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Research Article

Oxidative stress and DNA interactions are not involved in Enniatin- and Beauvericin-mediated apoptosis induction

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The fusariotoxins beauvericin (BEA) and the structurally related enniatins (ENN) are frequent contaminants of grain-based food and feed. They exert potent cytotoxic activities based on apoptosis induction. Since it is known, that reactive oxygen species (ROS) and DNA damage lead to apoptotic cell death, this study aimed to clarify whether oxidative stress and DNA interactions are involved in ENN- and BEA-induced cytotoxicity. Diverse cellular and molecular assays indicated that oxidative stress does not contribute to ENN- and BEA-induced cytotoxicity. In contrast, both fusariotoxins were shown to exert moderate antioxidative activities. Moreover, only at high concentrations (>100 μM) both mycotoxins were found to intercalate substantially into dsDNA and to inhibit the catalytic activity of topoisomerase I and II. Furthermore, the potent cytotoxic activity of ENN and BEA was shown to be widely independent of cellular mismatch- and nucleotide excision repair pathways. Also the ataxia-telangiectasia mutated (ATM) protein kinase, a well known DNA damage sensor, did not affect BEAs cytotoxic potential while in ENN-induced cytotoxicity ATM had a detectable but not a major modulating influence. Together, our data suggest that ROS and DNA damage are not key factors in ENN- and BEA-mediated cytotoxicity.

Keywords: Beauvericin / DNA damage / Enniatin / *Fusarium* mycotoxins / Oxidative stress

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1 Introduction

Several *Fusarium* strains, frequently found as contaminants of grain-based foodstuff, are capable of producing high levels of the cyclohexadepsipeptide metabolites enniatins (ENN) and beauvericin (BEA). Thus, human beings and animals are frequently exposed to both mycotoxins *via* food intake. Since ENN and BEA have been discovered only during the last decades, very limited data are available concerning their *in vivo* and *in vitro* toxicity [1]. So far, both cyclic hexadepsipeptides are reported to possess insecticidal, ionophoric, and antibiotic activities [2–4]. Moreover, they exert cytotoxic activities against normal and malignant cell lines through induction of mitochondrial modifications and cell cycle disruption, finally resulting in apoptotic cell death [5–7]. Most interestingly, resent research data suggested a potential quality of ENN and BEA as anticancer drugs [5, 8, 9]. For example, BEA was shown to inhibit the migration of metastatic prostate [8, 9] and breast cancer cells [9]. Moreover, it revealed antiangiogenic activity in human umbilical vein endothelial cells (HUVEC-2) at subtoxic concentrations [9]. With regard to the cyclic hexadepsipeptide ENN, we recently reported potent anticancer activity widely independent of the p53 and bcl-2 family status [5]. In general, several cyclic peptides such as Kahalalide F and FR901228 are currently in preclinical trials for

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Abbreviations: ATM, ataxia-telangiectasia mutated; **BEA,** beauvericin; **DAPI,** 4′-diamidino-2-phenylindole; **DCFH-DA,** 2′,7′-dichlorofluorescein diacetate; **ENN,** enniatins; **FACS,** fluorescence-activated cell sorting; **FB_1,** fumonisin B_1; **GSH,** glutathione; **H_2O_2,** hydrogen peroxide; **MMR,** mismatch repair; **MTT,** 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **NAC,** *N*-acetyl cysteine; **NER,** nucleotide excision repair; **ROS,** reactive oxygen species; **XPA,** xeroderma pigmentosum complementation group A; **XPD,** xeroderma pigmentosum complementation group D

cancer treatment and recent studies specifically focus on the identification of their mechanism of action [10, 11]. Thus, since ENN and BEA show promising pharmacological properties and not at least because of their salient feature as food contaminants, more detailed information on their cytotoxic mode of action is of importance.

In general, the generation of oxidative stress is a known trigger of apoptosis induction [12]. Under oxidative stress conditions, reactive oxygen species (ROS) such as hydroxyl radicals, superoxide anions and hydrogen peroxides (H_2O_2) have been shown to promote DNA damage and lipid peroxidation [13]. In case of several *Fusarium* mycotoxins as well as anticancer drugs oxidative stress is reported as one mechanism of action [14, 15]. For example, the fusariotoxin fumonisin B_1 (FB_1) induces ROS [16], lipid peroxidation [14] and glutathione (GSH) depletion [16]. Recently, Klaric *et al*. postulated a prooxidative activity of the *Fusarium* mycotoxin BEA in porcine kidney cells indicated by decreased GSH levels and lipid peroxidation [17]. Moreover, increased levels of H_2O_2 and a decreased ROS-scavenging enzyme catalase activity in tomato protoplasts after BEA-treatment suggested that this mycotoxin is able to impair the antioxidative status of plants [18]. To our knowledge, no data of ENN are available in this regard. However, we speculated that the toxicity of both BEA and the structurally related ENN might be due to the induction of oxidative stress.

Thus, this study was performed to elucidate whether ENN and BEA induce oxidative cell damage that might be relevant for their apoptosis-inducing properties in several human cell lines. Additionally, as it is known for numerous anticancer drugs and mycotoxins that their cytotoxic activities are based on interactions with DNA [19, 20], this study further addressed the question whether ENN and BEA induce direct or indirect DNA damage.

2 Materials and methods

2.1 Chemicals

ENN used in this study was the natural frequently occurring mixture of 3% A, 20% A1, 19% B, and 54% B1 homologues (ca. 97% purity), and was supplied together with BEA by Sigma – Aldrich GmbH (St. Louis, MO). As ENN and BEA are poorly soluble in H_2O stock solutions were frequently prepared in DMSO and stored at +4°C.

