1) and ABCC2 [13]. Consequently, this study aimed to investigate the interaction of BEA and ENNs with key members of this large family of efflux pumps known to play important roles as detoxifiers by translocating a large number of hydrophobic drugs including peptides as well as its metabolites across cellular membranes [14].

2 Materials and methods

2.1 Chemicals and drugs

ENN_{mix}, a mixture of 3% A, 20% A_1, 19% B and 54% B_1 homologues (*ca*. 97% purity) was supplied together with BEA by Sigma – Aldrich (St. Louis, MO). ENN B was purchased from Eubio (Vienna, Austria). ENN_{mix}, ENN B and BEA are poorly soluble in water, therefore, stock solutions were frequently prepared in DMSO and stored at 4°C. All final mycotoxin solutions used were microscopically checked for lack of drug precipitation. In case of fluorescence-imaging studies, BEA and ENN stock solutions were prepared with Tween-80/MeOH = 1:2. Pheophorbide A (PhA) was obtained from Frontier Science (Logan, UT). Verapamil was purchased from Abbott (IL, USA), gefitinib from Astra Zeneca (Devon, UK), imatinib from Novartis (Basel, Switzerland) and cyclosporine A (CSA) was purchased from Sandoz (Basel, Switzerland). The radiolabelled [^{125}I] iodoarylazidoprazosin (IAAP; 2200 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Billerica, MA). Fura 2AM and Pluronic F-127 were procured from Molecular Probes (Leiden, The Netherlands). Adenosine-5′-triphosphate disodium salt hydrate (5′-ATP-Na_2) was purchased from Fluka Bio Chemika (Switzerland). All other compounds were supplied by Sigma – Aldrich.

2.2 Cell culture

The following human cell lines and their chemoresistant sublines were used in this study: the epidermal carcinoma-derived cell line KB-3-1 and its ABCB1-overexpressing subline KBC-1 (generously donated by Dr. D. W. Shen, Bethesda, MD) [15]; the promyelocytic leukaemia cell line HL60 and its multidrug resistance-associated protein-1 (ABCC1)-overexpressing subline HL60/adr and ABCB1-overexpressing subline HL60/vinc (by Dr. M. Center, Kansas State University, Manhattan, KS) [16]; the small cell lung carcinoma cell line GLC-4 and its ABCC1- and MVP-overexpressing subline GLC-4/adr (by Dr. E. G. deVries, Groningen, The Netherlands) [17]; the alveolar epithelial cell carcinoma cell line SW-1573 and its MVP- and ABCC1-overexpressing subline SW-1573/2R120 and ABCB1-overexpressing subline SW-1573/2R160 (by Dr. H. J. Broxterman, Amsterdam, The Netherlands) [18] and the breast adenocarcinoma cell line MDA-MB-231 with the respective breast cancer resistance protein (ABCG2)-transfected subclone MDA-MB-231/bcrp (both by Prof. Ross, University of Maryland, Greenbaum Cancer Center, Baltimore, MD) [19]. Additionally, the non small cell lung cancer cell line A549 and the colon carcinoma cell line CaCo-2 (from American Type Culture Collection, Manassas, VA) were used. The alveolar epithelial cell carcinoma cell line SW-1573 and its respective sublines were grown in DMEM supplemented with 10% fetal calf serum (FCS). All other cell lines were grown in RPMI-1640 supplemented with 10% FCS (PAA, Linz, Austria). Cultures were regularly checked for *Mycoplasma* contamination. Additionally, ABC-transporter-overexpression was confirmed by Western blotting of membrane-enriched fractions.

To generate ENNs- and BEA-resistant cell lines, KB-3-1 cells were exposed over a 2-year period in continuous steps

to ENN_{mix} or BEA starting at 0.1 μM. In case of unaltered cell proliferation for at least six passages, drug concentration was incremented by 0.1 μM. The mycotoxins were administered to KB-3-1 cells twice a week at the day after passage, when cells had attached to the culture flasks. ENN- and BEA-selected cells were termed KB/ENN and KB/BEA, respectively.

2.3 Fluorescence imaging

Caco-2 cells were loaded with 4 μM Fura 2AM and an equivalent concentration of Pluronic 20%, at room temperature (18 – 25°C) for 30 – 45 min. After washout, cells were allowed to equilibrate for 30 min to 1 h before they were added to the bath of the setup. Next, ENN and BEA stock solutions were diluted in the bathing solution to an appropriate concentration. When a new plateau level of the signal was reached due to an increase in intracellular calcium concentration $[Ca^{2+}]_i$, 5 mM 5′-ATP-Na_2 (ATP) was added to the bathing solution. As reference compound the calcium ionophore ionomycin (100 μM) was used. The bath was located at the stage of an inverted epifluorescence microscope (Axiovert 100, Zeiss) equipped with a Fluar x40/1.30 NA oil immersion objective. The dyes were alternately excited at 340 and 380 nm and the emitted fluorescence was collected at 510 nm. Excitatory light (xenon arc lamp) was filtered to the relevant wavelengths by means of an optical filter changer (Lambda 10-2, Sutter Instruments, CA), and emitted light was acquired by an intensified CCD camera (Extended ISIS, Photonic Science, UK). Interposing neutral density filters in the light path and a shutter mechanism of the filter changer attenuated light intensity. In addition, illumination time was kept to minimum to prevent photodynamic damage to the cells. Optical and interference filters were provided from Omega Optical, VT. Acquired images were corrected for background fluorescence, which was measured at respective wavelengths from a neighbouring cell-free area. Background subtraction and analysis was performed using Axon Imaging Workbench 2.2 (Axon Instruments). Results are presented as the F_{340}/F_{380} signal ratio. Control experiments confirmed the absence of solvents' interference with fluorescence measurements.

2.4 Cytotoxicity assays

Cell lines (KB-3-1, MDA-MB-231, HL60, GLC-4 and SW-1573 cells as well as their respective chemoresistant sublines and A549 cells) were seeded in 96-well plates at a density of 2×10^3 cells *per* well and allowed to recover for 24 h. ENN_{mix} and BEA were added in 100 μL of growth medium. For modulator studies, the two fusarotoxins were added in 50 μL growth medium and modulators in another 50 μL medium. Afterwards, cells were exposed for 72 h. The proportion of viable cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay (MTT) following the manufacturer's recommendations (EZ4U; Biomedica, Vienna, Austria). Cytotoxicity was expressed as IC_{50} values calculated from full dose – response curves.

2.5 Clonogenic assay

10^3 cells *per* well were seeded into six-well plates. Following 24 h recovery, cells were treated with ENN_{mix} and BEA, respectively. At day 7 of exposure, cells were washed twice with PBS, fixed with methanol at −20°C and stained with crystal violet. The number of colonies containing at least 100 single cells was determined by counting microscopically using a Leica DMIL (Leica, Solms, Germany).

2.6 Quantification of glutathione (GSH)

A cap-LC-MSD-TOF system (Agilent Technologies, Palo Alto, CA, USA) was used for the quantification of GSH. The Atlantis dC18 separation column (0.32 mm × 150 mm, Waters Corporation, USA) was employed. The RP separation was accomplished using 0.1% HCOOH in water containing 1% methanol (A) and 0.1% HCOOH in methanol containing 1% of water (B). Initial conditions were 5% B going after 4 min to 95% B within 1 min. After 3 min, 95% B initial conditions were re-established. The eluent flow rate was 3 μL/min. A sample volume of 0.3 μL was injected.

2.7 Measurement of ABCB1 and ABCG2 ATPase activity

The ABCB1 ATPase assays as well as the preparation of plasma membrane vesicles from CCRF-ADR500 cells (gift of Dr. V. Gekeler) were performed exactly as described previously [20]. The known ABCB1 modulator verapamil was used as positive control. ABCG2 ATPase activity was determined using the SB-BCRP-HAM Predeasy™ ATPase Kit (SOLVO Biotechnology, Hungary) according to the manual. Activity of transportes was measured as the vanadate sensitive portion of the total ATPase activity. Sulphasalazine (10 μM) was used as positive control for activation studies and Hoechst 33342 provided the control to show inhibition of fully activated membranes.

2.8 Photo-crosslinking of ABCB1 and ABCG2 with [^{125}I]IAAP

The photoaffinity labelling of ABCB1 and ABCG2 with [^{125}I]IAAP was done as described previously with minor modifications [21]. Briefly, crude membranes (1 μg protein/μL) from ABCG2-overexpressing MDA-MB-231/bcrp cells and ABCB1-overexpressing KBC-1 cells were incubated with various concentrations (0.1, 1 and 10 μM) of ENN_{mix} and BEA for 10 min at room temperature in 50 mM

Tris-HCl (pH 7.5). Verapamil and imatinib as well as the respective parental cell lines were used as controls. The samples were incubated for additional 5 min under subdued light with 3 – 6 nM [^{125}I]IAAP (2200 Ci/mmol) and then crosslinked with an UV Stratalinker™ 1800 (Stratagene, La Jolla, CA) for 30 min on ice. Subsequently, 10 μL of 4 × SDS-PAGE sample buffer was added, and the samples were incubated at 37°C for 1 h. The samples were separated on a 7.5% SDS gel at 90 V. The gels were dried and exposed to KODAK Medical X-ray Films/General Purpose/Blue (Eastman Kodak, Rochester, NY) for 3 – 6 days at −80°C.

2.9 Mycotoxin accumulation assay

1×10^6 drug-sensitive cells and their drug-resistant sublines (KB-3-1 and KBC-1, GLC-4 and GLC-4/adr, MDA-MB-231 and MDA-MB-231/bcrp) were seeded into 75 cm^2 flasks and allowed to attach for 48 h. After 2 h incubation with 20 μM ENN_{mix} or BEA, cells were washed twice with PBS, and the pellets resuspended in 500 μL lysis buffer for 10 min. Subsequently, lysates were centrifuged at 18 188 × *g* for 15 min. The supernatants were analysed by ESI triple quadrupole MS (LC/ESI-MS/MS) as previously described [22]. Cell lysates deriving from control experiments were fortified with known amounts of ENNs and BEA to determine matrix-induced suppression of the analytical signal and all results were corrected for these effects.

2.10 Flow cytometric detection of topotecan efflux function

A549 cells (1×10^6) were preincubated for 15 min with 10 and 20 μM ENN_{mix} or BEA. Additionally, samples treated with 10 and 20 μM gefitinib were prepared as positive controls. All samples were exposed to 100 μM topotecan for another 30 min at 37°C. Fluorescence of topotecan was analysed with fluorescence-activated cell sorting (FACS) analysis. Topotecan-derived fluorescence was measured through a 488 nm bandpass filter and an excitation wave length of 585 nm. Cells without topotecan were included as autofluorescence control.

