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The naturally born fusariotoxin Enniatin B and Sorafenib exert synergistic activity against cervical cancer *in vitro* and *in vivo*

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Abstract

During the last decades substantial progress has been made in developing systemic cancer therapy. However, tumors are frequently intrinsically resistant against structurally and mechanistically unrelated drugs. Thus, it is of predominant interest to overcome drug resistance and to encourage the research for novel chemotherapeutic approaches. Recently, we have introduced Enniatins, naturally occurring cyclohexadepsipeptides produced by filamentous fungi of the genus *Fusarium*, as potential anticancer drugs. Here, we expand this approach by demonstrating antiangiogenic properties for Enniatin B (Enn B) indicated by a strong inhibition of human endothelial cell migration and tube formation. Moreover, combination of Enn B with the clinically approved multi-kinase inhibitor Sorafenib (Sora) displayed profound synergistic *in vitro* and *in vivo* anticancer effects against cervical cancer. Subsequent studies showed that this strong synergism is accompanied by a marked increase in mitochondrial injury and apoptosis induction reflected by mitochondrial membrane depolarization, caspase-7 activation, and subsequent cleavage of PARP. Additionally, cells were shown to stop DNA synthesis and accumulate in S and G₂/M phase of the cell cycle. The multifaceted characteristics underlying this strong synergism were suggested to be based on interference with the p38 MAPK as well as the ERK signaling pathways. Finally, also *in vivo* studies revealed that the combination treatment is distinctly superior to single drug treatments against the KB-3-1 cervix carcinoma xenograft model. Taken together, our data confirm the

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Conflict of interest

The authors declare no conflict of interest.

anticancer benefits of the naturally occurring fusariotoxin Enn B and further present Enn B/Sora as a novel combination strategy especially for the treatment of cervical cancer.

Keywords

Enniatin; cyclohexadepsipeptide; sorafenib; synergism; anticancer; angiogenesis

1. Introduction

Tyrosin kinase inhibitors (TKIs) are an important new class of targeted cancer therapy. These drugs inhibit or block oncogenic tyrosine kinases and thus, interfere with specific cell signaling pathways consequently, allowing target-specific therapy for defined malignancies. For instance, the clinically used multi-target inhibitor sorafenib (BAY 43-9006, Nexavar) is approved for the treatment of advanced renal cell carcinoma as well as unresectable hepatocellular carcinoma [1, 2]. Originally, sorafenib (Sora) was identified as a Raf kinase inhibitor, but was later found to inhibit also VEGFR-1/-2/-3, PDGF- β receptor (PDGFR- β), Fms-like tyrosine kinase-3 (FLT-3), c-Kit protein (c-Kit), and RET receptor tyrosine kinases. Sora has demonstrated potent anti-tumor activity by virtue of its antiangiogenic, antiproliferative and proapoptotic effects [1, 3]. However, such targeted therapies are not without limitations such as intrinsic lack of tumor response, the development of resistance as well as severe adverse effects. Depending on the targets and additional off target activities, tyrosine kinase inhibitors may cause a broad spectrum of adverse effects on skin and hair, haematological side reactions like anemia, thrombopenia, and neutropenia as well as extra-haematologic adverse events such as edema, nausea, hypothyroidism, vomiting, and diarrhea [4, 5]. The availability of newer inhibitors and combinations with other drugs should help to overcome these problems in the future. Generally, chemotherapeutics but also novel targeted anticancer drugs are frequently more effective when given not as mono-therapies but in combination schemes. The rationale for this strategy is to hit cancer cells at more than one target thereby decreasing the probability of resistance development. Moreover, when drugs with different effects are combined the occurrence of intolerable side effects might be limited [6].

In recent years, naturally occurring cyclic depsipeptides came into focus of interest as potential anticancer drugs. Within that substance class, enniatins (Enns) exert promising anticancer properties and the respective mechanisms of action have been recently addressed by several research groups including our own [7-10]. In general, Enns are cyclic hexadepsipetidic *Fusarium* metabolites [11]. As contaminants of cereals Enns are regularly found in food and feed [12, 13]. Remarkably, Enns are resistant to heat, acids and digestion, and they have been shown to propagate through the food and feed chain. Thus, possible impacts on human and animal health as food contaminates are discussed [14]. Moreover, during the last years Enns came into focus of interest as possible anticancer agents based on their widespread cytotoxic activity specifically against malignant cell types [7-10]. The primary toxic action of these cyclohexadepsipetides is considered to be based on their ionophoric properties. Due to transport of mono- and divalent cations through the cell membranes, Enns lead to disturbances of the physiological homeostasis, thus, leading to

apoptotic cell death. Moreover, Enns were shown to exert p53-dependent cytostatic and p53-independent cytotoxic activities against several cancer cell types, while normal proliferating cells remain widely unimpaired under identical conditions [7-10]. Already 24 h after Enns treatment at low micromolar concentrations DNA synthesis stop, cell cycle arrest and apoptotic cell death is induced in various cancer cell models [7, 8, 10]. Moreover, the potent cytotoxic effects of Enns are only weakly influenced by multidrug resistance (MDR) transport proteins which translocate a large number of hydrophobic drugs across cellular membranes consequently, leading to therapy resistance. In addition, several chemosensitizing properties were shown [9] which all together identify Enns as promising compounds for further studies. Thus, the aim of the present study was to gain deeper insights into the proposed anticancer activities of the naturally born cyclohexadepsipeptidic substance enniatin B (Enn B). Moreover, we intended to investigate possible interactions of Enn B with the clinically used multi-kinase inhibitor Sora and to elucidate the underlying modes of action.

2. Materials and methods

2.1. Enniatin B

For purification of Enn B *F. oxysporum* ETH 1536/9 was used. Cultivation conditions were adopted from Madry et al [15]. Cultures were harvested by suction filtration through miraclothes (Merck Milipore, Germany). Mycelium was freeze dried and extracted with ethyl acetate. Solvent was evaporated and the brownish residue dissolved in a minimum amount of ethyl acetate. Acetonitrile was added to the solution and kept overnight at -20°C . Crystals were separated from the mother liquor by suction filtration and washed with cold acetonitrile. Crystallizations were repeated until pure Enn B remained. Purity of the compound was verified by LCMS (ESI-Triple-Quadrupol-MS, 6460 Series, Agilent Technologies, Germany) and NMR (400 MHz-NMR mit Avance-Konsole, Bruker, Germany). Additionally, the cytotoxic potential was compared to Enn B obtained from Sigma Aldrich GmbH (St. Louis, MO, USA) and shown to be in a comparable range in all cell lines tested (compare IC_{50} values in KB-3-1 cells for the Sigma- and the self-produced derivative: $3.57\text{ }\mu\text{M} \pm 0.6$ and 3.49 ± 0.06 , respectively). For the experiments Enn B stock solutions were freshly prepared in DMSO and stored at 4°C . Concentration series for the experiments were prepared from DMSO stocks in fresh medium.

2.2. Chemicals

Sorafenib and the specific MAPK inhibitors (U0126, SB203580 and WP1066) were purchased from LC Laboratories (Woburn, USA). All other compounds were from Sigma-Aldrich GmbH.