2.2 Cell culture

The following human cancer cell lines were used in this study: the human lung cancer cell lines A549 and GLC-4 [21], the epidermal carcinoma-derived cell line KB-3-1 (generously donated by Dr. Shen, Bethesda, USA) [22] and the promyelocytic leukemia cell line HL-60 (by Dr. Center, Kansas State University, KS) [23]. These cell lines were cultivated in RPMI 1640 (Sigma-Aldrich GmbH St. Louis, MO). Moreover, the human colon carcinoma cell model HCT116 and the respective mismatch repair (MMR)-proficient subline HCT116+chr3, both cultured in McCoy's culture medium, were kindly supplied by C. R. Boland, Baylor University Medical Center (Dallas, Texas) [24]. Additionally, XP6BE(SV4O), a SV4O-immortalized skin fibroblast line established from a 19-year-old female with xeroderma pigmentosum complementation group D (XPD) and a SV4O-immortalized p2E-ER2-transfected DNA repair proficient cell line (XPD_{corr}) [25] as well as a fibroblast cell line from an XPA patient, XP2OS(SV4O) and a respective XPA corrected cell line [26] were obtained from Coriell cell repository (Camden, NJ). All fibroblasts were cultured in Dulbecos modified Eagle's medium supplemented with 20 mM L-glutamine (Gibco, UK). The XPD corrected cell line was additionally grown with 0.6 mg/ml geneticin. All culture media were supplemented with 10% fetal calf serum (PAA, Linz, Austria) and cultures were periodically checked for *Mycoplasma* contamination.

2.3 Morphological analysis of apoptosis by FITC and DAPI staining

KB-3-1 cells (1×10^5/well) were plated in six well plates and after 24 h recovery treated for another 24 h with the indicated ENN and BEA concentrations. 4′,6′-diamidino-2-phenylindole (DAPI) and FITC-phalloidin stainings were performed as described previously [5]. The rate of apoptosis of the two highest concentrations (5 and 10 μM) was determined as the percentage of apoptotic nuclei whereby at least 500 nuclei/experimental group were analyzed.

2.4 DCFH-DA assay for intracellular ROS determination

ROS levels were measured as described previously [27] using the cell permeable dye 2′,7′-dichlorofluorescein diacetate (DCFH-DA), purchased from Fluka AG (Buchs, Switzerland). Stock solutions of DCFH-DA (33.4 mM) in DMSO were stored at −20°C. KB-3-1 and HL60 cells (2.5×10^5 per sample) were treated with ENN, BEA or H_2O_2 for 15 min at 37°C in PBS containing Ca^{2+} and Mg^{2+}. This was followed by a 45 min exposure to DCFH-DA. If ENN and BEA were co-administered with H_2O_2, cells were pretreated with H_2O_2 15 min before adding the mycotoxins simultaneously with the dye. Mean fluorescence intensity was measured by fluorescence activated cell sorting (FACS) using a 488 nm excitation beam.

2.5 Cytotoxicity sssay

Cell lines (KB-3-1, HL60, HCT116, HCT116+chr3, XPA, XPA_{corr}, XPD and XPD_{corr}) were seeded in 96-well culture plates at a density of 2×10^3 cells/well and allowed to attach

for 24 h. Afterwards, cells were incubated with 100 µL of the two cyclic hexadepsipeptides with concentrations ranging from 0.5 to 10 µM. To investigate the impact of *N*-acetyl cysteine (NAC) or the protein kinase ATM on ENN- and BEA-induced cytotoxicity, cells were pretreated for 30 min at 37°C with 50 µL of a 1 and 2 mM NAC or a 0.5 and 1 mM caffeine solution. ENN and BEA (0.5 – 10 µM) were added in another 50 µL medium. Comparably, to determine the antioxidative effects of ENN and BEA, cells were pretreated for 30 min with low subtoxic ENN and BEA concentrations (1 µM) before addition of H_2O_2 (0.1 – 10 µM). In all experimental settings, the proportion of viable cells was determined after 72 h exposure by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based vitality assay (EZ4U, Biomedica, Vienna, Austria). Briefly, 100 µL of the supernatants were removed and replaced with 100 µL solution of the EZ4U assay. After 1-2 h incubation the absorbance was measured at 450 nm with 620 nm as reference to reduce unspecific background values. All experiments were performed at least twice in triplicates.

2.6 Alkaline comet assay

The induction of DNA strand breaks by oxidative stress was determined using the alkaline comet assay, according to the method described [28 – 30]. Briefly, HL-60 cells were treated with the two cyclic hexadepsipeptides for 1 h. Subsequently, they were mixed with 100 µl of 0.5% low melting point agarose at 37°C and transferred to agarose-coated slides. The agarose was allowed to solidify for 2 min on a cooled tray and then immersed in ice-cold lysing solution (2.5 M NaCl; 100 mM Na_2EDTA; 10 mM Tris – HCl; 1% Triton X-100, pH 10) for another hour. Following lysis, DNA was allowed to unwind for 30 min in electrophoresis buffer. Electrophoresis was conducted for 20 min at 25 and 300 mA in a chamber cooled on ice. After DNA staining with 2 µg/mL ethidium bromide coded slides were examined with a fluorescence microscope (NIKON Eclipse E600) equipped with an automated digital imaging system running COMET Assay IV software (Perceptive Instruments, UK). Captured data were calculated by image analysis (Perceptive Instruments) and the extent of DNA migration indicative for DNA damage is expressed as percentage DNA in the tail (% tail intensity), defined as the ratio between total tail intensity and total comet (head + tail) intensity multiplied by 100 [31]. Per experimental group, three slides were prepared and from each 50 cells were analysed. Cell viability at the time of assay was in all cases >90%.