2.11 Flow cytometric detection of PhA efflux function

Trypsinized A549 cells were incubated for 30 min in RPMI supplemented with 10% FCS and ENN_{mix} or BEA (5 and 10 μM) at 37°C in 5% CO_2. Imatinib (10 μM), a known ABCG2 inhibitor, was used as positive control. The chlorophyll catabolite PhA (10 μM) was added and the samples were incubated for another hour. Cells were washed subsequently with cold complete medium and then incubated for 1 h at 37°C in PhA-free complete medium. Samples were analysed by flow cytometry. PhA fluorescence was detected with a 488 nm argon laser and a 585 nm bandpass filter. Cells without PhA were included as autofluorescence control.

2.12 Rhodamine 123 (Rh123) accumulation assay

HL60 cells (5×10^5) and their ABCB1-overexpressing subline HL60/vinc were treated with rising concentrations of ENN_{mix} and BEA (0.25 – 5 μM) in FCS-free RPMI/HEPES medium, supplemented with MOPS. Rh123 was added and the samples were incubated for another hour at 37°C. Fluorescence of the cellular Rh123 accumulation was visualized by flow cytometry through a 530/30 nm bandpass filter using FACS Calibur (Becton Dickinson, Palo Alto, CA). The known ABCB1 modulator CSA was used as control.

2.13 Western blot analyses

Cell fraction, protein separation and Western blotting were performed as described [23]. Additionally, the same antibodies and dilutions were used.

3 Results

3.1 Protective effect of ATP against BEA- and ENN-induced Ca^{2+} overload

Previously, BEA was shown to raise the intracellular calcium concentration $[Ca^{2+}]_i$ in myocytes and in CaCo-2 cells [7]. Within a few minutes after addition of the positive control ionomycin at 100 μM (Fig. 1E) as well as 100 μM BEA and ENN_{mix} (Figs. 1A and C), the intracellular calcium concentrations $[Ca^{2+}]_i$ increased in Caco-2 cells. This effect was concentration-dependent (data not shown) and irreversible. However, addition of 5′-ATP-Na_2 (5 mM) to the bathing solution shortly after $[Ca^{2+}]_i$ had reached a new steady-state level led to a marked decrease of $[Ca^{2+}]_i$ (Figs. 1B and D). This effect was more pronounced for ENN (Fig. 1D) as compared to BEA (Fig. 1B) but in both cases clearly detectable. Since exogenous ATP addition led to a significant decline of the ENN- and BEA-induced $[Ca^{2+}]_i$ levels, we hypothesized that ATP-consuming processes might impair the ionophoric properties but probably also other cellular activities of these two cyclohexadepsipeptides. Since the ABC transport proteins ABCB1 and ABCC2 are expressed at the apical pole in Caco-2 cells and implicated in the active efflux of drugs and xenobiotics [24], we set out to investigate the role of multidrug resistance (MDR)-mediating ABC transports on the cytotoxic activity of ENN and BEA.

3.2 Cytotoxicity of ENN and BEA in chemosensitive and chemoresistant cell lines

In a first approach to investigate the impact of ABC-transporters on short-term BEA- and ENN-induced cytotoxicity,

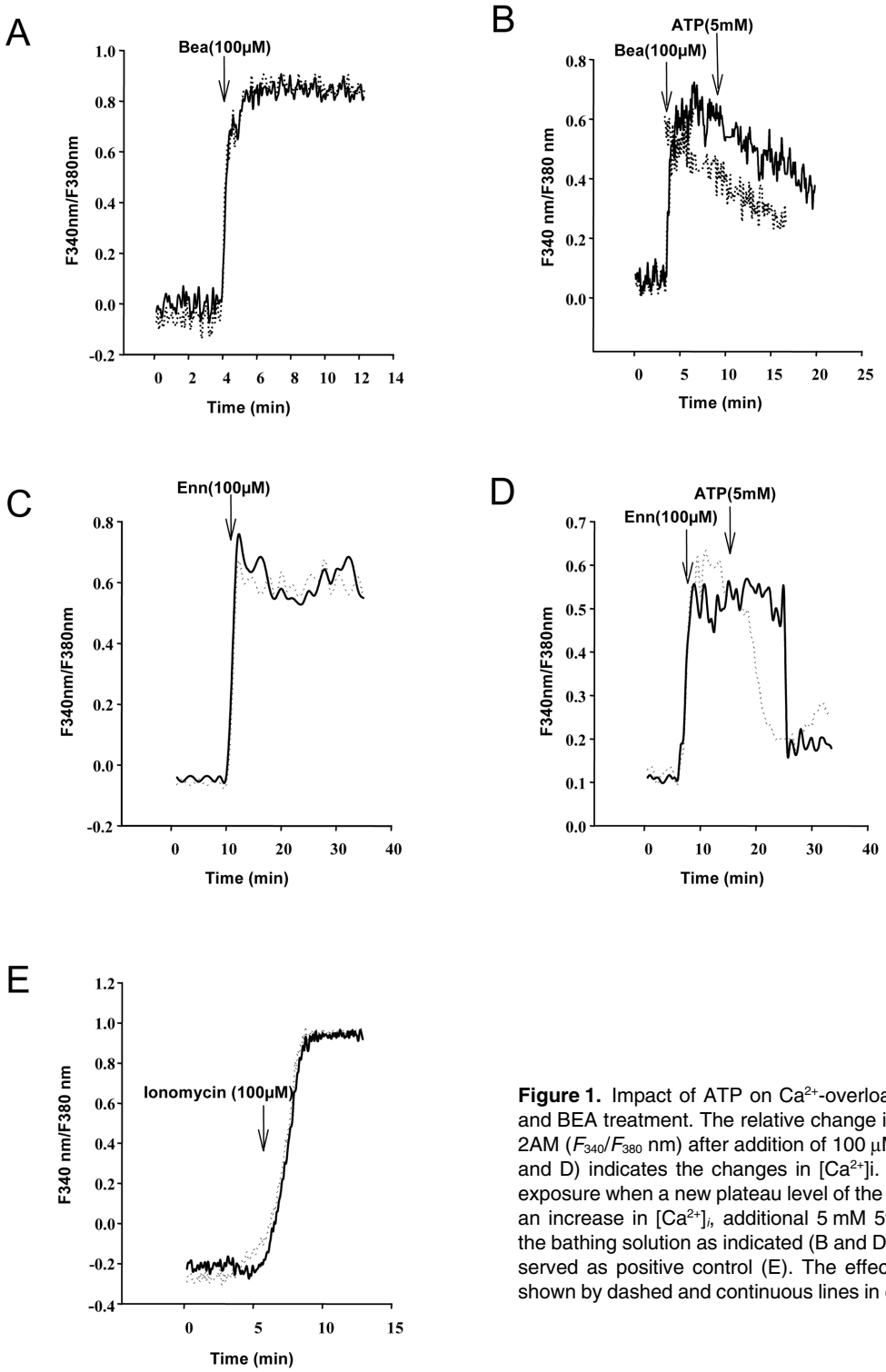

Figure 1. Impact of ATP on Ca^{2+}-overload of CaCo-2 cells after ENN_{mix} and BEA treatment. The relative change in the fluorescence ratio of Fura 2AM (F_{340}/F_{380} nm) after addition of 100 μM BEA (A and B) and ENN_{mix} (C and D) indicates the changes in $[Ca^{2+}]i$. After a few minutes mycotoxin exposure when a new plateau level of the signal had been reached due to an increase in $[Ca^{2+}]_i$, additional 5 mM 5′-ATP-Na_2 (ATP) was added to the bathing solution as indicated (B and D). Addition of 100 μM ionomycin served as positive control (E). The effect of two representative cells is shown by dashed and continuous lines in each graph.

concentration–response curves (1–10 μM; 72 h exposure time) were generated using chemoresistant sublines and their chemosensitive parental cell lines. The results are summarized in Tables 1–3. In general, the sensitivity of parental, unselected tumour cell lines against ENN_{mix} ranged from IC_{50} values of 1.74 μM (HL60) to 2.4 μM (GLC-4) and for ENN B from 2.13 μM (HL60) to 4.50 μM (MDA-MB-231). In case of BEA treatment, GLC-4 was the most sensitive and SW-1573 the most resistant parental cell line (IC_{50} 1.88 and 3.17 μM, respectively). With regard to chemoresistance, ABCG2-overexpression had a moderate but significant (~1.7-fold) protective effect against BEA

Table 1. Cytotoxic activity of ENN mixture against chemoresistant sublines compared to their chemosensitive parental lines

Cell line	IC_{50} (μM) (mean[a] ± SD)		Relative Resistance (-fold)[b]	Resistance mechanism[c]
KB-3-1	1.95	0.12		
KBC-1	1.77	0.04	0.9	ABCB1
HL60	1.74	0.2		
HL60/vinc	2.4	0.14	1.4	ABCB1
HL60/adr	2.1	0.12	1.2	ABCC1
GLC-4	2.4	1.53		
GLC-4/adr	1.41	0.83	0.6	ABCC1, MVP
MDA-MB-231	2.36	1.57		
MDA-MB-231/bcrp	3.18	1.70	1.3	ABCG2
SW-1573	2.16	0.12		
SW-15732/R120	2.50	0.74	1.2	ABCC1, MVP
SW-15737/2R160	2.69	1.06	1.2	ABCB1

a) IC_{50} values were calculated from whole dose–response curves. Values are given as mean ± SD out of three experiments, performed in triplicates.
b) Differences in ENN_{mix} sensitivity calculated by dividing IC_{50} values of the MDR subline by those of the parental cell lines.
c) ABCB1: *P*-glycoprotein (*P*-gp) acts as a transmembrane active efflux pump for a variety of drugs. ABCC1: The multidrug resistance protein 1 (MRP1) preferentially cotransports amphipathic organic anions, hydrophobic drugs or other compounds that are conjugated or complexed to GSH, to glucuronic acid, or to sulphate. ABCG2: breast cancer resistance protein (BCRP) functions as a high capacity drug transporter with broad substrate specificity. MVP: the major vault protein (MVP) also known as lung resistance protein (LRP) is expressed preferentially in epithelial cells as well as cells chronically exposed to xenobiotics and following antineoplastic drug exposure.