2.3. Cell culture

The following human cancer cell lines were used in this study: the epidermal carcinoma-derived cell line KB-3-1 (generously donated by Dr. Shen, Bethesda, USA) [16], the cervical carcinoma cell lines C4-I and CaSki, the epithelial cervix carcinoma cell line HTB-31, the hepatocellular carcinoma cell line Hep3B and the renal carcinoma cell line Caki-2 (all from American Type Culture Collection (ATCC), Manassas, VA). Additionally,

human umbilical vein endothelial cells (HUVEC) (Lonza, Verviers, Belgium) were used. KB-3-1, CaSki and C4-I cells were grown in RPMI 1640 medium. Caki cells were grown in McCoy's and Hep3B cells in EMEM culture medium. For HTB-31 cells, minimal essential medium modified with non-essential amino acids and pyruvate (MNP) was used and HUVEC were maintained in endothelial basal medium (EBM)-2 (Lonza) supplemented according to the instructions of the manufacturer. All other culture media were purchased from Sigma-Aldrich and supplemented with 10% fetal calf serum (PAA, Linz, Austria). Cultures were regularly controlled for *Mycoplasma* contamination.

2.4. Cell viability assays

The antiproliferative effects of Enn B combined with diverse TKIs were evaluated by MTT assays, as previously described [17]. Cells ($2-6 \times 10^4$ /ml) were seeded in 100 μ l/well into 96-well plates. After 24 h recovery, cells were concurrently treated with rising concentrations of Enn B and TKIs, for each substance using a test volume of 50 μ l. In cell viability assays using ERK, p38, and STAT3 inhibitors, cells were pretreated 30 min with the inhibitors before the test drug was added. After the indicated treatment durations, for all experiments the proportion of viable cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based vitality assay according to the manual (EZ4U, Biomedica, Vienna, Austria). All experiments were performed at least twice in triplicates and data evaluated using the Graphpad Prism 5 software.

2.5. Synergy calculations

The presence of synergy was determined using the Calcu Syn software (Biosoft, Ferguson, MO, USA) according to the Chou-Talalay method [18] and expressed by the combination index (CI). CI of < 0.9 represents synergism, CI = $0.9 - 1.1$ indicates pure additivity and a CI greater than 1.1 points to antagonism.

2.6. Apoptosis detection

To visualize apoptosis-related morphological changes of KB-3-1 cells, treated 24 h with Enn B alone or in combination with Sora, Hoechst 33258 (HÖ)/propidium iodide (PI) co-stainings were performed according to Dornetshuber et al. [17]. In HÖ/PI stainings, the Hoechst dye stains the DNA of live and dead cells, while PI uptake indicates the loss of cell membrane integrity which is characteristic for dead cells as a consequence of necrosis or apoptosis ("late apoptosis"). Moreover, to investigate the impact of the test substances on the mitochondrial membrane potential, KB-3-1 cells (5×10^5 cells/well) were exposed to the indicated Enn B and Sora concentrations for 24 h before staining with the fluorescent dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide (JC-1; Mitochondrial Membrane Potential detection Kit; Stratagene, La Jolla, CA, USA). Subsequently, FACS analysis was performed as described [7].

2.7. Cell cycle analyses

Cells (3.5×10^5 cells/sample) were incubated in 6-well plates at 37°C with Enn B and TKIs as single agents or in drug combination. Afterwards, cells were collected and fixed with 70% ethanol at -20°C overnight. DNA staining was performed using a solution with PI

(0.01 mg/ml) and RNase (0.2 mg/ml). Cell cycle progression was examined by flow cytometry using FACS Calibur (Becton Dickinson, Palo Alto, CA, USA) as described [17]. CellQuest Pro software (Becton Dickinson) was used to analyze the resulting DNA histograms.

2.8. ³H-thymidine incorporation

For these experiments 5×10^4 KB-3-1 cells were seeded into a 96-well plate. After a 24 h recovery, cells were treated with the test substances for another 24 h. After exposure, the culture medium was replaced for 4 h by a 2 nM ³H-thymidine solution (diluted in full culture medium; 25 Ci/mM) and cells were incubated at 37°C. Afterwards, cells were washed three times with phosphate-buffered saline (PBS) and cell lysates were prepared. Radioactivity was determined as described [17]. Experiments were carried out in triplicate, and results were expressed as counts per minute (cpm).

2.9. Western blot analyses

After 48 h drug exposure cell proteins were isolated, resolved by sodium dodecyl sulfate (SDS)/PAGE and transferred onto a polyvinylidene difluoride membrane for Western blotting as described [7]. Western blots were performed three-times and band intensities were quantified using the Image J program and normalized to the respective β -actin loading controls. Statistical analyses were performed using one-way ANOVA. Antibodies from Antibody Sampler kits purchased from Cell Signalling Technology (Beverly, MA) were used as follows: PARP, caspase 7, and cleaved caspase 7 polyclonal rabbit (Apoptosis Sampler kit); bax, bak and bim polyclonal rabbit (Pro-Apoptosis Bcl-2 Family Sampler kit), bcl-xL and Mcl-1 polyclonal rabbit (Pro-Survival Bcl-2 Family Sampler kit), VEGFR-2 and pVEGFR-2 (Phospho-VEGF Receptor 2 Antibody Sampler kit) as well as cyclin A2, B1, D1, and E1 (Cyclin Antibody Sampler kit). Additionally, the following antibodies were used: pERK, ERK, p38, pp38, pCREB, STAT3, pSTAT3 (all polyclonal rabbit), pHsp27 (Ser 82) monoclonal rabbit and Hsp27, the cyclic AMP response element binding protein (CREB) monoclonal mouse (all from Cell Signaling Technologies) as well as β -actin monoclonal mouse AC-15 from Sigma. All primary antibodies were diluted as recommended by the manufactures. Secondary, horseradish-peroxidase-labeled antibodies (DakoCytomation, Denmark A/S) were used at dilutions of 1:10000.

2.10. Endothelial cell migration and tube formation

To investigate the impact of Enn B or Sora as single substances or in combination on capillary-like tube formation and on VEGF-induced endothelial cell migration of HUVEC cells, tube formation and scratch assays were performed according to Dornetshuber-Fleiss et al. [17]. In brief, for the tube formation assays HUVEC cells were seeded on angiogenesis slides (Ibi, Martinsried, Germany) coated with matrigel (Growth Factor Reduced, Becton Dickinson). Subsequently, cells were incubated for 20 h with Enn B and/or Sora at the indicated concentrations. Cells were stained with 1 μ M calcein (Sigma) and micrographs of fluorescent cells were taken at 4-fold magnification using a Nikon Eclipse Ti, the FITC filter set of the instrument, and a Nikon Digital Sight DS-Fi1C camera. All experiments were carried out in triplicate.

Scratch assays were performed with HUVEC cells seeded in 24-well culture plates and grown to 90% confluence. In the center of the monolayer a denuded zone (gap) of constant width was scratched with a micropipette tip and cells were exposed for 15 h to 10 ng/ml VEGF, Enn B and/or Sora at the indicated concentrations. Pictures of the artificial wounds were taken at 4-fold magnification at the beginning and at the end of the experiment (after 15 h) using a Nikon Eclipse Ti and a Nikon Digital Sight DS-Fi1C camera. Changes of the open area during the experiment were measured using the Image J programme and statistical significances were evaluated using the Student's t-test.