2.7 DNA intercalation assay

Intercalation of the tested substances into salmon sperm DNA (Sigma – Aldrich GmbH, St. Louis, MO) was determined using the methyl green assay as described [28, 29]. Briefly, after 1 h incubation with 30 µM methyl green, salmon sperm DNA (10 mg/mL) was treated for 2 h with the indicated ENN and BEA concentrations up to 150 µM. Absorbance was measured at 642 nm. Treatment with the intercalators and minor groove binders ethidium bromide [32] and doxorubicin [33] served as positive controls.

2.8 Topoisomerase DNA-cleavage assays

Topoisomerase I activity in presence of ENN and BEA was determined as relaxation of supercoiled pGEM1 plasmid DNA by nucleic extract from MCF-7 cells as described previously [34]. Briefly, 250 ng plasmid DNA (pGEM1) was incubated for 30 min at 37°C with rising concentrations of ENN and BEA (1 nM – 1 mM) in a final volume of 30 µL containing 0.1 µL of nucleic extract, 10 mM Tris (pH 7.9), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, and 0.03 mg/mL BSA. Topotecan (33.3 nM), a well-known topoisomerase I inhibitor, was used as positive control. The reaction was stopped by incubation with 5 µL 5% SDS containing 1 mg/mL proteinase K at 37°C for 30 min. Subsequently, samples were separated by submarine 1% agarose gel electrophoresis (60 V, 2 h) and gels were stained with 10 µL/100 mL ethidium bromide for 20 min. UV-transilluminated gels were documented by Multi-Analyst software.

Effects of ENN and BEA on the catalytic activity of topoisomerase II were determined using a decatination assay [34]. 0.2 µg catenated kinetoplast DNA (KDNA) (TopoGEN, Ohio) was incubated at 37°C for 1 h in presence of ENN and BEA in a final volume of 20 µL containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 10 mM $MgCl_2$, 5 mM ATP, 0.5 mM DTT and 30 µg/ml BSA. Doxorubicin at 5 µM was used as positive control. The reaction was stopped by further 30 min incubation at 37°C with 3 µL SDS containing 1 mg/mL proteinase K. Gel electrophoresis and detection were performed as described above.

3 Results and discussion

3.1 Apoptotic and morphological changes after BEA treatment

BEA induces significant cell death in several cell lines derived from healthy and malignant tissues of different origin [6, 7, 19, 35 – 38]. This BEA-induced cell death is based on apoptosis induction in a dose- and time-dependent manner [6, 7, 37]. As reported previously, also ENN treatment leads to time- and dose-dependent apoptosis induction and characteristic apoptotic features like chromatin condensation and apoptotic body formation in KB-3-1 cells (Fig. 1a) [5]. Additionally, changes in the microfilament system of KB-3-1 cells were shown (Fig. 1b) [5]. To test whether BEA leads to similar apoptotic changes in KB-3-1 cells, used as main test model for our further experiments, DAPI-

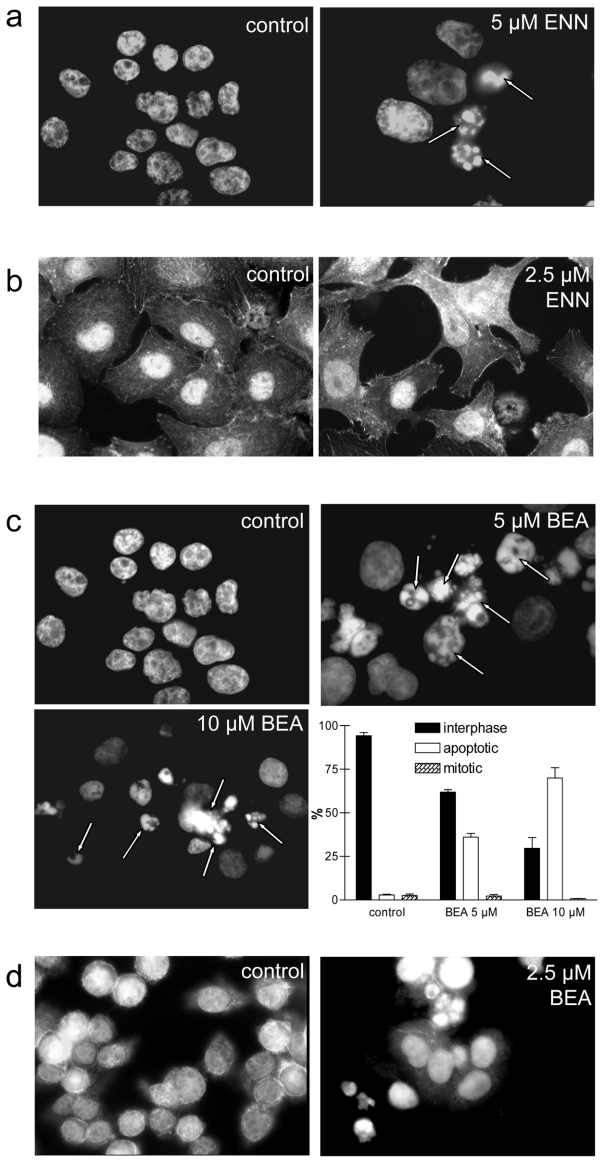

Figure 1. Apoptotic morphological changes after ENN and BEA treatment. Induction of apoptosis is shown in KB-3-1 cells after (a) 5 µM ENN (c) 5 and 10 µM BEA treatment for 24 h using DAPI stainings. Apoptotic bodies are indicated by arrows. With regard to BEA (c), DAPI-stained nuclei were classified in interphase, mitotic, and apoptotic. At least 500 nuclei from three independent experiments were counted and means ± SD calculated. (b, d) KB-3-1 cells were plated on chamber slides and treated with 2.5 µM ENN and BEA, respectively. Photomicrographs were taken using 40x oil objectives. DNA and microfilaments were visualized by DAPI and FITC phalloidin stainings, respectively.

and FITC-phalloidin staining experiments of BEA-treated KB-3-1 cells were performed. Comparable to ENN, nuclear DAPI staining and fluorescence microscopy revealed typical nuclear features of apoptosis in KB-3-1 cells after 24 h BEA incubation (Fig. 1c). When counted microscopically, the amount of nuclei indicating apoptotic characteristics increased from 3% in the control to 36% at 5 µM BEA exposure and to 75% at 10 µM. Additionally, changes in the microfilament system of KB-3-1 cells treated with 2.5 µM BEA were visualized by FITC-phalloidin staining (Fig. 1d). As compared to the untreated control, loss of life cells and cell–cell contacts, cell shrinkage, and the accumulation of microfilaments at the cell borders were observable. These data indicate that both, ENN and BEA lead to apoptosis induction in KB-3-1 cells in a comparable manner.