Table 2. Cytotoxic activity of ENN B against chemoresistant sublines compared to their chemosensitive parental lines

Cell line	IC_{50} (μM) (mean[a] ± SD)		Relative resistance (-fold)[b]	Resistance mechanism
KB-3-1	3.57	0.6		
KBC-1	4.18	0.5	1.2	ABCB1
HL60	2.13	0.2		
HL60/vinc	2.2	0.3	1.0	ABCB1
HL60/adr	2.6	0.0	1.0	ABCC1
GLC-4	3.21	0.7		
GLC-4/adr	2.90	0.5	0.9	ABCC1, MVP
MDA-MB-231	4.50	0.4		
MDA-MB-231/bcrp	4.57	0.05	1.2	ABCG2
SW-1573	4.28	0.18		
SW-1573/2R120	4.29	0.16	1.1	ABCC1, MVP
SW-1573/2R160	4.43	0.32	0.8	ABCB1

a) IC_{50} values were calculated from whole dose–response curves. Values are given as mean ± SD out of three experiments performed in triplicates.
b) Differences in ENN B sensitivity calculated by dividing IC_{50} values of the MDR subline by those of the parental cell lines.

but not against ENN in MDA-MB-231 cells. The expression of ABCB1 and ABCC1 had no significant impact on both cyclohexadepsipeptides. Additionally, presence of MVP, another protein up-regulated in multiple MDR cells not overexpressing ABCB1 [25], did not influence ENN-or BEA-induced cytotoxicity.

3.3 Impact of ENN and BEA on colony formation capacity in chemosensitive and chemoresistant cell lines

To investigate the effect of prolonged ENN_{mix} and BEA treatment, the capacity of single tumour cells to form colonies was analysed by clonogenic assays. Treatment with ENN_{mix} distinctly reduced colony formation in a dose-dependent manner in both KB-3-1 and ABCB1-overexpressing KBC-1 cells (Fig. 2A). In more detail, while no further colony formation occurred at 1 μM in KB-3-1 cells, the ABCB1-overexpressing subline was demonstrated to be significantly less sensitive at this concentration. In contrast, BEA treatment displayed a weaker effect on colony formation of KB-3-1 and KBC-1 cells but again ABCB1-overexpressing cells were less sensitive (Fig. 2B). A profound dose-dependent inhibition of colony formation after ENN_{mix} incubation was also found in MDA-MB-231 cells. The anticlonogenic activity of ENN_{mix} (Fig. 2C) and to a lesser extent of BEA (Fig. 2D) was significantly reduced in the ABCG2-transfected subline.

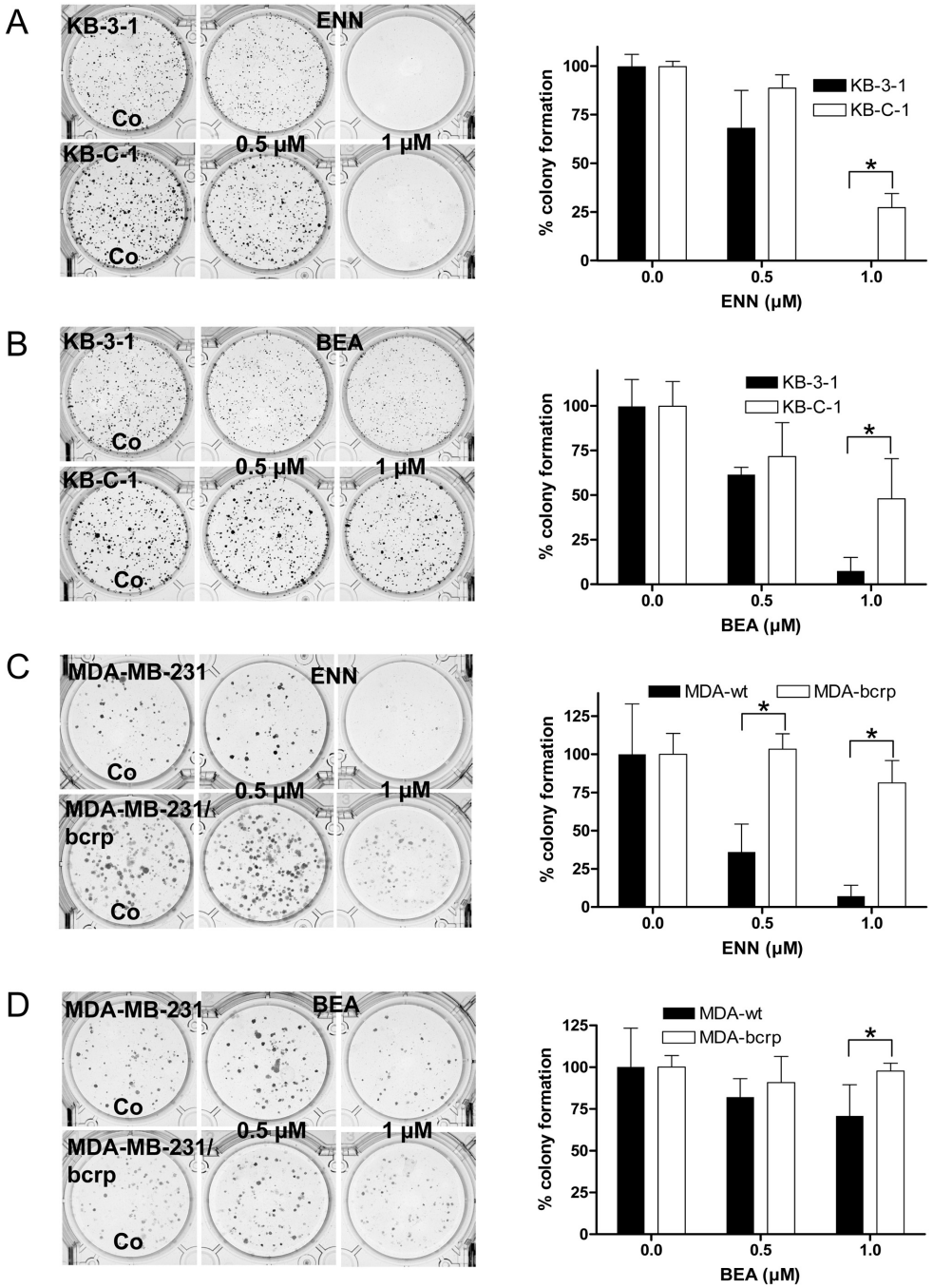

Figure 2. Anticlonogenic potential of ENN and BEA. Clonogenic survival after ENN_{mix} and BEA treatment of KB-3-1 and KBC-1 cells (A and B) as well as MDA-MB-231 and MDA-MB-231/bcrp cells (C and D) was determined. Cell colonies were visualized after 7 days fusariotoxin exposure at the indicated concentrations by crystal violet staining. One representative experiment out of three is shown in the left panels. In the right panels, numbers of colonies have been calculated as mean ± SD of three independent experiments and are expressed relatively to the respective controls (100%). * Differences of the two different cell lines were statistically significant; $p < 0.05$ (two way ANOVA, followed by Bonferroni test).

3.4 Impact of diverse ABC-transporter modulators on cytotoxicity of ENN and BEA

Several substances are known to inhibit ABC protein-mediated transport functions, and are consequently termed resistance modulators [26]. The impact of well-defined MDR modulators on the cytotoxic potential of ENN_{mix} and BEA was investigated in short-term MTT assays. The ABCG2 inhibitors imatinib and gefitinib were found to

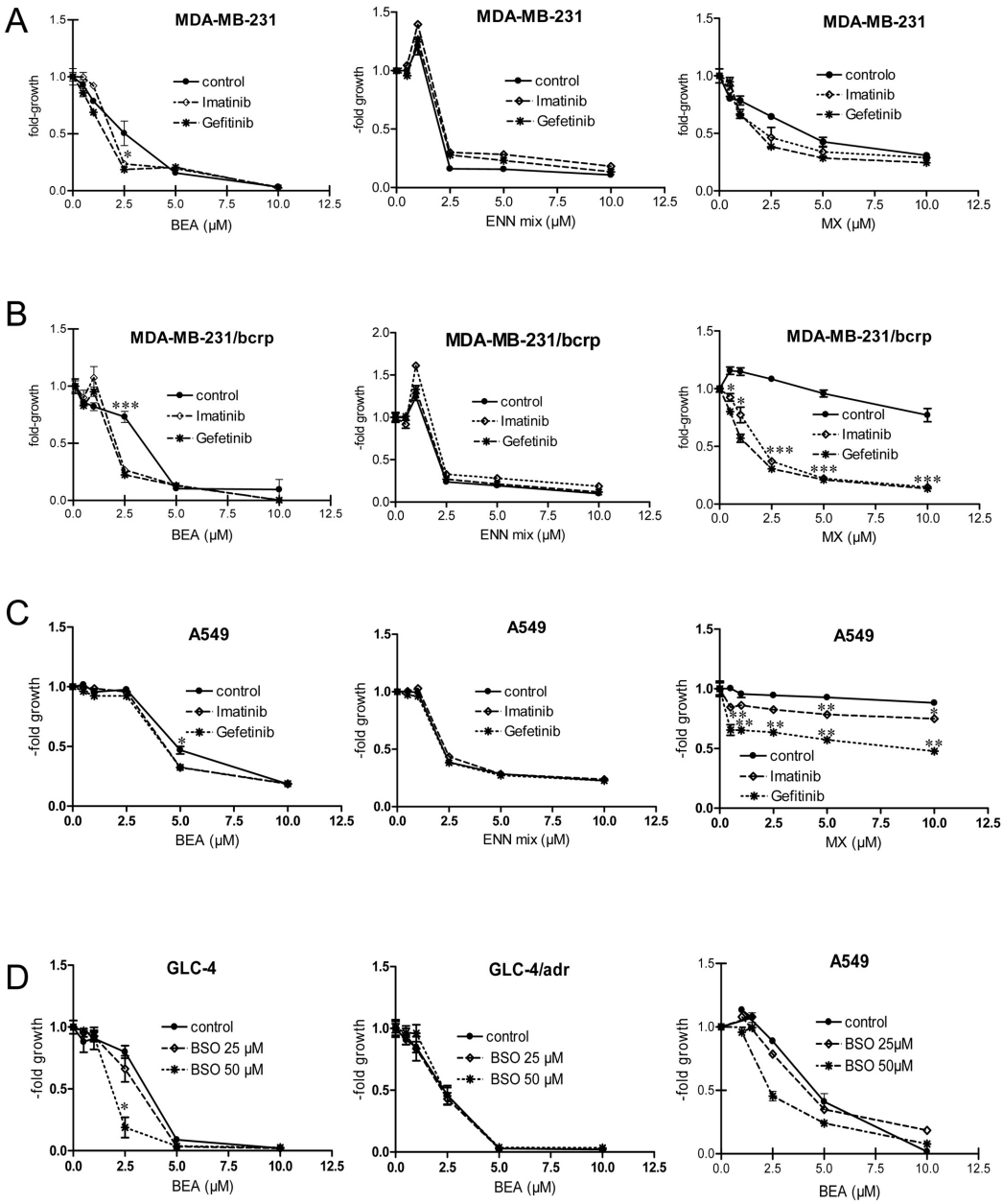

Figure 3. Impact of MDR modulators on the cytotoxic potential of ENN and BEA. MDA-231 (A), MDA-MB-231/bcrp (B) as well as A549 cells (C) were incubated for 72 h with the indicated BEA, ENN_{mix} and MX concentrations coadministered with the ABCG2 modulators imatinib (10 μM) and gefitinib (10 μM). A549, GLC4 and GLC-4/adr cells were pretreated for 24 h with 25 and 50 μM BSO (D). The secondary metabolites were administered at the indicated concentrations and vitality was measured by MTT assays after 72 h. Values given are mean ± SD of one representative experiment performed in triplicate. At least three experiments were done delivering comparable results. *, **, *** Significantly ($p < 0.05$, <0.01, <0.001, respectively) different from the control cells (one way ANOVA, followed by Dunett's test).

enhance BEA sensitivity in all tested ABCG2-positive cell lines with a significant effect in ABCG2-overexpressing MDA-MB-231 cells (Figs. 3A and B). Additionally, a minor BEA-sensitizing effect of both modulators was observed in endogenously ABCG2-overexpressing A549 cells (Fig. 3C). Comparable effects of the ABCG2 modulators were not obvious for ENN_{mix}. Further, ABCB1 and ABCC1 modulators like verapamil or CSA did not influence the cytotoxic activity of both *Fusarium* mycotoxins at this experimental setting (data not shown).