2.11. Xenograft experiments

Animal experiments were authorized by the Ethics committee (BMWF-66.009/0084-II/36/1013) and carried out according to the Austrian and the Federation of Laboratory Animal Science Associations (FELASA) as well as to the Arrive guidelines for animal care and protection. Sixteen eight-week-old male CB-17 scid/scid (SCID) mice were purchased from Harlan Laboratories (San Pietro al Natisone, Italy). Four animals per cage were kept in a pathogen-free environment and every procedure was done in a laminar airflow cabinet. For xenograft experiments 1×10^6 KB-3-1 cells (diluted in RPMI) were injected subcutaneously into the right flank. When tumor nodules reached a mean size of 25 mm^3 , animals were treated with 25 mg Sora/kg bodyweight orally (dissolved in DMSO, further diluted 1:1 in Cremophor EL:ethanol and again 1:10 with deionized water), 5 mg Enn B/kg body weight intraperitoneally (in 10% DMSO) or both. Animals in the control group received the Cremophor EL solvent orally and DMSO intraperitoneally. The experiment was terminated after 14 days from transplantation by cervical dislocation. Following parameters were evaluated: daily bodyweight, tumor growth (assessed daily by caliper measurement), and final tumor weight. Tumor volume was calculated using the formula $\text{length} \times \text{width}^2 / 2$.

2.12. Immunohistochemistry

Tumor tissues were formalin-fixed, paraffin-embedded and sectioned at $3 \mu\text{m}$. Afterwards, the tissue section was deparaffinised and rehydrated. To visualize histology and to check for live and dead cells, the tissue sections were stained with haematoxylin and eosin (H&E). To evaluate percentage of apoptotic/necrotic and mitotic cells, at least four visual fields per slide were counted microscopically. The proliferative fraction of the tumors was shown by staining with the Ki-67 (clone MiB-1) antibody from DAKO (Glostrup, Denmark; 1: 100) for 1 h at room temperature. Binding of primary antibodies was detected with the UltraVision LP detection system according to the manufacturer's instructions (Thermo Fisher Scientific), followed by incubation with 3,3'-diaminobenzidine and counterstaining with haematoxylin [19].

3. Results

3.1. Cytotoxic synergy between Enn B and Sora

To evaluate the anticancer effects of Enn B in combination with the multikinase inhibitor Sora, concentration-response curves were established. Since Sora is approved for patients with hepatocellular carcinoma and advanced renal cancer, the hepatocellular carcinoma cell line Hep3B and the renal carcinoma cell line Caki-2 were used in a first approach. In Hep3B

cells a profound dose- and time-dependent growth inhibition was observed for Sora as well as for Enn B monotreatment (Fig. 1). In contrast, Caki-2 cells were only weakly affected by the two agents even after 72 h treatment (compare IC_{50} values shown in Tab. 1A). Moreover, concomitant administration of the two drugs potentiated their single anticancer activities in Hep3B cells indicated by a decrease in IC_{50} values of Enn B up to 2.3-fold. This was further confirmed by CI values showing at least additive effects for the drug combination in Hep3B cells (Fig. 1).

As a next step, the combination schedule was tested in the Enn B-sensitive cervix cancer cell line KB-3-1 (carrying genomic HPV 18 copies) [7] and the HPV-negative cervix carcinoma cell line HTB-31 at different time points. Additionally, the cervix cancer cell lines C4-I and CaSki (carrying genomic HPV 18 and 16 copies, respectively) [20] were used as test models for long-term treatment (72 h). In all cell lines tested a profound dose-dependent growth inhibition was observed for Sora as well as for Enn B monotreatment with HTB-31 cells being most sensitive (Tab.1A and Fig. 2A-C). Moreover, concomitant administration of the two drugs potentiated their single anticancer activities indicated by a decrease in the respective IC_{50} values of Enn B up to 13.7-fold (KB-3-1), 11.92-fold (CaSki), 3.12-fold (C4-I), and 2-fold (HTB-31) (Fig. 2A-C, Tab. 1B). Moreover, CI analyses indicated that the Enn B/Sora combination regimen was synergistic in all cervix cancer cell lines tested. Best effects were observed in KB-3-1 with the combination regimen 3 μ M Enn B + 5 μ M Sora (CI = 0.37), in CaSki cells at 2.5 μ M Enn B + 1-5 μ M Sora (CI = 0.38) and in C4-I cells at 2.5-5 μ M Enn B + 10 μ M Sora (CI = 0.45). Synergistic effects of Sora and Enn B were also shown in HTB-31 cells but to a lesser extent in the combination regimen 1-3 μ M Enn B and 1 μ M Sora (CI = 0.74-0.89). Taken together, our results reveal that co-treatment with Enn B and Sora synergistically increased cytotoxicity and improved antiproliferative effects especially in cervix cancer cells.

3.2. Apoptosis induction by Enn B and Sora

To gain more insights into the molecular mechanisms underlying the strong synergistic effects of Enn B and Sora in cervix cancer cells, we further investigated apoptosis induction by these drugs either alone or in combination. Therefore, KB-3-1 cells were used as a representative cell model. FACS analyses of KB-3-1 cells demonstrated that Enn B and Sora monotreatment induced a concentration-dependent increase of cells with collapsed mitochondrial membrane potential (Fig. 3A). This is indicated by JC-1 which remains in its monomeric, green-fluorescent form in the cytoplasm of dead cells or cells with collapsed mitochondrial membrane potential while in intact cells the dye accumulates in the mitochondria and exhibits red fluorescence [7]. In more detail, the number of dead cells with depolarized mitochondrial membrane potential, changed from 3.4% in the control to 24.3% and 11.7% after single treatment with 3 μ M Enn B and 5 μ M Sora, respectively. Remarkably, when cells were cotreated with the two substances, the number of dead cells with distorted mitochondrial membrane increased up to 41%. In accordance, H₂O₂/PI stainings of the combination treatment revealed a distinctly higher amount of PI-permeable cells indicative for late apoptotic/necrotic cells compared to single substance treatment (Fig. 3B). This was accompanied by enhanced PARP cleavage paralleled by the loss of the respective uncut molecule (Fig. 3C). Additionally, the apoptotic cleavage of caspase 7 was

more pronounced in the combinational treatment. Among the other pro-apoptotic factors studied, bak and bim tended to be upregulated in the combination schedule while the pro-apoptotic bax remained widely unchanged. In contrast, the pro-survival bcl-2 family members bcl-xL and Mcl-1 were reduced by the highest Enn B concentration as well as in the combination treatment (Fig. 3C). Overall, these data indicate that the synergism between Enn B and Sora in killing cervical cancer cells might be achieved through intrinsic apoptosis induction.