3.2 The role of oxidative stress in ENN- and BEA-induced apoptosis

In a first approach, possible pro-oxidative properties of BEA and ENN in two different cell lines (KB-3-1 and HL-60) were investigated. For this purpose, the cell permeant dye DCFH-DA which becomes highly fluorescent after oxidation was used. The intensity of fluorescence is equivalent to the intracellular ROS levels [39]. In general, no detectable increase in the amount of ROS with respect to control after ENN or BEA exposure in both cell lines tested was observable (Fig. 2a and b). Accordingly, in viability assays pretreatment with the established radical scavenger NAC showed no significant protective effects on the cytotoxic actions against ENN and BEA in KB-3-1 (Fig. 2c), GLC-4 and A549 cells (data not shown). Since ROS are involved in the generation of DNA strand breaks, the alkaline comet assay, a quick and versatile method for assessing DNA damage in individual cells was used [30]. This method allows the detection of single and double DNA strand breaks, as well as the presence of alkali labile sites [40]. No changes of the mean comet tail intensity were detectable after 1 h treatment of HL60 (Fig. 2e) and KB-3-1 cells (data not shown) with apoptosis-inducing concentrations of ENN and BEA. In contrast, H_2O_2 used as positive control at identical conditions increased DNA migration significantly ($p < 0.0001$). Together, these data obtained by three independent well-established assays indicate that the cytotoxic effects of both mycotoxins are not a result of ROS-induced DNA strand breaks. This is in contrast to Klaric *et al*. [17] who speculated that the ionophoric action of BEA might impair lipid membrane structures and produces H_2O_2, which in turn decreases the cellular anti-oxidative capacity. Moreover, we have previously suggested oxidative properties of BEA based on enhanced cytotoxicity when cells were pretreated with the specific GSH synthesis inhibitor buthionine sulfoximine leading to reduced cellular GSH levels [41]. However, this study unambiguously demonstrated that BEA-induced lipid peroxidation and GSH reduction [17] are not a consequence of oxidative stress. Comparable effects were shown for the fusariotoxin FB1 inducing lipid peroxidation without an increase of ROS-generation in human neuroblastoma cells [16]. Moreover, FB1 treatment induced DNA damage without increased ROS production [42, 43]. Thus, the authors suggested that the cytotoxic