Unexpectedly, cotreatment with buthionine sulphoximine (BSO), known to inhibit ABCC1-mediated resistance by GSH depletion, sensitized parental GLC-4 cells but not its ABCC1-overexpressing subline GLC-4/adr against BEA

Table 3. Cytotoxic activity of BEA against chemoresistant sublines compared to their chemosensitive parental line

Cell line	IC_{50} (μM) (mean[a)] ± SD)		Relative resistance (-fold)[b)]	Resistance mechanism
KB-3-1	2.85	1.02		
KBC-1	3.79	0.20	1.3	ABCB1
HL60	2.27	0.04		
HL60/vinc	2.19	0.02	1.0	ABCB1
HL60/adr	3.23	0.74	1.4	ABCC1
GLC-4	1.88	0.03		
GLC-4/adr	2.06	0.29	1.1	ABCC1, MVP
MDA-MB-231	2.4	0.79		
MDA-MB-231/bcrp	4	0.76	1.7*	ABCG2
SW-1573	3.17	0.03		
SW-1573/2R120	3.53	0.74	1.1	ABCC1, MVP
SW-1573/2R160	3.00	1.1	0.9	ABCB1

a) IC_{50} values were calculated from whole dose–response curves. Values are given as mean ±SD out of three experiments, performed in triplicates.
b) Differences in BEA sensitivity calculated by dividing IC_{50} values of the MDR subline by those of the parental cell lines.* Significantly different from parental cell line ($p<0.05$).

treatment. Pretreatment (24 h) with 25 and 50 μM BSO caused a 1.2- and 1.7-fold sensitization to BEA in GLC-4 cells, respectively (Fig. 3D). A comparable sensitizing effect of BSO was observable in A549 cells (Fig. 3D). Hypothesizing that the disparity in the GLC-4 cell model might be based on different amounts of GSH, the GSH levels of the two cell lines were measured by a cap-LC-MSD TOF system. Indeed GLC-4 cells contain distinctly higher GSH levels than GLC-4/adr (20.1 *vs*. 11 μM/μg protein).

3.5 Impact of drug transporter overexpression on intracellular accumulation of ENN and BEA

Next, the influence of ABC-transporters on the cellular ENN and BEA accumulation was examined. For this purpose, the uptake of the tested depsipeptides was measured by LC/ESI-MS/MS using a respective cell model for each ABC transporter. In general, a more efficient (up to tenfold) accumulation was shown for BEA compared to ENN_{mix} in all tested cell lines (data not shown). Interestingly, ABC transport protein-overexpression had no significant effect on the accumulation potential of BEA (Fig. 4A) and ENN_{mix} (Fig. 4B). However, ABCG2 expression induced at least a tendency to reduce the intracellular levels of both cyclic peptides. When comparing the different ENN subtypes, the highest intracellular amount was measured in all tested cell lines for ENN A_1 (Figs. 4C–E). This is of interest because ENN A_1 comprises only 20% in the used mixture while ENN B_1 is represented with more than 50%. Consequently, this suggests a distinctly different uptake of ENN subtypes. Again no significant differences between parental and drug-resistant sublines were observed.

3.6 Effect of ENN and BEA on ABCB1 and ABCG2 ATPase activity

To investigate a direct interaction of the mycotoxins with ABCB1 and ABCG2, ATPase assays were performed. The ABCB1 ATPase activity was measured as previously described [20]. Compared to the positive control verapamil, both fusariotoxins showed a similar ATPase stimulating activity of ABCB1 (Fig. 5A). The dose–response curve of ENN_{mix} revealed a biphasic interaction pattern with maximal stimulation at 1 μM (1.48-fold) and inhibition at 100 μM (Fig. 5B). ENN B showed lower ATPase stimulation (1.3-fold) than ENN_{mix} reaching a plateau at about 3 μM (data not shown). ABCB1 ATPase measurements after BEA treatment revealed a weaker stimulation (1.29-fold) compared to ENN_{mix}. Again the maximum was observed at 1 μM BEA while a reduction of ATPase activity below basal was observed already at 30 μM (Fig. 5C). Both cyclic depsipeptides dose-dependently inhibited the ABCG2 ATPase activity (Figs. 5D and E). The activation test control sulphasalazine stimulated vanadate sensitive ATPase activity from 21.14 nmol Pi/mg protein/min to 31.4 nmol Pi/mg protein/min. In contrast, the inhibitor Hoechst 33342 inhibited the maximal vanadate sensitive ATPase activity to –16.16 nmol Pi/mg protein/min (data not shown).

3.7 ENN and BEA as ABCG2 modulators

In order to investigate whether ENN_{mix} and BEA reverse ABCG2-mediated drug resistance, the cyclic hexadepsipeptides were administered at low, subtoxic concentrations (0.5 and 1 μM) to ABCG2-overexpressing cells together with the well known ABCG2 substrate drug mitoxantrone (MX). After 72 h incubation in these subtoxic concentrations neither ENN_{mix} nor BEA had any effect on ABCG2-mediated resistance (data not shown).

To test short-term effects of ENN_{mix} and BEA on the ABCG2 transport function, efflux assays with the ABCG2-specific substrates PhA and topotecan in endogenously ABCG2-overexpressing A549 cells were performed. In contrast to topotecan, which is reported to be effluxed by multiple ABC transporters including ABCG2, ABCB1 as

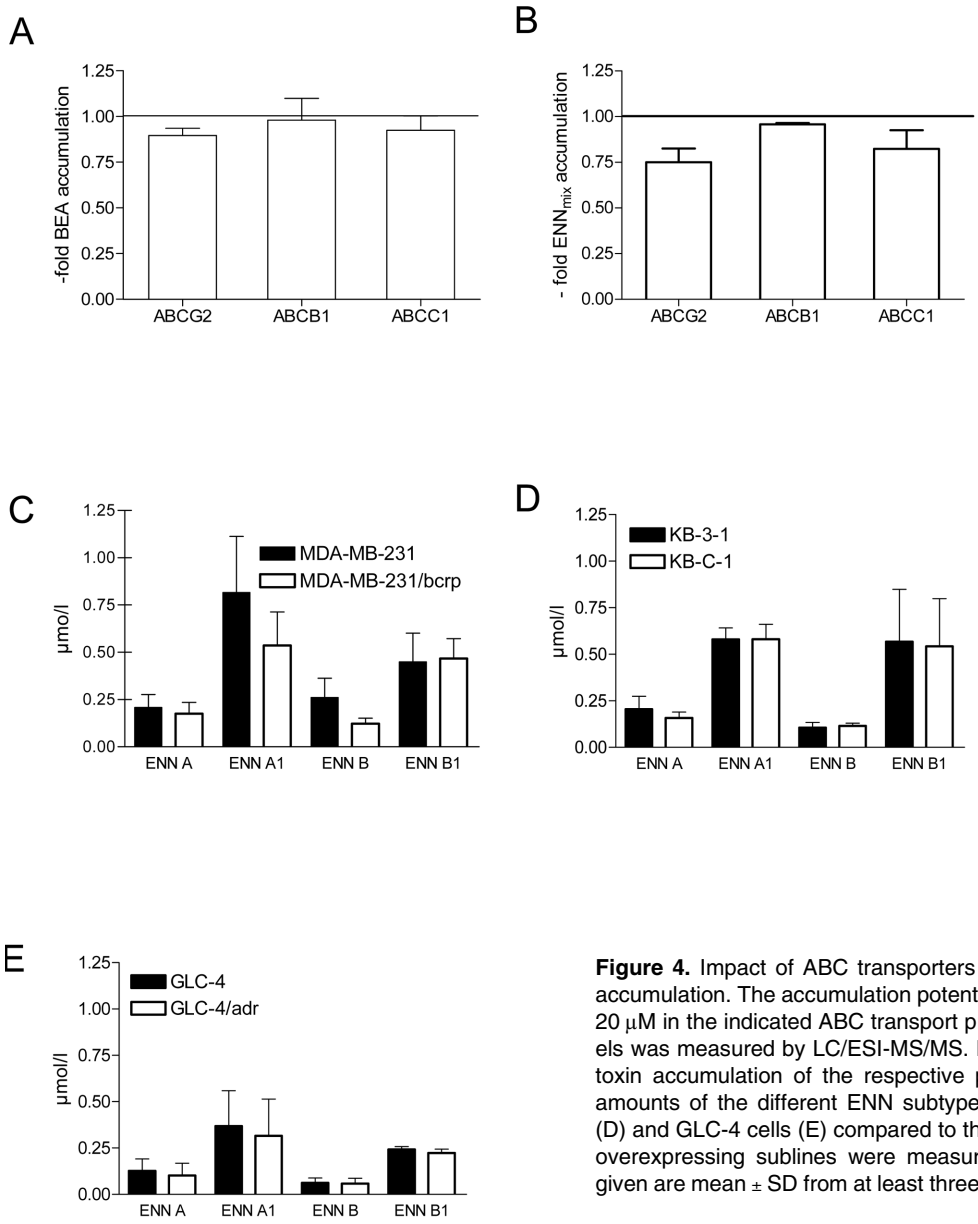

Figure 4. Impact of ABC transporters on intracellular ENN and BEA accumulation. The accumulation potential of BEA (A) and ENN_{mix} (B) at 20 µM in the indicated ABC transport protein-overexpressing cell models was measured by LC/ESI-MS/MS. Data were normalized to mycotoxin accumulation of the respective parental cell lines. Intracellular amounts of the different ENN subtypes in MDA-MB-231 (C), KB-3-1 (D) and GLC-4 cells (E) compared to their respective ABC transporter-overexpressing sublines were measured by LC/ESI-MS/MS. Values given are mean ± SD from at least three independent experiments.

well as ABCC1, the chlorophyll catabolite PhA is an ABCG2-specific substrate [27]. As shown in Fig. 6A, topotecan accumulation was significantly increased in A549 cells treated with BEA compared to untreated cells, while ENN_{mix} had no effects. The modulating effect of BEA at 10 µM was comparable to the one of the known ABCG2 modulator gefitinib. Comparable results for BEA were obtained using the ABCG2-specific substrate PhA (Fig. 6B). Interestingly, in this setting also ENN_{mix} inhibited the drug efflux function of ABCG2, indicating that both secondary metabolites are ABCG2 efflux pump inhibitors, however, with slightly differing interaction characteristics.