3.3. Effects of Enn B and Sora cotreatment on cell cycle progression

To further investigate the effects of Enn B and Sora on DNA synthesis, ³H-thymidine assays were performed revealing a profound DNA synthesis stop in cells with the combination regimen treatment, while single drug-treated cells were only minimally affected (Fig. 4A). This block of DNA synthesis was also accompanied by changes in the cell cycle distribution. Enn B as well as Sora mono-treatment for 24 h (Fig. 4B, left panel) resulted in a moderate increase of cells in G₀/G₁ cell cycle phase. Remarkably, the combined treatment exhibited a completely different effect on cell cycle distribution, namely a S and G₂/M phase arrest paralleled by loss of cells in G₀/G₁ phase. Also prolonged (48 h) drug treatment (Fig. 4B, left panel) slightly enhanced these effects. Interestingly, the increase of G₂/M cells at the combination of 1 μM Enn B and 5 μM Sora after 24 h was not observable any more. To further characterize these alterations in cell cycle distribution, changes in the cyclin expression patterns were analysed by Western blotting (Fig. 4C). The Enn B/Sora-induced S-phase increase and the respective loss of cells in G₀/G₁ phase correlated well with a reduction in protein levels of cyclin D1 and E1. In parallel, activation of the G₂/M checkpoint was indicated by induction of the G₂/M checkpoint cyclins B1 and A2 specifically at the higher Enn B concentrations [21]. These data suggest that the synergistic interaction between Enn B and Sora might involve impaired DNA replication in S-phase and arrest at the G₂/M checkpoint. Considering the high apoptosis rate already after 24 h exposure (compare Fig. 3A and B), the persisting accumulation of cells in G₂/M phase might also reflect preferential survival of a non-cycling cell subpopulation at least for 48 h.

3.4. Impact of Enn B and Sora treatment on MAPK signaling

Since Sora is well-known to interact with several kinases involved in the mitogen-activated protein kinase (MAPK) pathways [1, 3], the effects of Enn B and Sora on MAPK signaling were investigated by Western blot analysis (Fig. 5A and B). Concisely, the phosphorylation of ERK, which is in general associated with cell survival, proliferation and differentiation, was significantly decreased by Enn B monotreatment while this effect was significantly abrogated in combination with Sora. Regarding the stress kinase p38, a strong activation was observed under Enn B single drug treatment and to a lesser extend in the combination regimen (Fig. 5A). A comparable pattern was observed for the p38-downstream target CREB, while STAT3 was significantly inhibited by both, Enn B monotreatment and EnnB/Sora combination treatment (Fig. 5B). Consequently, it was further tested whether the specific MAPK inhibitors U0126 (an ERK upstream MEK 1/2 inhibitor), SB203580 (p38 inhibitor), and WP1066 (STAT3 inhibitor) interact with Enn B anticancer activity. As shown in Figure 5C, inhibition of MEK1/2 distinctly increased the cytotoxicity of Enn B. Additionally, p38 inhibition significantly enhanced the cytotoxic potential of the cyclic

depsiptide while inhibition of the p38 downstream target STAT3 showed only minor effects. Since p38 MAPK is known to activate the anti-apoptotic heatshock protein (Hsp) 27 by phosphorylation [22], we further investigated the impact of p38 inhibition by SB203580 on the phosphorylation levels of Hsp27 after Enn B treatment. Correlating with the increased cytotoxicity of Enn B after p38 inhibition (Fig. 5D), a reduced phosphorylation of Hsp27 (Fig. 5D) was observed when cells were concomitantly treated with the p38 inhibitor and Enn B. Collectively, these data imply that a complex interference with the MAPK signaling is underlying the synergistic effects of Enn B and Sora.

3.5. Enn B and Sora inhibit migration and tube formation of human endothelial cells

VEGFR and PDGFR signaling is involved in endothelial cell mitogenesis and migration. Thus, we investigated the impact of Enn B and Sora on HUVEC behavior. Human endothelial cells normally form tubes and branching networks, when cultured in the presence of a three dimensional supportive matrix [23]. Thus, we evaluated whether this characteristic feature of endothelial cells is affected by Enn B and Sora as mono- or combination treatments at subtoxic concentrations (IC₅₀ values of Enn B and Sora after 48 h treatment: 7.7 μ M and 2.45 μ M, respectively). For Sora inhibitory effects on cell migration and tube formation were already described in the literature at concentrations between 0.001 μ M and 0.01 μ M [24]. Thus, according to literature 0.01 μ M Sora were used to investigate the impact on endothelial cell behavior. Representative networks of tube structures shown in Figure 6A reveal that both substances strongly inhibit capillary-like tube formation of HUVEC already within 20 h. When Enn B and Sora were co-administered, this inhibitory effect tended to be a bit stronger. To further test the effect of the substances on VEGF-induced migration activity of HUVEC, *in vitro* wound healing assays were performed. While VEGF-supplemented cells were able to close the artificial wound within 15 h, 57.5% and 75% of the initial gap remained open when Sora or Enn B were added to the cells, respectively. In the combination regimen even 86.8% open wound area persisted (Fig. 6B). Additionally, the impact on VEGFR-2 signaling which is associated with cell migration was investigated. Unexpectedly, although Enn B was shown to inhibit migration and tube formation of HUVEC, no effect on VEGF-induced VEGFR-2 phosphorylation was observed (Fig. 6C). However, for Sora and the combination regimen a significant abrogation of the VEGFR-2 activation was detected.

These results suggest possible antiangiogenic effects of Enn B and further indicate that the fusariotoxin at least in part supports the well-known inhibitory effects of Sora on VEGF-induced migration and tube formation of endothelial cells.

3.6. Enn B and Sora synergistically reduce tumor growth *in vivo*

Finally, the anticancer activities of Enn B and Sora were validated in a KB-3-1 cervix carcinoma xenograft model. As *in vivo* toxicity data for Enn B are fragmentary, we started the experiments with toxicity analysis in tumor-free mice (data not shown). Based on these studies and according to recent data on the structurally-related destruxin B in a colon cancer xenograft model [25], we decided to use for *in vivo* application a 5 mg/kg bw/day i.p. scheme. The Sora concentration was selected according to Heffeter et al. [26]. To assess the effects of treatments on toxicity, body weight and animal behavior were monitored

throughout the study. None of the treatment schedules was associated with any apparent signs of toxicity such as reduced food and fluid consumption, fatigue (data not shown) or body weight alterations (Fig. 7A). With regard to the anticancer activity of the test substances, solvent- and Enn B-treated mice exhibited similar tumor burden as reflected by approximately equal tumor volume and tumor weight at day 14 (Fig. 7B, C). However, in Sora-treated animals tumor progression appeared to be reduced as shown by a 1.8-fold and 3-fold decreased tumor volume and tumor weight, respectively. Remarkably, the combined treatment with Sora and Enn B distinctly enhanced the antitumor effect indicated by a 4.7-fold and 10.8-fold reduced final tumor volume and tumor weight, respectively. Additionally, H&E stainings of the collected tumor samples depicted loss of cell-cell contacts and tissue disintegration in tumors treated with the drug combination (Fig. 7E). Furthermore, a significant increase of apoptotic/necrotic cells (from 10% to 56%) as well as a moderate decrease in cells displaying mitotic features were found for the combination treatment compared to solvent controls (Fig. 7D, E). Accordingly, the fraction of Ki-67-positive proliferating cells was distinctly reduced (Fig. 7E). Taken together, the data obtained from the *in vivo* experiments are further emphasizing the potential of the combination regimen against cervix carcinoma.

4. Discussion and Conclusion

In an attempt to search for new and better anticancer drug combinations, we investigated the activity of the cyclohexadepsipeptide Enn B [11], which recently came into focus of interest as possible anticancer agent [7-10], together with Sora, a well-established TKI approved for treatment of renal cell carcinoma and hepatocellular carcinoma [1, 2]. *In vitro* experiments performed in this study demonstrated that combination of Sora with Enn B had particular synergistic activity in cervix carcinoma cell lines. This synergistic effect was further confirmed *in vivo* using a SCID mouse xeno-transplantation model. Thus, an Enn B and Sora combination treatment might be especially attractive for this tumor entity.