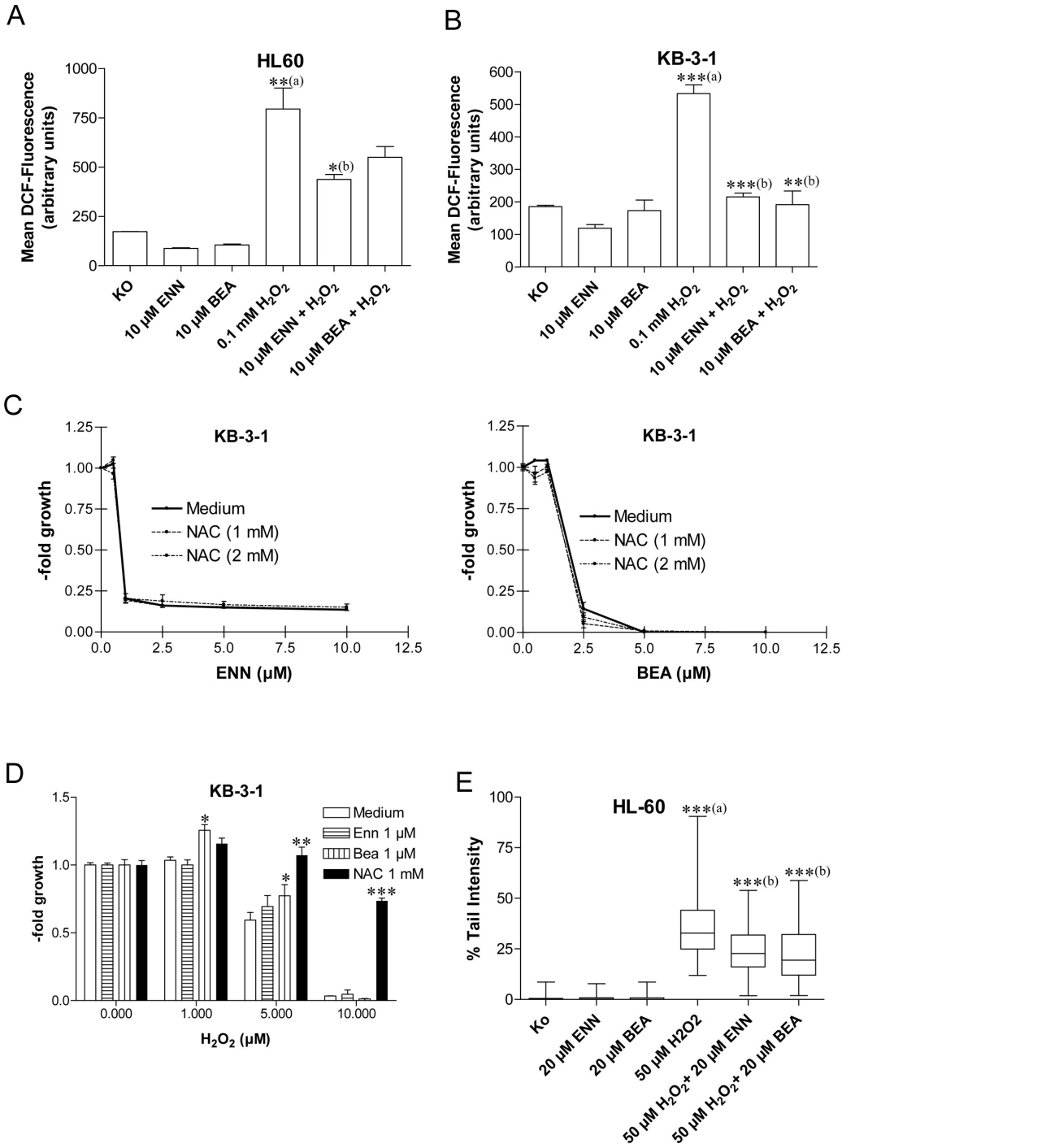

Figure 2. The role of ROS in ENN- and BEA-induced cytotoxicity. Production of intracellular ROS by incubation with the indicated ENN and BEA concentrations was determined in (A) HL60 and (B) KB-3-1 cells using the ROS indicator DCFH-DA. Fluorescence was measured by flow cytometry. (a) Significantly different from control, (b) Significantly different from H_2O_2 treatment, *,**,*** p <0.05, <0.01, <0.001 (Students t-test). (C) Effects of 30 min pretreatment with the radical scavenger NAC on the cytotoxic activity of ENN and BEA in KB-3-1 cells were analysed after 72 h mycotoxin exposure by MTT-based survival assays. (D) Protective effects of 30 min pretreatment with subtoxic ENN and BEA concentrations on H_2O_2-induced cytotoxicity was measured after 72 h total exposure time by MTT assays. NAC was used as positive control. *,**,*** Significantly (p <0.05, <0.01, <0.001, respectively) different from medium control (Students t-test). (E) Induction of DNA migration after 1 h treatment with the indicated concentrations of ENN, BEA and H_2O_2 were analyzed by comet assays performed as described in Section 2.6. (a)*** Significantly different from control, p <0.0001 (Students t-test); (b)*** Significantly different from H_2O_2 treatment, p <0.0001 (Students t-test).

effects of FB1 are rather exerted by modulating the expression of some early genes than by activating pathways involving ROS production. Moreover, lipid peroxidation was supposed to be rather a consequence than a causative event of FB1-damage [42, 43] which might also be the case for BEA-induced injury. With regard to GSH, detoxification of xenobiotics is frequently based on the generation of GSH-S-conjugates by GSH S-transferases. Consequently, consumption of GSH during this process might result in GSH depletion [44]. In accordance, for the structurally related cyclic hexadepsipeptide destruxin E, GSH conjugation was observed during detoxification [45, 46]. The existence of BEA-GSH conjugates is, however, hypothetical and matter of ongoing investigations in our lab.

3.3 Antioxidant activities of ENN and BEA

Most interestingly, results obtained in this study give profound evidence that ENN and BEA do not induce oxidative injury of human cells but in contrast, possess antioxidative properties. For instance, DCFH-DA assays using HL-60 and KB-3-1 cells revealed that ROS levels produced by H_2O_2 were reduced if co-administered with ENN and BEA, respectively (Fig. 2a and b). These antioxidative properties were further confirmed in cell viability assays with KB-3-1 cells (Fig. 2d). Pretreatment with 1 μM BEA 30 min before H_2O_2 exposure displayed a significant protective effect against 1 and 5 μM H_2O_2 after 72 h total incubation time which was, however, moderate as compared to the positive control NAC. Attendance similar to BEA was shown for ENN pretreatment but not reaching statistical significance. Additionally, in the alkaline comet assay 30 min ENN and BEA pretreatment (20 μM) reduced H_2O_2-induced comet tail intensity by 30 and 34%, respectively (Fig. 2e). Comparable effects were shown by Theumer *et al*. for the *Fusarium* mycotoxin FB_1 [47]. FB_1 treatment decreased the H_2O_2 production in peritoneal macrophages of rats *in vivo* and *in vitro* suggesting immunsuppressive effects, which could be related to a lower antitumour activity [47]. So far, this study gives evidence that ENN and BEA possess antioxidative properties which have to be further characterised in more detail.