As a final approach to elucidate the interaction of the two *Fusaria* mycotoxins with ABCG2, ENN_{mix} and BEA competition for [^{125}I]IAAP labelling of ABCG2 was investigated. As indicated in Fig. 6C, ENN_{mix} potently inhibited the photoaffinity labelling of ABCG2 with [^{125}I]IAAP at 1 µM. Similar results were obtained for BEA but changes were less pronounced.

3.8 ENN and BEA as ABCB1 modulators

The ABCB1-modulating effects of ENN_{mix} and BEA were analysed using the ABCB1 substrate daunomycin. Also in this setting, subtoxic concentrations of the two secondary metabolites administered for 72 h did not modulate ABCB1 mediated resistance (data not shown).

Next, it was tested whether ENN_{mix} or BEA impair the Rh123-efflux of ABCB1-overexpressing HL60/vinc cells. As shown in Fig. 6D, both cyclic peptides caused a minor

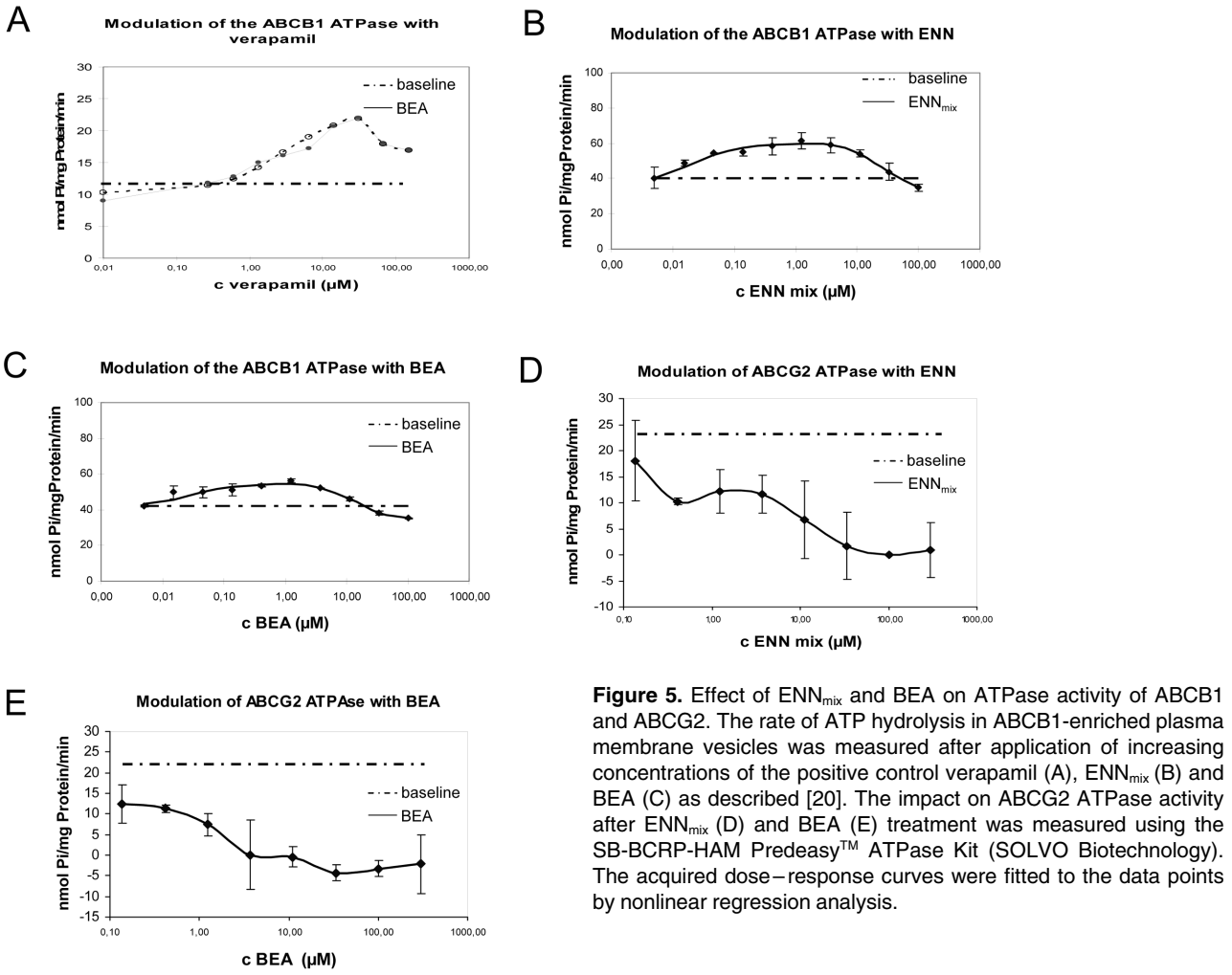

Figure 5. Effect of ENN_{mix} and BEA on ATPase activity of ABCB1 and ABCG2. The rate of ATP hydrolysis in ABCB1-enriched plasma membrane vesicles was measured after application of increasing concentrations of the positive control verapamil (A), ENN_{mix} (B) and BEA (C) as described [20]. The impact on ABCG2 ATPase activity after ENN_{mix} (D) and BEA (E) treatment was measured using the SB-BCRP-HAM Predeasy™ ATPase Kit (SOLVO Biotechnology). The acquired dose–response curves were fitted to the data points by nonlinear regression analysis.

but significant inhibition of the ABCB1-mediated Rh123 efflux at lower concentrations (0.5–1 µM) in the ABCB1-expressing subline. In general, BEA was a more effective inhibitor of ABCB1 than ENN_{mix} in this short-term assay. Surprisingly, this inhibitory effect was abolished at higher concentrations >1 µM, probably based on cytotoxic effects of ENN_{mix} and BEA.

Comparable to the results obtained with ABCG2, ENN_{mix} slightly inhibited the photoaffinity labelling of ABCB1 at 0.1 µM whereas the addition of ≥1 µM potently abolished crosslinking (Fig. 6E). As in case of ABCG2, BEA was less effective than ENN_{mix} in this aspect.

3.9 Generation and characterization of ENN- and BEA-resistant cell lines

Continuous and long-term exposure to low up to critical concentrations of a cytotoxic compound may result in selection of a drug-resistant cell population [28]. Thus, we aimed to establish a BEA- as well as an ENN_{mix}-resistant KB-3-1 subline by stepwise selection against increasing concentrations of the two secondary metabolites. After 18 months of selection, the cells were growing at the highest achievable concentration of 1.95 µM and displaying a significant (~two-fold) resistance against ENN_{mix} (Fig. 7A). Further increase of the selection pressure resulted repeatedly in cell death. In contrast, creating a significantly BEA-resistant cell model widely failed (Fig. 7B).

Interestingly, the resulting KB-3-1 sublines (KB/ENN and KB/BEA) displayed significant resistance against anticancer drugs including cisplatin (6- and 1.9-fold, respectively) and daunomycin (21- and 10-fold, respectively) (Figs. 7A and B). In addition, Western blot analysis was performed to characterize changes in the expression levels of several drug resistance proteins (ABCB1, ABCC1, ABCC2, ABCG2 and MVP) in KB/ENN and KB/BEA cells compared to the one of parental KB-3-1 cells. Consistent with the acquired cisplatin and daunomycin resistance, a profound induction of ABCC1 and ABCC2 in the resistant cell lines was detectable (Fig. 7C). Moreover, a strong up-