Cervical carcinoma is a major gynecological cancer especially in developing countries which is nearly almost (~ 90%) caused by HPV infections [27]. Notably, the Enn B/Sora combination was particularly potent in the HPV-positive KB-3-1, CaSki and C4-I cell lines while the synergistic effects in HTB-31 cell was less pronounced. Thus, the different susceptibility of the diverse cervical cancer cell lines tested in this study might be based on their different p53 and pRb status. While in KB-3-1, CaSki and C4-I cells the virally encoded proteins E6 and E7 of HPV16 and 18 bind and inactivate p53 and pRb, the HPV-negative HTB-31 cells harbour a p53 “loss-of-function” mutation and an in-frame deletion in the pRb protein [28].

So far, cytotoxic treatment options for advanced cervical cancer are very limited. Most commonly, a cisplatin-based cytotoxic chemotherapy is used with response rates ranging from 20% to 30% and an overall survival of less than 10 months [29, 30]. Thus, the search for novel and advanced chemotherapeutic drugs or drug combinations is of utmost importance. Remarkably, Sora is currently tested in different clinical trials in combination with radiotherapy and cisplatin for advanced cervical carcinoma but conclusive data are not available so far [29, 30]. Recently, Mao et al. reported cytotoxic activity of Sora in HeLa

cells by directly blocking the VEGFR-2 phosphorylation and the downstream MEK/ERK pathway [31]. This is in accordance with the data obtained in this study demonstrating comparable IC₅₀ values and MAPK inhibition indicated by reduced phosphorylation of ERK as well as p38 and the p38 down-stream targets CREB and STAT3. Additionally, apoptotic and cytostatic activities were shown in the KB-3-1 cell model. Taken together, data from literature and our results suggest that Sora might have potential for treatment of cervical cancer.

In addition, our study revealed that the activity of Sora against cervix cancer is further increased by combination with the cyclohexadepsipeptidic *Fusarium* metabolite Enn B. Accordingly, synergistic anti-proliferative and migratory effects have been shown for the combination of the structurally related cyclohexadepsipeptide destruxin B and Sora in a hepatocellular carcinoma cell line [32]. Consequently, we here elucidate several molecular mechanisms underlying this profound synergism. Initially, combination treatment resulted in a marked increase in apoptotic cell death via the mitochondrial pathway which was further confirmed by *in vivo* data revealing more than 50% apoptotic cells in the tumor tissues of Enn B/Sora-treated mice. Additionally, impaired DNA replication in S-phase and accumulation of cells in G₂/M phase was observed. These findings were accompanied by several biochemical changes which altogether suggest the Enn B/Sora regimen as potential strategy for cervical cancer treatment.

In order to dissect molecular factors underlying the observed synergism between Enn B and Sora, cellular stress signal pathway analyses were performed. In general, the MAPKs ERK, JNK, and p38 play an important role in controlling cellular responses to the environment (xenobiotics) and in regulating gene expression, cell growth, and apoptosis. Thus, ERK, JNK, and p38 pathways are promising molecular targets for anticancer drug development [33, 34]. Collectively, data obtained in this study revealed at least a partially molecular interplay between Enn B/Sora and the ERK and p38 signaling molecules. In contrast, the Jun amino-terminal kinases (JNK) does not play a major role in the synergistic effects of Enn B and Sora as phosphorylation of this MAPK remained widely unchanged (data not shown).

In particular, Western blot analyses showed that Enn B single substance treatment induces a concentration-dependent activation of the p38 MAPK which was further corroborated by hyperphosphorylation of its major cytosolic target, the cyclic AMP response CREB. Sora in turn particularly inhibited Enn B-mediated p38 and to a lesser extent CREB phosphorylation. In contrast, phosphorylation of STAT3, another down-stream target of p38, was distinctly reduced by both substances. For Sora comparable inhibitory effects on STAT3 have already been described [35, 36] and are also underlying the synergism with the anti-platelet agent YC-1 [37]. Frequently, the p38 MAPK kinase pathway is activated in response to a wide range of cellular stress stimuli such as chemotherapeutic drugs [38] or other food-borne fusariotoxins like ochratoxin [39], T-2 toxin [40], or deoxynivalenol [41]. Additionally, p38-stimulating effects were shown for the structurally related and currently preclinically investigated romidepsin (FR901228) [42] which might suggest that p38 induction is characteristic for cyclic depsipeptides and/or mycotoxins from food borne fungi. Originally, the p38 MAPK was identified as stress kinase stimulating rather than protecting

from stress-induced cell death. However, during the last years there was increasing evidence that enhanced survival instead of death is promoted by p38 MAPK activation [26, 38]. Corroboratingly, our data revealed that treatment with the specific p38 inhibitor SB203580 resulted in significantly enhanced Enn B-induced cell death. Since p38 MAPK is known to phosphorylate the anti-apoptotic Hsp27 [22], our data further indicate that p38 MAPK inhibition prevents the phosphorylation of Hsp27 by Enn B. This strongly suggests a protective function of p38 against Enn B which is inhibited by Sora. Accordingly, a shift from G₀/G₁ cell cycle arrest in single drug-treated cells to G₂/M in combi-treated cells was observed. As the p38 pathway is known to induce a G₁/S checkpoint-mediated cell cycle arrest [38], it is feasible that overcoming the Enn B-induced G₀/G₁ arrest might be one mechanism underlying the observed synergistic activity of Enn B with Sora. Summarizing these data suggest that attenuation of the Enn B-induced p38 MAPK pathway by Sora supports synergistic anticancer activities by negatively regulating cell survival and proliferation. Comparable synergistic effects were reported for the ruthenium compound KP1339 with Sora showing p38 inhibitory properties of Sora on KP1339-induced p38 signaling [43]. However, as specific p38 inhibition only modestly enhanced the anticancer activity of Enn B, it was concluded that interfering with the p38 signaling might not be the only mechanism underlying the observed synergistic activity of Enn B with Sora.

Indeed, a central role was also shown for ERK. However, in contrast to p38, an inhibitory effect of Enn B was found for the ERK signaling pathway. This is in accordance with data from Waetjen et al. [10] reporting a comparable disruption of the ERK signaling pathway by Enn B and the derivatives Enn A1 and B1. Since ERK is generally associated with cell proliferation and constitutively active in several tumors, [44] inhibition of this pathway by Enn B might be of interest for tumor therapy. Moreover, specific ERK upstream inhibition by the MEK1/2 inhibitor U0126 potentially sensitized cells to Enn B further suggesting that ERK is involved in cell survival and proliferation. Unexpectedly, in the Sora/Enn B combination regimen ERK phosphorylation was restored. As cells recognize and respond to cytotoxic stimuli by engaging specific intracellular programs, we suggest that the observed restoration of the ERK signaling by Enn B/Sora combination treatment – especially at high concentrations – might be a stress-induced compensatory mechanism of a surviving cells subpopulation. Alternatively, the combination of Enn B and Sora may block a negative control pathway responsible for inhibition of specific MAPK phosphatases which are induced due to environmental stress and selectively down-regulate ERK activity [45]. Thus, we hypothesize that beside p38 signaling also the modulation of ERK pathway is involved in the strong synergism of Enn B and Sora.