3.4 DNA intercalation by ENN and BEA

For a number of anticancer drugs and mycotoxins, DNA intercalation is believed to be one of the main mechanisms underlying their cytotoxic activity [19, 48]. In general, functional intercalation requires at least partial planarity and is facilitated by the formation of at least one hydrogen bond [49]. Since the three dimensional structure of ENN and BEA resembles a disc [1] and DNA intercalation was described for the structurally related actinomycin D [50], methyl green competition assays were performed to analyse whether ENN and BEA possess the ability to intercalate into dsDNA. In general, ENN showed higher intercalating properties compared to BEA. Incubation with 50 μM ENN or BEA led to about 20% reduced methyl green staining. Enhanced drug concentrations (up to 150 μM) further decreased the signal significantly to 75 and 37% for ENN and BEA, respectively (Fig. 3a). However, compared to the positive controls doxorubicin and ethidium bromide, highly active already at 15 and 25 μM, respectively, the intercalation properties of ENN and BEA were proved to be rather weak. These data indicate that ENN and BEA exhibit low affinity to DNA leading to substantial intercalation only at high concentrations. Consequently, this suggested that interaction with DNA is of minor importance for their cytotoxic activity which is already present at low micromolar concentrations (1 – 10 μM) [5]. In contrast to our findings that ENN and BEA only interact with DNA at high concentrations, previous cell free studies using BEA-oligonucleotide mixtures with different stoichiometrics revealed BEA adduct formation at low mycotoxin concentrations. Moreover, it was shown that BEA does not have strongly preferred base sequences or sites in the DNA. Consequently, the authors suggested that BEA does not target the minor groove preferentially [51]. However, it has to be considered that Pocsfalvi *et al*. did not use whole genomic DNA. Thus, their experimental settings did not evaluate the impact of the tertiary structure on the interaction of BEA with DNA. Additionally, the methyl green assay is a reliable method for assessing DNA intercalation but not DNA adduct formation. Finally, it has to be kept in mind that it is unknown in how far ENN and BEA reach DNA in living cell nuclei as all these experiments were performed in cell-free systems. So far, no DNA damage could be observed by either BEA or ENN exposure of cell cultures or organisms. Consequently, we currently aim to determine mycotoxin levels in subcellular fractions of human cells exposed to different concentrations of BEA and ENN.

3.5 Effects of ENN and BEA on topoisomerases I and II

In an attempt to elucidate the mechanisms underlying the reaction of ENN and BEA with DNA, possible impacts on DNA-modifying enzymes were investigated. One consistent finding of DNA intercalators is the suppressed topoisomerase-mediated cleavage of DNA at sufficiently high drug concentrations [48]. DNA topoisomerases are nuclear enzymes required to overcome topological problems encountered during DNA replication, transcription, recombination and maintenance of genomic stability. Moreover, since toposiomerases are activated in growing cancer cells, these enzymes are important targets for cancer therapy [52]. In accordance to the intercalating properties of ENN and BEA at high concentrations, plasmid relaxation assays revealed detectable inhibition of the catalytic activity of topoisomerase I only at concentrations ≥100 μM ENN or

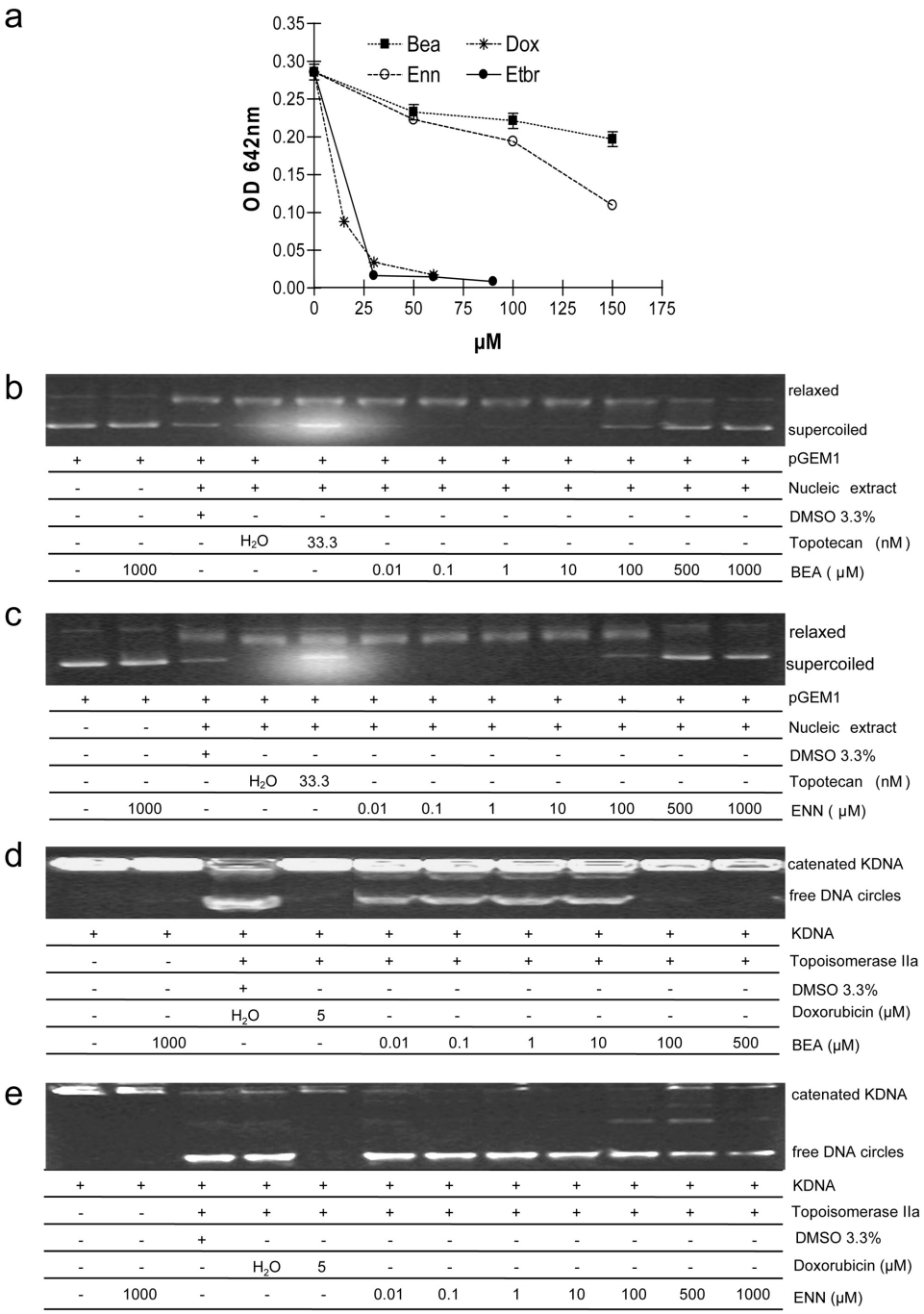

Figure 3. DNA interactions by ENN and BEA. (a) DNA intercalation was determined by methyl green assays using salmon sperm DNA (10 mg/mL) treated for 2 h with the indicated drug concentrations. Dose–response curves derived from two independent experiments in triplicates are shown. Catalytic activity of topoisomerase I in presence of (b) BEA and (c) ENN was measured by pGEM1 DNA relaxation. Impact of (d) BEA and (e) ENN on Topoisomerase II catalytic activity was determined as decatenation of kinetoplast DNA. One representative gel out of three identical experiments with similar outcomes is shown.