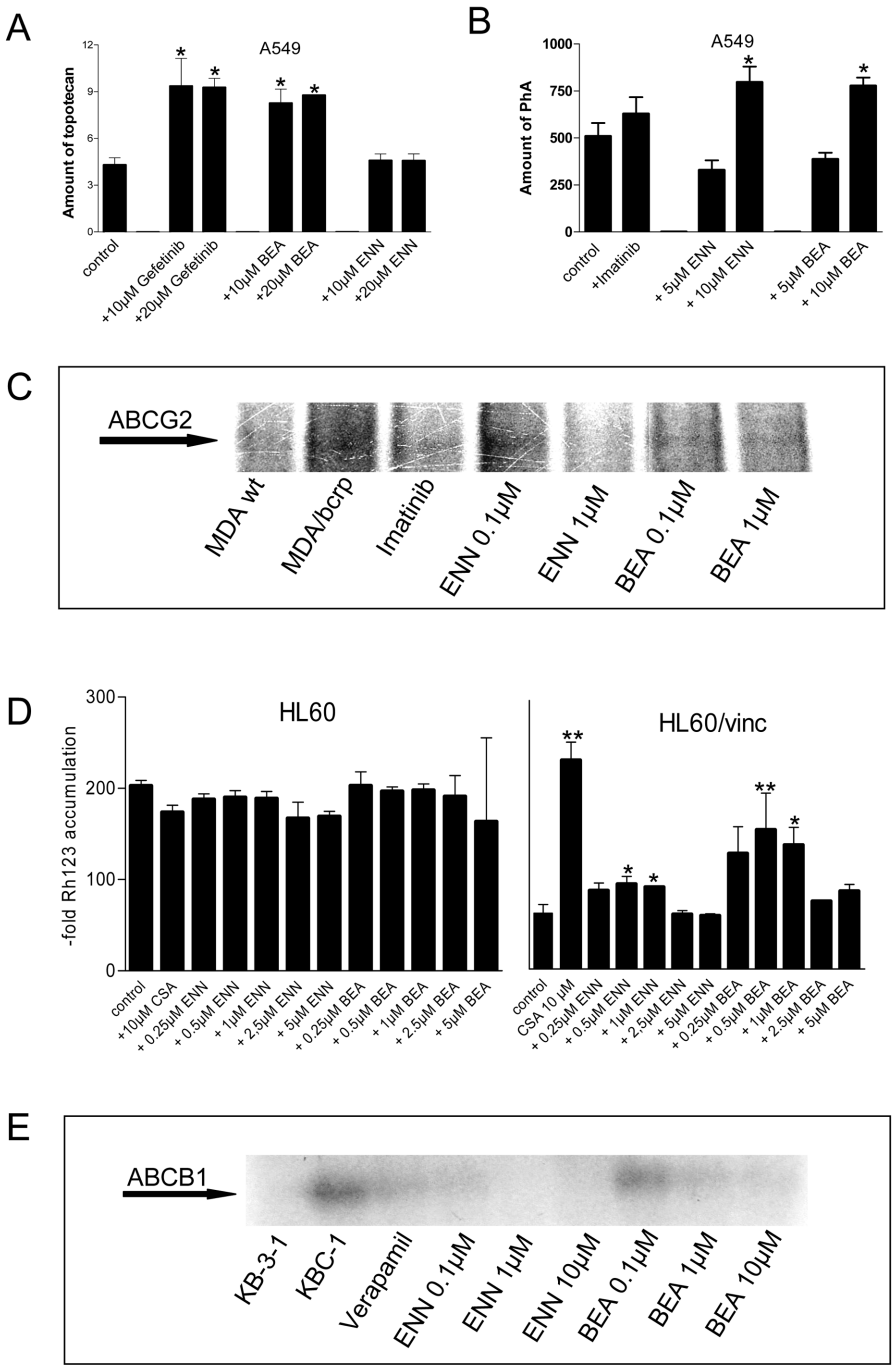

Figure 6. Interaction of ENN_{mix} and BEA with ABCG2 and ABCB1 transport functions. The modulating effect of ENN_{mix} and BEA compared to the positive controls imatinib or gefitinib on ABCG2-mediated transport in A549 cells was analysed by flow cytometry using the fluorescent substrates topotecan (A) and PhA (B). Values given are mean ± SD from three independent experiments. *Significantly ($p < 0.05$) different from the control cells (one way ANOVA, followed by Dunett's test). (C) Crude membranes (1 μg/mL) of ABCG2-overexpressing MDA-MB-231/bcrp cells were photoaffinity labelled with 3 – 6 nM [^{125}I]IAAP (2200 Ci/mmol) as described under Section 2 in the presence of the indicated drugs for 10 min at room temperature. The autoradiogram from one representative experiment out of three is shown. (D) Rh123 accumulation in HL60 and ABCB1-overexpressing HL60/vinc cells with and without coadministration of ENN_{mix}, BEA and the positive control CSA at the indicated concentrations was measured after 1 h drug exposure by FACS analysis. Values represent the mean of three experiments ± SD **, * Significantly ($p < 0.01$ and $p < 0.05$, respectively) different from the control cells (one way ANOVA, followed by Dunett's test. (E) Crude membranes (1 μg/mL) from ABCB1-overexpressing KBC-1 cells were photoaffinity labelled as described under (C). One of three experiments delivering comparable results is shown.

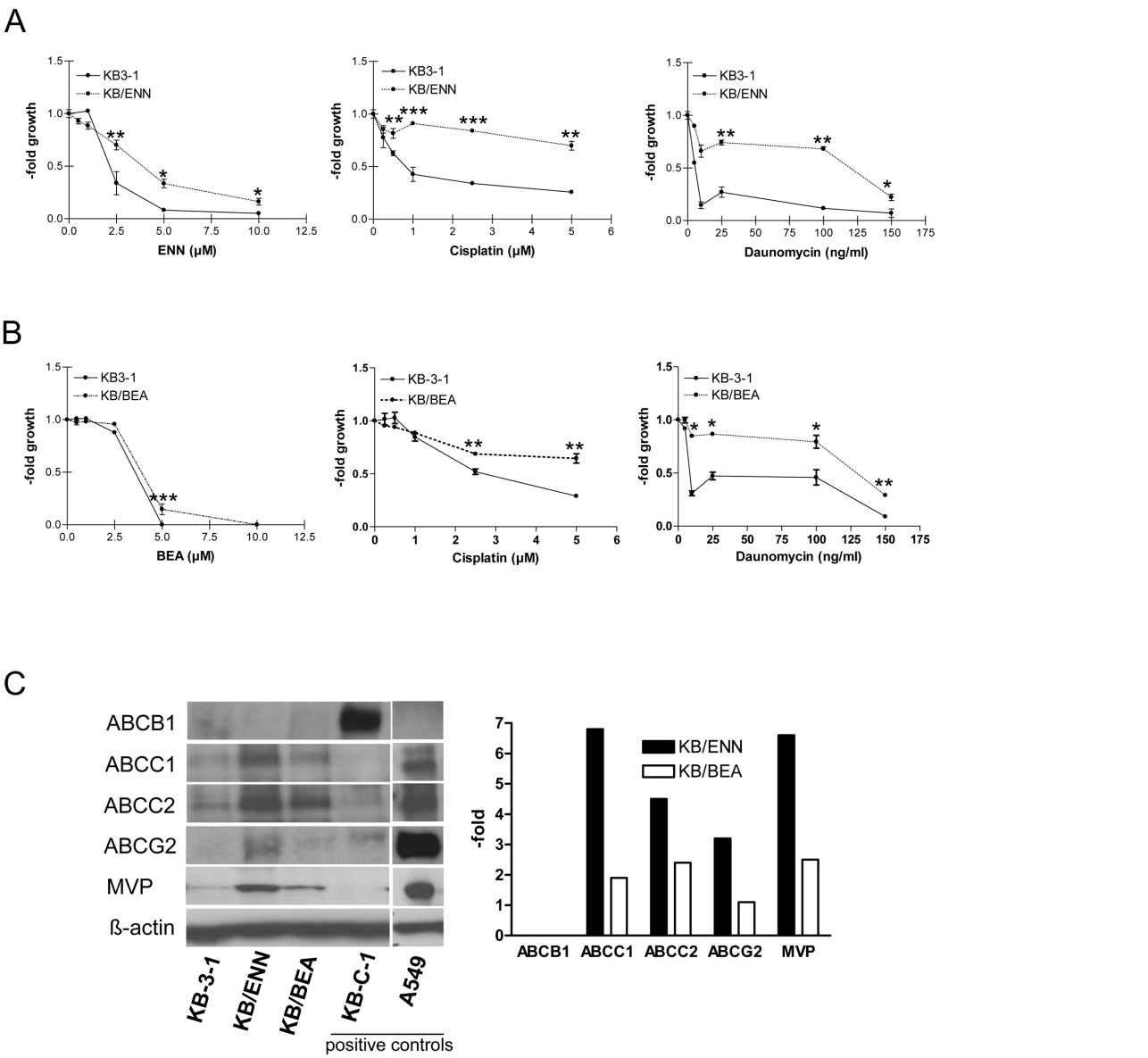

Figure 7. Characterization of ENN_{mix}- and BEA-selected KB-3-1 cells. Concentration–response curves were established for (A) ENN_{mix}, cisplatin and daunomycin with the selected KB/ENN cells compared to the parental cell line. Following 72 h drug exposure, cell viability was determined by MTT assays. (B) Concentration–response curves of the selected KB/BEA compared to the parental cells after 72 h drug exposure with BEA, cisplatin and daunomycin are shown as mean ± SD of triplicates. ***, **, * Significant ($p <$ 0.001, <0.01 and <0.05, respectively) difference between the tested cell lines (*t*-test with Welch's correction). (C) ABCB1, ABCC1, ABCC2, ABCG2 and MVP expression levels of the indicated cell lines were measured in membrane-enriched fractions by Western blotting. In the right panel, the expression levels quantified by Molecular Analyst software (BioRad) are shown. The control was set as one and β-actin expression served as loading control.

regulation of MVP as well as a weak expression of ABCG2 was obvious. In contrast, ABCB1 was not induced by ENN_{mix} or BEA treatment.

4 Discussion

ENNs and BEA, two secondary metabolites of the genus *Fusarium*, are worldwide contaminants of grain-based foodstuff [29]. Especially in Norwegian and Finnish grain samples, a high prevalence of ENNs and BEA was shown with a maximum concentration of 18 300 ppb for ENN B in the year 2001 [30]. Such contamination levels raise serious concerns about potential impacts of these fusariotoxins on human and animal health. However, toxicological and toxicokinetic data on BEA and ENNs are fragmentary. On the one hand, low to negligible effects of BEA-contaminated

Table 4. Interaction of ENN and BEA with ABCB1 and ABCG2 in different test systems

Test system	ENN		BEA	
	ABCB1	ABCG2	ABCB1	ABCG2
MTT assays	–	–	–	+
Clonogenic assays	+	++	+	+
Modulation assays	–	–	–	+
Accumulation assays	–	–	+	–
ATPase activity	+	+	+	+
Efflux assays	+	+	++	++
Photoaffinity labelling	++	+	+	+

–, No interaction; +, moderate interaction; ++, strong interaction.

grain have been indicated by several feeding studies in poultry [31, 32]. On the other hand, intraperitoneal injection of ENNs into mice was accompanied by severe cytotoxicity [33]. Accordingly, several *in vitro* studies including our own, demonstrated potent cytotoxic activities of BEA and ENNs at low μM doses [10, 12, 34]. These data implicate that at least avian organisms might be protected from these secondary metabolites by the intestinal epithelial barrier mainly executed by ATP-driven efflux pumps, *e.g.* ABCB1, exporting toxins back into the luminal space of the gut [35]. Consistently, several structurally related hydrophobic peptides and ionophores like valinomycin, gramicidin D, CSA [36] and aureobasidins [37, 38] are known to be substrates for the human ABC transporter ABCB1. Moreover, it has been suggested previously that ENNs are substrates for the human ABCB1 functional ortholog Pdr5p in *Saccharomyces cerevisiae* [39].

A second indication that energy-dependent transport systems might interact with the investigated cyclic peptides came from electrophysiological experiments. ENNs and BEA possess pore-forming and ionophoric properties which have been connected to their cytotoxic activities [5, 40, 41]. Our initial experiments demonstrated that the impact of ENNs and BEA on ion homeostasis in CaCo-2 cells can be partially reversed by addition of ATP. Thus, we hypothesized that ATP-consuming processes interact with ENNs- and BEA-induced cytotoxicity, especially, since ABCB1, ABCC1-6 and ABCG2 are known to be localized in the luminal membranes of CaCo-2 cells [42]. Moreover, some of these ABC-transport proteins have already been shown to counteract absorption and enhance excretion of other fusariotoxins like nivalenol, deoxynivalenol and fumonisin B1 [13, 43, 44].