Finally, also antiangiogenic activities might be involved in the strong synergism. *In vitro* data showed that Enn B exerts potent antiangiogenic effects indicated by an abrogated VEGF-induced HUVEC migration and tube formation after Enn B treatment. Interestingly, we found no effect on VEGF-induced VEGFR-2 phosphorylation. Similar results were reported for the chemotherapeutic drug Taxotere [46]. This microtubule-targeting agent prevents signaling from VEGFR via ubiquitination and subsequent proteasomal degradation of Hsp 90 which is important to stabilize signaling molecules associated with VEGF-mediated endothelial cell migration [46]. Thus, similar mechanisms could be suggested for

Enn B. Moreover, accumulating data suggest the importance of Wnt signaling in endothelial cell proliferation, migration and survival. Especially for Wnt3a canonical and non-canonical VEGFR-independent promotion of HUVEC proliferation and migration was reported [47]. Since for the structurally related destruxins interferences with the Wnt signaling pathway are reported [25, 32] inhibitory effects of Enn B on Wnt3a might be assumed. In summary, the mechanisms underlying the inhibitory effects on HUVEC cell migration and tube formation might be multifarious and matter of ongoing studies. However, to our knowledge, this is the first report showing angiogenesis inhibiting effects for Enn B while comparable effects were already reported for the structurally related cyclopeptide YSNSG [48]. Additionally, the cyclohexadepsipeptides beauvericin and destruxins were shown to exert antiangiogenic activities in HUVEC-2 [17, 49]. As these properties tended to be more pronounced in combination with the well-known angiogenesis inhibitor Sora, it indicates that angiogenesis inhibition by down-regulation of endothelial cell migration and capillary-like structure formation might also play at least in part a role in the observed *in vivo* synergism. Further *in vitro* and *in vivo* studies are currently underway to fully elucidate the antiangiogenic potential of this drug combination.

Taken together, our data indicate that Enn B and Sora exert promising synergistic activities *in vitro* and *in vivo* against human cervix carcinoma and that this synergism is based on a complex interference with MAPK signaling and angiogenesis inhibition. Thus, combining Enn B with Sora represents a novel therapeutic strategy for further clinical development in advanced cervical cancer.

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Abbreviations

Bax	Bcl-2-associated X protein
bw	body weight
CI	combination index
Ci	curie
c-Kit	c-Kit receptor tyrosine kinase
cpm	counts per minute
CREB	cyclic AMP response element binding protein
EBM-2	endothelial basal medium
Enn	enniatin
ERK	extracellular signal-regulated kinase

ESI	electrospray ionisation
FACS	fluorescence-activated cell sorter
FITC	fluorescein isothiocyanate
FLT-3	Fms-like tyrosine kinase-3
H&E	haematoxylin and eosin
HÖ/PI	Hoechst 33258/propidium iodide
Hsp27	heat shock protein 27
HPV	human papillomavirus
HUVEC	human umbilical vein endothelial cells
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolylcarbocyanine iodide
JNK	Jun amino-terminal kinases
LCMS	liquid chromatography mass spectrometry
MAPK	mitogen-activated protein kinase
MDR	multidrug resistance
MEK 1/2	mitogen-activated protein kinase kinase
MNP	minimal essential medium modified with non-essential amino acids and pyruvate
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	nuclear magnetic resonance
PARP	poly(ADP-ribose) polymerases
PBS	phosphate-buffered saline
pCREB	phosphorylated cyclic AMP response element binding protein
PDGF	platelet-derived growth factor
Raf	v-raf murine sarcoma 3611 viral oncogene homolog
RET	rearranged during transfection receptor tyrosine kinase
SCID	severe combined immunodeficiency
SD	standard deviation
SDS/PAGE	sodium dodecyl sulfate/polyacrylamide gel electrophoresis
SEM	standard error mean
Sora	sorafenib
STAT3	signal transducers and activators of transcription 3
TKI	tyrosine kinase inhibitors

VEGFR

vascular endothelial growth factor receptor

References

- [1]. Iyer R, Fetterly G, Lugade A, Thanavala Y. Sorafenib: a clinical and pharmacologic review. Expert opinion on pharmacotherapy. 2010; 11:1943–55. [PubMed: 20586710]
- [2]. Shen YC, Hsu C, Cheng AL. Molecular targeted therapy for advanced hepatocellular carcinoma: current status and future perspectives. J Gastroenterol. 2010; 45:794–807. [PubMed: 20567987]
- [3]. Yu J, Zhang L. The transcriptional targets of p53 in apoptosis control. Biochem Biophys Res Commun. 2005; 331:851–8. [PubMed: 15865941]
- [4]. Hartmann JT, Haap M, Kopp HG, Lipp HP. Tyrosine Kinase Inhibitors - A Review on Pharmacology, Metabolism and Side Effects. Curr Drug Metab. 2009; 10:470–81. [PubMed: 19689244]
- [5]. Arora A, Scholar EM. Role of tyrosine kinase inhibitors in cancer therapy. Journal of Pharmacology and Experimental Therapeutics. 2005; 315:971–9. [PubMed: 16002463]
- [6]. Ali R, Mirza Z, Ashraf GMD, Kamal MA, Ansari SA, Damanhour GA, et al. New Anticancer Agents: Recent Developments in Tumor Therapy. Anticancer research. 2012; 32:2999–3005. [PubMed: 22753764]
- [7]. Dornetshuber R, Heffeter P, Kamyar MR, Peterbauer T, Berger W, Lemmens-Gruber R. Enniatin exerts p53-dependent cytostatic and p53-independent cytotoxic activities against human cancer cells. Chem Res Toxicol. 2007; 20:465–73. [PubMed: 17326668]
- [8]. Dornetshuber R, Heffeter P, Lemmens-Gruber R, Elbling L, Marko D, Micksche M, et al. Oxidative stress and DNA interactions are not involved in Enniatin- and Beauvericin-mediated apoptosis induction. Mol Nutr Food Res. 2009; 53:1112–22. [PubMed: 19653228]
- [9]. Dornetshuber R, Heffeter P, Sulyok M, Schumacher R, Chiba P, Kopp S, et al. Interactions between ABC-transport proteins and the secondary Fusarium metabolites enniatin and beauvericin. Mol Nutr Food Res. 2009; 53:904–20. [PubMed: 19517454]
- [10]. Watjen W, Debbab A, Hohlfeld A, Chovolou Y, Kampkotter A, Edrada RA, et al. Enniatins A1, B and B1 from an endophytic strain of Fusarium tricinctum induce apoptotic cell death in H4IIE hepatoma cells accompanied by inhibition of ERK phosphorylation. Mol Nutr Food Res. 2008; 53:431–40. [PubMed: 19065580]
- [11]. Sussmuth R, Muller J, von Dohren H, Molnar I. Fungal cyclooligomer depsipeptides: from classical biochemistry to combinatorial biosynthesis. Nat Prod Rep. 2011; 28:99–124. [PubMed: 20959929]
- [12]. Jestoi M. Emerging fusarium-mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin: a review. Crit Rev Food Sci Nutr. 2008; 48:21–49. [PubMed: 18274964]
- [13]. Uhlig S, Jestoi M, Parikka P. Fusarium avenaceum -- the North European situation. Int J Food Microbiol. 2007; 119:17–24. [PubMed: 17884217]
- [14]. Faeste CK, Ivanova L, Uhlig S. In vitro metabolism of the mycotoxin enniatin B in different species and cytochrome p450 enzyme phenotyping by chemical inhibitors. Drug metabolism and disposition: the biological fate of chemicals. 2011; 39:1768–76. [PubMed: 21622627]
- [15]. Norbert Madry RZ, Kleinkauf Horst. Enniatin Production by Fusarium oxysporum in Chemically Defined Media. European Journal of Applied Microbiology and Biotechnology. 1983; 17:75–9.
- [16]. Shen DW, Cardarelli C, Hwang J, Cornwell M, Richert N, Ishii S, 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; 261:7762–70. [PubMed: 3711108]
- [17]. Dornetshuber-Fleiss R, Heffeter P, Mohr T, Hazemi P, Kryeziu K, Seger C, et al. Destruxins: Fungal-derived Cyclohexadepsipeptides with Multifaceted Anticancer and Antiangiogenic Activities. Biochem Pharmacol. 2013; 86:361–77. [PubMed: 23747344]