BEA (Fig. 3b and c). In contrast, concentrations <100 µM did not affect the topoisomerase I activity. Similar effects were obtained in topoisomerase II decatination assays for BEA (Fig. 3d). BEA was found to be a potent inhibitor of topoisomerase II at concentrations ≥100 µM. In contrast, ENN revealed a weaker but concentration-dependent inhibition of the catalytic activity of topoisomerase II (Fig. 3e). This is comparable to effects reported for the structurally related cyclic depsipeptide sansalvamide A, produced by a marine *Fusarium* species, which inhibits the topoisomerase

enzyme of the pathogenic poxvirus molluscum contagiuosum virus with a relative high IC_{50} value of 124 μM [53]. The frequent observation that at sufficiently high drug concentrations DNA intercalating substances can suppresses topoisomerase-mediated DNA cleavage was suggested to be dependent on two different mechanisms: i) poisoning the enzyme by trapping it on the DNA as a cleavable complex or ii) inhibiting the enzyme from ever binding to DNA when drug concentrations are high enough [48]. In how far this holds true for cytotoxic mode of action of ENN and BEA has to be investigated in further studies.

3.6 The Role of DNA repair pathways in ENN- and BEA-mediated cytotoxicity

Once DNA adducts are formed, cells must be able to counteract DNA damage to carry out normal DNA transactions. Therefore, mammalian cells are equipped with several repair mechanisms. One of such repair mechanisms is the MMR pathway which acts as kind of "damage sensor". The human MMR pathway was suggested to be involved in the cytotoxic effects of several different anti-neoplastic agents [20]. This pathway initiates a series of p53-dependent as well as p53-independent events that finally result in cell cycle arrest and apoptosis [54, 55]. One objective of this study was to determine impact of MMR on ENN- and BEA-induced cytotoxicity. For this purpose, MMR deficient parental HCT116 cells and the MMR-proficient cells HCT116+chr3 were tested for their ENN and BEA sensitivity. Based on MTT assays, no major differences in the IC_{50} values of MMR-proficient and -deficient cells could be detected after a mycotoxin incubation period of 72 h. In more detail, ENN showed IC_{50} values of 1.9 and 1.8 μM in HCT116 and HCT116+chr3, respectively (Fig. 4a). BEA treatment of HCT116 and HCT116+chr3 cells resulted in IC_{50} values of 3.7 and 3.6 μM, respectively (Fig. 4b).

In addition to MMR, nucleotide excision repair (NER) is known as an important DNA repair pathway. Basic steps of NER reaction include distortion recognition, damage verification, repair complex assembly, dual incision and damage excision, DNA resynthesis and ligation [56]. Since NER is activated by cells at an early stage of apoptosis to repair endogenous- and exogenous-induced DNA damage, the impact of DNA excision repair on ENN- and BEA-mediated cell death was investigated. For efficient NER seven repair complementation groups (XPA through G) are known. Genetic disruptions of XPA, for example, are a most common defect in the orphan disease XP [57]. To determine the contribution of NER in ENN- and BEA-induced apoptosis XPD- and XPA-mutant fibroblast cell lines from XP patients were compared to the respective complemented cell lines. As shown in MTT assays, no major differences between the IC_{50} values of the tested xeroderma pigmentosum (XP) cell lines were observed for both mycotoxins (Fig. 4c-f). The IC_{50} values for ENN were as follows: 1.9, 2, 1.8, and 2 μM for XPA, XPA_{corr}, XPD and XPD_{corr}, respectively. The IC_{50} values obtained for BEA for all cell lines tested were 2.0 μM. Noteably, at higher ENN concentration (2.5 – 10 μM) a weak but significant difference was observed between the XPA cell lines suggesting a minor impact of NER on ENN-induced apoptosis at high concentrations.

Another critical DNA damage checkpoint is the ataxia-telangiectasia mutated (ATM) protein kinase. This enzyme activates cellular damage response by phosphorylation of several key proteins involved in cell cycle regulation and DNA repair [56]. ATM is involved in homologous recombinational repair and signals downstream to p53 further leading to activation of bax [56, 58]. Since ENN exposure significantly stimulates p53 as well as bax expression [5], the impact of ATM on ENN- and BEA-induced cell death in KB-3-1 cells was investigated. For this purpose, cells were co-treated with both mycotoxins and the known ATM kinase inhibitor caffeine (0.5 and 1 mM) [59]. In general, 1 mM caffeine weakly but significantly diminished the cytotoxic potential of ENN at 2.5 μM (Fig. 4g). In contrast, equal caffeine concentrations did not affect BEA-induced cytotoxicity (Fig. 4h). This suggests that ATM kinase is only a minor player in ENN-induced cytotoxicity and probably not involved in BEA-induced cell death.

Since many chemotherapeutic drugs act *via* DNA interactions, one major confounding factor for successful anticancer treatment is resistance to DNA damage which can be acquired *via* a plethora of different mechanisms [60]. Thus, since MMR and NER only weakly reduce the cyctotoxic potential of ENN and BEA and, both mycotoxins induce apoptosis widely irrespective of the cellular ATM and, in case of ENN, additionally of the p53 status, these compounds are interesting candidates for further development as anticancer drugs.