Based on these observations, we set out to investigate possible interactions of ENNs and BEA with several important detoxifying ABC transporters (Table 4). Interestingly, the short-term (72 h) cytotoxicity of ENNs and BEA was not significantly influenced by overexpression of ABCB1, ABCC1 or the non-ABC transporter-MDR protein MVP [25]. In contrast, overexpression of ABCG2 weakly but significantly reduced BEA- but not ENNs-induced cytotoxicity against human breast cancer cells in these experiments. Corroboratingly, the ABCG2 inhibitors gefitinib and imatinib [45] significantly sensitized ABCG2-positive cells against BEA only. Interestingly, BSO known to inhibit ABCC1-mediated resistance by GSH depletion-sensitized several cell models against BEA in an ABCC1-independent manner. In contrast, this sensitizing activity seemed to be dependent on cellular GSH levels. Thus, for example the GLC4 parental cell line with high GSH content but negligible ABC transporter expression responded to BSO preexposure while the highly ABCC1- and MVP-positive subline GLC/adr showed no effect. This suggests an efflux-independent protective effect of GSH against BEA-induced cytotoxicity which might be based on the proposed oxidative function of BEA leading to increased lipid peroxidation and decreased cellular GSH levels [46].

In long-term exposure (clonogenic) assays, ABCG2 conferred low but significant resistance against both secondary metabolites. Interestingly, in this experimental setting also ABCB1-overexpressing KBC-1 cells exhibited significant insensitivity against ENNs and BEA. These data further indicate that ABCG2 and to a lesser extend ABCB1 might protect from toxic impacts of ENNs and BEA, fitting well with the ability of ABCG2 to extrude preferentially large hydrophobic molecules. Moreover, ABCG2 is believed as a main barrier of the body against harmful xenotoxins because of its broad substrate specificity and its highest expression levels in potentially toxin-exposed tissues including intestine, liver and placenta [47]. Consequently, the functional expression of ABCG2 in the intestinal epithelium suggests a reduced bioavailability of ENNs and BEA *via* food intake.

Interestingly, although both ABCG2 and to a less extent ABCB1 significantly protected human cells against the toxic impacts of BEA and/or ENNs we could only detect a trend towards reduced cellular accumulation in overexpressing cell models. This might suggest that the interaction of ENNs and BEA with the ABC transporters at cell membranes interfere with the mycotoxins pore-forming properties or prevent them from reaching other intercellular targets than to generally reduce intracellular accumulation.

These effects might be enhanced by the high lipophilicity of these compounds [48, 49]. These hypotheses clearly need to be tested and are topic of ongoing investigations.

Generally, a ten-fold higher cellular accumulation potential for BEA as compared to ENNs was observed. This is consistent with animal data from the Jestoi group [49]. In their study, BEA levels were higher as compared to ENNs in Finnish poultry tissues [49] although their relative amounts in the respective grain samples were ENN B > ENN B1 > ENN A1 > ENN A > BEA [30]. Comparably, different accumulation potentials for the distinct ENN subtypes were observed in our study. ENN A_1 demonstrated the highest intracellular accumulation in all cell lines tested although this ENN subtype comprises only 20% in the used ENN_{mix} while ENN B_1 represents 50%. Consequently, this suggests a distinctly different uptake of ENN subtypes and BEA probably based on their divergent lipophilic characters: BEA (phenylmethyl-side chains) > ENN A_1 (two *N*-methylisoleucine, one *N*-methylvaline) > ENN B_1 (one *N*-methylisoleucine, two *N*-methylvaline) [48, 49]. Thus, a more potential health hazard of BEA-contaminated food and feed compared to ENNs might be assumed.

The low BEA and ENNs transporting/resistance efficacy became even more surprising by demonstrating potent molecular interaction of both mycotoxins with ABCG2 and ABCB1. Both substances strongly inhibited photoaffinity labelling of the two transporters with [^{125}I]IAAP already at 1 μM. Moreover, both secondary metabolites inhibited the ABCB1- and ABCG2-mediated efflux transport of substrate drugs including Rh123, topotecan and PhA. Additionally, the ATPase activity of both transporters was modulated by the cyclic peptides. In case of ABCB1, a biphasic stimulation/inhibition pattern of ATPase modulation was obtained resembling those of the known ABCB1 modulator verapamil. Comparable data for BEA have also been reported by Sharom *et al*. [50]. Interestingly, for the structurally related peptide ionophore valinomycin ATPase stimulation without transport inhibition was described [36]. In contrast, both BEA and ENNs dose-dependently inhibited the ABCG2 ATPase activity in our study. Comparable observations have also been published for the mycotoxins fumitremorgin C and cyclosporin A [51, 52]. With regard to the importance of ABC-transporters for excretion of xenobiotics and drugs, our data suggest possible unwanted interactions of the fusariotoxins with detoxification processes of other toxic compounds [53]. This is even more important as these inhibitory effects occur at low mycotoxin concentrations which might be reached due to food consumption and based on tissue accumulation [49]. Moreover, with regard to food intake, ENNs and BEA might increase the absorption of commonly co-occurring *Fusarium* mycotoxins probably leading to enhanced toxicity.

Chronic exposure of KB-3-1 cells to stepwise increasing ENNs or BEA concentrations resulted in a cell model displaying two-fold ENNs resistance, while development of BEA resistance widely failed. This low resistance values are in sharp contrast to published data about selection against diverse chemotherapeutics [15, 54]. However, while lacking strong resistance against the selection drugs, the resulting sublines KB/ENN and KB/BEA exhibited a chemoresistance phenotype against, *e.g*. cisplatin and daunomycin. Accordingly, Western blot analyses demonstrated in both sublines profound induction of several proteins mediating, *e.g*. daunomycin (ABCC1, ABCG2) and cisplatin resistance (ABCC2, MVP) [14, 55, 56] while no induction of ABCB1 expression could be observed. Taken together, these results suggest that exposure to low, subtoxic ENNs and BEA concentrations activates diverse cellular stress-response and protection systems. Although these changes were insufficient to protect from the cytotoxic effects of higher ENNs and BEA concentrations (>1.95 μM), they conferred distinct resistance against chemotherapeutic drugs. This indicates that also continuous exposure to ENNs and BEA might lead to altered bioavailability and pharmacokinetic properties of other toxic compounds or pharmaceuticals.

In summary, our data demonstrate that some ABC transport proteins moderately but significantly protect human cells against the impacts of the fusariotoxins ENNs and BEA. In contrast, the potent inhibition of drug efflux pumps and the induction of drug resistance protein expression by the fusariotoxins suggest that these food contaminants might exert substantial impacts on the bioavailability, pharmaco(toxico)kinetics and efficacy/toxicity of both remedies and environmental toxins.

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5 References

[1] Klyszejko, A., Kubus, Z., Zakowska, Z., Mycological analysis of cereal samples and screening of Fusarium strains' ability to form deoxynivalenole (DON) and zearalenone (ZEA) mycotoxins – A pilot study, *Pol. J. Microbiol*. 2005, *54 (Suppl.)*, 21 – 25.

[2] Jestoi, M., Rokka, M., Yli-Mattila, T., Parikka, P., *et al*., Presence and concentrations of the Fusarium-related mycotoxins beauvericin, enniatins and moniliformin in finnish grain samples, *Food Addit. Contam*. 2004, *21*, 794 – 802.

[3] Wilson, J. P., Jurjevic, Z., Hanna, W. W., Wilson, D. M., *et al*., Host-specific variation in infection by toxigenic fungi and contamination by mycotoxins in pearl millet and corn, *Mycopathologia* 2006, *161*, 101 – 107.

[4] Tomoda, H., Huang, X. H., Cao, J., Nishida, H., *et al*., Inhibition of acyl-CoA: Cholesterol acyltransferase activity by cyclodepsipeptide antibiotics, *J. Antibiot. (Tokyo)* 1992, *45*, 1626 – 1632.

[5] Kamyar, M., Rawnduzi, P., Studenik, C. R., Kouri, K., Lemmens-Gruber, R., Investigation of the electrophysiological properties of enniatins, *Arch. Biochem. Biophys.* 2004, *429*, 215 – 223.

[6] Kouri, K., Kamyar, M. R., Lemmens-Gruber, R., Actions of the antibiotic mycotoxins beauvericin and enniatin on mammalian tissue, *Forum Nutr*. 2003, *56*, 413 – 414.

[7] Kamyar, M. R., Kouri, K., Rawnduzi, P., Studenik, C., Lemmens-Gruber, R., Effects of moniliformin in presence of cyclohexadepsipeptides on isolated mammalian tissue and cells, *Toxicol. In Vitro* 2006, *20*, 1284 – 1291.

[8] Calo, L., Fornelli, F., Ramires, R., Nenna, S., *et al*., Cytotoxic effects of the mycotoxin beauvericin to human cell lines of myeloid origin, *Pharmacol. Res*. 2004, *49*, 73 – 77.

[9] Calo, L., Fornelli, F., Nenna, S., Tursi, A., *et al*., Beauvericin cytotoxicity to the invertebrate cell line SF-9, *J. Appl. Genet*. 2003, *44*, 515 – 520.

[10] Jow, G. M., Chou, C. J., Chen, B. F., Tsai, J. H., Beauvericin induces cytotoxic effects in human acute lymphoblastic leukemia cells through cytochrome c release, caspase 3 activation: The causative role of calcium, *Cancer Lett.* 2004, *216*, 165 – 173.

[11] Zhan, J., Burns, A. M., Liu, M. X., Faeth, S. H., Gunatilaka, A. A., Search for cell motility and angiogenesis inhibitors with potential anticancer activity: Beauvericin and other constituents of two endophytic strains of Fusarium oxysporum, *J. Nat. Prod*. 2007, *70*, 227 – 232.

[12] Dornetshuber, R., Heffeter, P., Kamyar, M. R., Peterbauer, T., *et al*., Enniatin exerts p53-dependent cytostatic and p53-independent cytotoxic activities against human cancer cells, *Chem. Res. Toxicol.* 2007, *20*, 465 – 473.

[13] Tep, J., Videmann, B., Mazallon, M., Balleydier, S., *et al*., Transepithelial transport of fusariotoxin nivalenol: Mediation of secretion by ABC transporters, *Toxicol. Lett.* 2007, *170*, 248 – 258.

[14] Gottesman, M. M., Fojo, T., Bates, S. E., Multidrug resistance in cancer: Role of ATP-dependent transporters, *Nat. Rev. Cancer* 2002, *2*, 48 – 58.