- [18]. Chou TC. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacological reviews*. 2006; 58:621–81. [PubMed: 16968952]
- [19]. Lotsch D, Steiner E, Holzmann K, Spiegl-Kreinecker S, Pirker C, Hlavaty J, et al. Major vault protein supports glioblastoma survival and migration by upregulating the EGFR/PI3K signalling axis. *Oncotarget*. 2013; 4:1904–18. [PubMed: 24243798]
- [20]. Wang H, Gao P, Zheng J. Arsenic trioxide inhibits cell proliferation and human papillomavirus oncogene expression in cervical cancer cells. *Biochem Biophys Res Commun*. 2014; 451:556–61. [PubMed: 25117446]
- [21]. Takita M, Furuya T, Sugita T, Kawauchi S, Oga A, Hirano T, et al. An analysis of changes in the expression of cyclins A and B1 by the cell array system during the cell cycle: comparison between cell synchronization methods. *Cytometry Part A: the journal of the International Society for Analytical Cytology*. 2003; 55:24–9. [PubMed: 12938185]
- [22]. Combaret V, Boyault S, Iacono I, Brejon S, Rousseau R, Puisieux A. Effect of bortezomib on human neuroblastoma: analysis of molecular mechanisms involved in cytotoxicity. *Molecular cancer*. 2008; 7:50. [PubMed: 18534018]
- [23]. Donovan D, Brown NJ, Bishop ET, Lewis CE. Comparison of three in vitro human ‘angiogenesis’ assays with capillaries formed in vivo. *Angiogenesis*. 2001; 4:113–21. [PubMed: 11806243]
- [24]. Mangiameli DP, Blansfield JA, Kachala S, Lorang D, Schafer PH, Muller GW, et al. Combination therapy targeting the tumor microenvironment is effective in a model of human ocular melanoma. *Journal of translational medicine*. 2007; 5:38. [PubMed: 17640369]
- [25]. Yeh CT, Rao YK, Ye M, Wu WS, Chang TC, Wang LS, et al. Preclinical evaluation of destruxin B as a novel Wnt signaling target suppressing proliferation and metastasis of colorectal cancer using non-invasive bioluminescence imaging. *Toxicology and applied pharmacology*. 2012; 261:31–41. [PubMed: 22465936]
- [26]. Heffeter P, Atil B, Kryeziu K, Groza D, Koellensperger G, Korner W, et al. The ruthenium compound KP1339 potentiates the anticancer activity of sorafenib in vitro and in vivo. *Eur J Cancer*. 2013; 49:3366–75. [PubMed: 23790465]
- [27]. Choo KB, Chong KY. Absence of mutation in the p53 and the retinoblastoma susceptibility genes in primary cervical carcinomas. *Virology*. 1993; 193:1042–6. [PubMed: 8384745]
- [28]. Wesierska-Gadek J, Borza A, Walzi E, Krystof V, Maurer M, Komina O, et al. Outcome of treatment of human HeLa cervical cancer cells with roscovitine strongly depends on the dosage and cell cycle status prior to the treatment. *Journal of cellular biochemistry*. 2009; 106:937–55. [PubMed: 19180585]
- [29]. del Campo JM, Prat A, Gil-Moreno A, Perez J, Parera M. Update on novel therapeutic agents for cervical cancer. *Gynecologic oncology*. 2008; 110:S72–6. [PubMed: 18544460]
- [30]. Gonzalez-Cortijo L, Carballo N, Gonzalez-Martin A, Corraliza V, Chiva de Agustin L, Lapuente Sastre F, et al. Novel chemotherapy approaches in chemoradiation protocols. *Gynecologic oncology*. 2008; 110:S45–8. [PubMed: 18678399]
- [31]. Mao WF, Shao MH, Gao PT, Ma J, Li HJ, Li GL, et al. The important roles of RET, VEGFR2 and the RAF/MEK/ERK pathway in cancer treatment with sorafenib. *Acta pharmacologica Sinica*. 2012; 33:1311–8. [PubMed: 22941289]
- [32]. Huynh TT, Rao YK, Lee WH, Chen HA, Do-Quyen Le T, Tzeng DT, et al. Destruxin B inhibits hepatocellular carcinoma cell growth through modulation of the Wnt/beta-catenin signaling pathway and epithelial-mesenchymal transition. *Toxicology in vitro: an international journal published in association with BIBRA*. 2014; 28:552–61. [PubMed: 24434019]
- [33]. Xu C, Li CY, Kong AN. Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch Pharm Res*. 2005; 28:249–68. [PubMed: 15832810]
- [34]. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science*. 2002; 298:1911–2. [PubMed: 12471242]
- [35]. Gu FM, Li QL, Gao Q, Jiang JH, Huang XY, Pan JF, et al. Sorafenib inhibits growth and metastasis of hepatocellular carcinoma by blocking STAT3. *World journal of gastroenterology: WJG*. 2011; 17:3922–32. [PubMed: 22025881]