4 Concluding remarks

In conclusion, our results showed that generation of ROS is not involved in ENN- and BEA-induced apoptotic cell death. In contrast, both secondary metabolites showed antioxidative properties which have to be further investigated in more detail. Since no ENN- and BEA-induced DNA strand breaks were detectable in comet assays and DNA intercalation as well as topoisomerase I and II inhibition was only observed at high ENN and BEA concentrations, it can be concluded that DNA might not be the primary target of the two cyclic hexadepsipeptides. Comparably, also experiments with diverse repairtest cell models indicate that DNA damage plays only a minor role in ENN- and BEA-induced cytotoxicity. Hence, further studies must be encouraged to precisely define the molecular mechanisms underlying the distinct cytotoxic activities of these two cyclic hexadepsipetide mycotoxins – especially, since these compounds

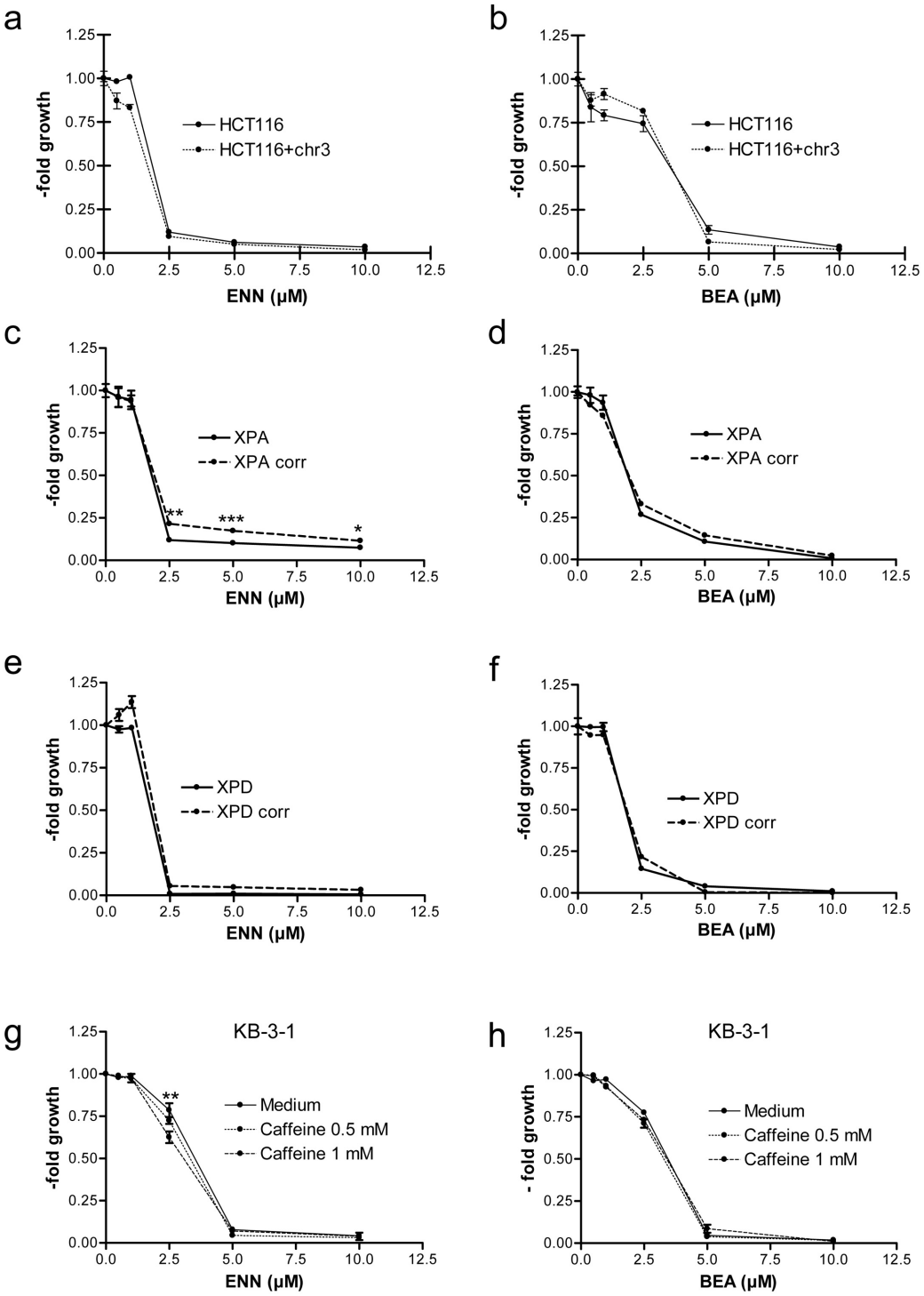

Figure 4. Impact of DNA repair pathways on ENN- and BEA-mediated cytotoxicity. Impact of (a) ENN and (b) BEA on cell viability of MMR-deficient HCT116 and MMR-proficient HCT116+chr3 cells was determined by MTT-based vitality assays. Contribution of NER to ENN- and BEA-induced cytotoxicity was evaluated using (c, d) an XPA-deficient and a respective XPA corrected cell model as well as (e, f) XPD-deficient and XPD-proficient cells. *, **, *** significant (p <0.05, <0.01, <0.001, respectively) difference between the tested cell lines (Students t-test). Effects of ATM on (g) ENN- and (h) BEA-induced cytotoxicty in KB-3-1 cells were determined by co-treatment with the ATM inhibitor caffeine at the indicated concentrations. Cell viability was measured by EZ4U after 72 h drug exposure. ** Significantly (p <0.01) different to medium control (Students t-test).

constitute a serious risk factor in animals and humans nutrition and are of interest as potential anticancer drugs.

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