[15] Shen, D. W., Cardarelli, C., Hwang, J., Cornwell, M., *et al*., Multiple drug-resistant human KB carcinoma cells independently selected for high-level resistance to colchicine, adriamycin, or vinblastine show changes in expression of specific proteins, *J. Biol. Chem*. 1986, *261*, 7762 – 7770.

[16] McGrath, T., Center, M. S., Mechanisms of multidrug resistance in HL60 cells: Evidence that a surface membrane protein distinct from P-glycoprotein contributes to reduced cellular accumulation of drug, *Cancer Res*. 1988, *48*, 3959 – 3963.

[17] Zijlstra, J. G., de Vries, E. G., Mulder, N. H., Multifactorial drug resistance in an adriamycin-resistant human small cell lung carcinoma cell line, *Cancer Res*. 1987, *47*, 1780 – 1784.

[18] Elbling, L., Berger, W., Weiss, R. M., Printz, D., *et al*., A novel bioassay for P-glycoprotein functionality using cytochalasin D, *Cytometry* 1998, *31*, 187 – 198.

[19] Doyle, L. A., Yang, W., Abruzzo, L. V., Krogmann, T., *et al*., A multidrug resistance transporter from human MCF-7 breast cancer cells, *Proc. Natl. Acad. Sci. USA* 1998, *95*, 15665 – 15670.

[20] Schmid, D., Ecker, G., Kopp, S., Hitzler, M., Chiba, P., Structure-activity relationship studies of propafenone analogs based on P-glycoprotein ATPase activity measurements, *Biochem. Pharmacol.* 1999, *58*, 1447 – 1456.

[21] Shi, Z., Peng, X. X., Kim, I. W., Shukla, S., *et al*., Erlotinib (Tarceva, OSI-774) antagonizes ATP-binding cassette subfamily B member 1 and ATP-binding cassette subfamily G member 2-mediated drug resistance, *Cancer Res*. 2007, *67*, 11012 – 11020.

[22] Sulyok, M., Berthiller, F., Krska, R., Schuhmacher, R., Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize, *Rapid Commun. Mass Spectrom.* 2006, *20*, 2649 – 2659.

[23] Heffeter, P., Pongratz, M., Steiner, E., Chiba, P., *et al*., Intrinsic and acquired forms of resistance against the anticancer ruthenium compound KP1019 [indazolium trans-[tetrachlorobis(1H-indazole)ruthenate (III)] (FFC14A), *J. Pharmacol. Exp. Ther*. 2005, *312*, 281 – 289.

[24] Hirohashi, T., Suzuki, H., Chu, X. Y., Tamai, I., *et al*., Function and expression of multidrug resistance-associated protein family in human colon adenocarcinoma cells (Caco-2), *J. Pharmacol. Exp. Ther*. 2000, *292*, 265 – 270.

[25] Steiner, E., Holzmann, K., Elbling, L., Micksche, M., Berger, W., Cellular functions of vaults and their involvement in multidrug resistance, *Curr. Drug Targets* 2006, *7*, 923 – 934.

[26] Fojo, T., Bates, S., Strategies for reversing drug resistance, *Oncogene* 2003, *22*, 7512 – 7523.

[27] Robey, R. W., Steadman, K., Polgar, O., Morisaki, K., *et al*., Pheophorbide a is a specific probe for ABCG2 function and inhibition, *Cancer Res*. 2004, *64*, 1242 – 1246.

[28] Heffeter, P., Jakupec, M. A., Korner, W., Chiba, P., *et al*., Multidrug-resistant cancer cells are preferential targets of the new antineoplastic lanthanum compound KP772 (FFC24), *Biochem. Pharmacol*. 2007, *73*, 1873 – 1886.

[29] Logrieco, A., Rizzo, A., Ferracane, R., Ritieni, A., Occurrence of beauvericin and enniatins in wheat affected by Fusarium avenaceum head blight, *Appl. Environ. Microbiol*. 2002, *68*, 82 – 85.

[30] Uhlig, S., Jestoi, M., Parikka, P., Fusarium avenaceum – The North European situation, *Int. J. Food Microbiol*. 2007, *119*, 17 – 24.

[31] Leitgeb, R., Lew, H., Khidr, R., Böhm, J., *et al*., Influence of *Fusarium* toxins on growth and carcass characteristics of turkeys, *Die Bodenkultur* 2000, *52*, 171 – 178.

[32] Leitgeb, R., Lew, H., Wetscherek, W., Böhm, J., Quinz, A., Influence of fusariotoxins on growing and slaughtering performance of broilers, *Die Bodenkultur* 1999, *50*, 67 – 66.

[33] McKee, T. C., Bokesch, H. R., McCormick, J. L., Rashid, M. A., *et al*., Isolation and characterization of new anti-HIV and cytotoxic leads from plants, marine, and microbial organisms, *J. Nat. Prod*. 1997, *60*, 431 – 438.

[34] Lin, H. I., Lee, Y. J., Chen, B. F., Tsai, M. C., *et al*., Involvement of Bcl-2 family, cytochrome c and caspase 3 in induction of apoptosis by beauvericin in human nonsmall cell lung cancer cells, *Cancer Lett.* 2005, *230*, 248 – 259.

[35] Fricker, G., Miller, D. S., Relevance of multidrug resistance proteins for intestinal drug absorption in vitro and in vivo, *Pharmacol. Toxicol*. 2002, *90*, 5 – 13.

[36] Litman, T., Druley, T. E., Stein, W. D., Bates, S. E., From MDR to MXR: New understanding of multidrug resistance systems, their properties and clinical significance, *Cell Mol. Life Sci*. 2001, *58*, 931 – 959.

[37] Kino, K., Taguchi, Y., Yamada, K., Komano, T., Ueda, K., Aureobasidin A, an antifungal cyclic depsipeptide antibiotic, is a substrate for both human MDR1 and MDR2/P-glycoproteins, *FEBS Lett.* 1996, *399*, 29 – 32.

[38] Tiberghien, F., Kurome, T., Takesako, K., Didier, A., *et al*., Aureobasidins: Structure-activity relationships for the inhibition of the human MDR1 P-glycoprotein ABC-transporter, *J. Med. Chem*. 2000, *43*, 2547 – 2556.

[39] Hiraga, K., Yamamoto, S., Fukuda, H., Hamanaka, N., Oda, K., Enniatin has a new function as an inhibitor of Pdr5p, one of the ABC transporters in *Saccharomyces cerevisiae*, *Biochem. Biophys. Res. Commun*. 2005, *328*, 1119 – 1125.

[40] Jestoi, M., Emerging fusarium-mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin: A review, *Crit. Rev. Food Sci. Nutr*. 2008, *48*, 21 – 49.

[41] Kouri, K., Lemmens, M., Lemmens-Gruber, R., Beauvericin-induced channels in ventricular myocytes and liposomes, *Biochim. Biophys. Acta* 2003, *1609*, 203 – 210.

[42] Prime-Chapman, H. M., Fearn, R. A., Cooper, A. E., Moore, V., Hirst, B. H., Differential multidrug resistance-associated protein 1 through 6 isoform expression and function in human intestinal epithelial Caco-2 cells, *J. Pharmacol. Exp. Ther.* 2004, *311*, 476 – 484.

[43] De Angelis, I., Frigge, G., Raimondi, F., Stammati, A., *et al*., Absorption of fumonisin B1 and aminopentol on an in vitro model of intestinal epithelium; the role of P-glycoprotein, *Toxicon* 2005, *45*, 285 – 291.

[44] Videmann, B., Tep, J., Cavret, S., Lecoeur, S., Epithelial transport of deoxynivalenol: Involvement of human P-glycoprotein (ABCB1) and multidrug resistance-associated protein 2 (ABCC2), *Food Chem. Toxicol*. 2007, *45*, 1938 – 1947.

[45] Ozvegy-Laczka, C., Cserepes, J., Elkind, N. B., Sarkadi, B., Tyrosine kinase inhibitor resistance in cancer: Role of ABC multidrug transporters, *Drug Resist. Updat.* 2005, *8*, 15 – 26.

[46] Klaric, M. S., Pepeljnjak, S., Domijan, A. M., Petrik, J., Lipid peroxidation and glutathione levels in porcine kidney PK15 cells after individual and combined treatment with fumonisin B(1), beauvericin and ochratoxin A, *Basic Clin. Pharmacol. Toxicol*. 2007, *100*, 157 – 164.

[47] Polgar, O., Robey, R. W., Bates, S. E., ABCG2: Structure, function and role in drug response, *Expert Opin. Drug Metab. Toxicol*. 2008, *4*, 1 – 15.

[48] Ivanov, V. T., Evstratov, A. V., Sumskaya, L. V., Melnik, E. I., *et al*., Sandwich complexes as a functional form of the enniatin ionophores, *FEBS Lett.* 1973, *36*, 65 – 71.

[49] Jestoi, M., Rokka, M., Peltonen, K., An integrated sample preparation to determine coccidiostats and emerging Fusarium-mycotoxins in various poultry tissues with LC-MS/MS, *Mol. Nutr. Food Res*. 2007, *51*, 625 – 637.

[50] Sharom, F. J., Lu, P., Liu, R., Yu, X., Linear and cyclic peptides as substrates and modulators of P-glycoprotein: Peptide binding and effects on drug transport and accumulation, *Biochem. J.* 1998, *333*, 621 – 630.

[51] Ozvegy, C., Litman, T., Szakacs, G., Nagy, Z., *et al*., Functional characterization of the human multidrug transporter, ABCG2, expressed in insect cells, *Biochem. Biophys. Res. Commun*. 2001, *285*, 111 – 117.

[52] Robey, R. W., Honjo, Y., van de Laar, A., Miyake, K., *et al*., A functional assay for detection of the mitoxantrone resistance protein, MXR (ABCG2), *Biochim. Biophys. Acta* 2001, *1512*, 171 – 182.

[53] Sarkadi, B., Ozvegy-Laczka, C., Nemet, K., Varadi, A., ABCG2 – A transporter for all seasons, *FEBS Lett.* 2004, *567*, 116 – 120.

[54] Kok, J. W., Veldman, R. J., Klappe, K., Koning, H., *et al*., Differential expression of sphingolipids in MRP1 overexpressing HT29 cells, *Int. J. Cancer* 2000, *87*, 172 – 178.

[55] Stewart, D. J., Mechanisms of resistance to cisplatin and carboplatin, *Crit. Rev. Oncol. Hematol*. 2007, *63*, 12 – 31.

[56] Berger, W., Elbling, L., Micksche, M., Expression of the major vault protein LRP in human nonsmall-cell lung cancer cells: Activation by short-term exposure to antineoplastic drugs, *Int. J. Cancer* 2000, *88*, 293 – 300.