- [36]. Zhao W, Zhang T, Qu B, Wu X, Zhu X, Meng F, et al. Sorafenib induces apoptosis in HL60 cells by inhibiting Src kinase-mediated STAT3 phosphorylation. *Anti-cancer drugs*. 2011; 22:79–88. [PubMed: 20881478]
- [37]. Kong J, Kong F, Gao J, Zhang Q, Dong S, Gu F, et al. YC-1 enhances the anti-tumor activity of sorafenib through inhibition of signal transducer and activator of transcription 3 (STAT3) in hepatocellular carcinoma. *Molecular cancer*. 2014; 13:7. [PubMed: 24418169]
- [38]. Thornton TM, Rincon M. Non-classical p38 map kinase functions: cell cycle checkpoints and survival. *International journal of biological sciences*. 2009; 5:44–51. [PubMed: 19159010]
- [39]. Rumora L, Grubisic TZ. A journey through mitogen-activated protein kinase and ochratoxin A interactions. *Arhiv za higijenu rada i toksikologiju*. 2009; 60:449–56. [PubMed: 20061246]
- [40]. Kruber P, Trump S, Behrens J, Lehmann I. T-2 toxin is a cytochrome P450 1A1 inducer and leads to MAPK/p38-but not aryl hydrocarbon receptor-dependent interleukin-8 secretion in the human intestinal epithelial cell line Caco-2. *Toxicology*. 2011; 284:34–41. [PubMed: 21453744]
- [41]. Lucioi J, Pinton P, Callu P, Laffitte J, Grosjean F, Kolf-Clauw M, et al. The food contaminant deoxynivalenol activates the mitogen activated protein kinases in the intestine: Interest of ex vivo models as an alternative to in vivo experiments. *Toxicon*. 2013; 66:31–6. [PubMed: 23403092]
- [42]. Song P, Wei JX, Wang HCR. Distinct roles of the ERK pathway in modulating apoptosis of Ras-transformed and non-transformed cells induced by anticancer agent FR901228. *FEBS letters*. 2005; 579:90–4. [PubMed: 15620695]
- [43]. Heffeter P, Atil B, Kryeziu K, Groza D, Koellensperger G, Korner W, et al. The ruthenium compound KP1339 potentiates the anticancer activity of sorafenib in vitro and in vivo. *European Journal of Cancer*. 2013; 49:3366–75. [PubMed: 23790465]
- [44]. Kang S, Kim HS, Seo SS, Park SY, Sidransky D, Dong SM. Inverse correlation between RASSF1A hypermethylation, KRAS and BRAF mutations in cervical adenocarcinoma. *Gynecologic oncology*. 2007; 105:662–6. [PubMed: 17360030]
- [45]. Chuang SM, Wang IC, Yang JL. Roles of JNK, p38 and ERK mitogen-activated protein kinases in the growth inhibition and apoptosis induced by cadmium. *Carcinogenesis*. 2000; 21:1423–32. [PubMed: 10874022]
- [46]. Murtagh J, Lu H, Schwartz EL. Taxotere-induced inhibition of human endothelial cell migration is a result of heat shock protein 90 degradation. *Cancer Res*. 2006; 66:8192–9. [PubMed: 16912198]
- [47]. Samarzija I, Sini P, Schlange T, Macdonald G, Hynes NE. Wnt3a regulates proliferation and migration of HUVEC via canonical and non-canonical Wnt signaling pathways. *Biochem Biophys Res Commun*. 2009; 386:449–54. [PubMed: 19523451]
- [48]. Thevenard J, Ramont L, Devy J, Brassart B, Dupont-Deshorgue A, Floquet N, et al. The YSNSG cyclopeptide derived from tumstatin inhibits tumor angiogenesis by down-regulating endothelial cell migration. *International journal of cancer Journal international du cancer*. 2010; 126:1055–66. [PubMed: 19551865]
- [49]. Zhan J, Burns AM, Liu MX, Faeth SH, Gunatilaka AA. 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; 70:227–32. [PubMed: 17286429]

Figure 1. Anticancer activity of Enn B combined with Sora in Hep3B cells

Hep3B cells were treated for the indicated time points with Enn B and Sora. Viability was evaluated by MTT assay. Values given are means $\pm$ SEM of three independent experiments performed in triplicates. Combination indexes (CI) for the 72 h analyses were calculated using CalcuSyn software. CI < 0.9, CI = 0.9 - 1.1 or CI > 1.1 represent synergism, additive effects, and antagonism, respectively.

Figure 2. Anticancer activity of Enn B combined with Sora in cervix cancer cells

(A) KB-3-1, (B) HTB-31, (C) C4-I and CaSki cells were treated for the indicated time points with Enn B and Sora. Viability was evaluated by MTT assay. Values given are means $\pm$ SEM of three independent experiments performed in triplicates. (D) Combination indexes (CI) for the 72 h analyses were calculated using CalcuSyn software. CI < 0.9, CI = 0.9 - 1.1 or CI > 1.1 represent synergism, additive effects and antagonism, respectively.

Figure 3. Apoptosis induction by Enn B in combination with Sora in KB-3-1 cells

(A) Loss of mitochondrial membrane potential was measured at the indicated concentrations by JC-1 stainings after 24 h drug treatment. Cells with depolarised mitochondria (green fluorescent, FL-1) are indicated in the graph. (B) Percentages of PI-positive nuclei of at least 500 HÖ/PI-stained cells treated 24 h with the indicated Enn B/Sora concentrations, were calculated from three independent experiments. Statistical significances were evaluated using one-way ANOVA followed by Dunnett posttest. Significantly different from medium control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). (C) Impact of 48 h Enn B/Sora

treatment at the indicated concentrations on caspase-induced cleavage of PARP and caspase 7 as well as expression of the Bcl-2 family members bax, bak and bim as well as the pro-survival proteins bcl-xL and Mcl-1 was determined via Western blotting of three biological experiments. β -actin served as loading control. Representative Western blots and quantification analysis are shown. Asterisks indicate significant differences in the protein expression (significantly different to * control; # 2.5 μ M Sora; ° 5 μ M Sora). P values < 0.05; < 0.01; < 0.001 were designated with one, two or three asterisks, respectively.

Figure 4. Impact of Enn B combined with Sora on DNA synthesis and cell cycle distribution
 (A) DNA synthesis of KB-3-1 cells was determined by ^3H -thymidine incorporation after 24 h Enn B and Sora exposure at the indicated concentrations. Data are expressed as mean $\pm$ SD of triplicates. Respective CI values were calculated from incorporated ^3H -thymidine in cells treated with Enn B/Sora alone-or in combination. (B) PI stainings and flow cytometry analyses were performed on KB-3-1 cells after 24 h or 48 h Enn B and Sora incubation at the indicated drug concentrations. Percentages of values G₀/G₁, S, and G₂/M phase are indicated. (C) Representative Western blots of three individual experiments and

quantification analyses are shown for the impact of Enn B and Sora treatment at the indicated doses on the expression pattern of cyclins in KB-3-1 cells. Loading controls were performed using β -actin. Asterisks indicate significant differences in the protein expression (significantly different to * control; # 5 μ M Sora). $p < 0.05$; $p < 0.01$; and $p < 0.001$ was designated with one, two or three asterisks, respectively.

Figure 5. Impact of Enn B in combination with Sora on MAPK signaling

Changes of expression and/or phosphorylation levels of the indicated proteins involved in (A) MAPK and (B) p38-downstream pathway after 48 h Enn B and/or Sora exposure are shown as representative Western blots of three individual experiments and quantification analyses. β-actin served as loading control. Asterisks obtained by one-way ANOVA followed by Dunnett posttest indicate significant differences in the protein expression levels (significantly different to * control; # 2.5 μM Sora; ° 5 μM Sora). P values < 0.05; < 0.01; < 0.001 were designated with one, two or three asterisks, respectively. (C) The impact of

MEK1/2 inhibition by U0126, p38 inhibition by SB203580 or STAT3 inhibition by WP1066 on the anticancer activity of Enn B was tested in KB-3-1 cells by MTT assay after 72 h treatment. Values given are relative means $\pm$ SD from at least 2 independent experiments performed in triplicates. (D) Down regulation of Hsp27 phosphorylation by inhibition of p38 is shown using SB203580 prior to culture with different concentrations of the indicated test substances. Representative Western blots of three individual experiments and quantification analyses are shown (significantly different to control (one-way ANOVA followed by Bonferroni test): *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

Figure 6. Inhibitory effects of Enn B combined with Sora on VEGF-induced HUVEC proliferation, migration, and tubular structure formation

(A) HUVEC cultured on angiogenesis slides coated with matrigel were treated with VEGF alone (control) or in presence of Enn B and/or Sora for 20 h at the indicated concentrations. Photomicrographs shown were taken after calcein staining at 4-fold magnification using a Nikon Digital Sight DS-Fi1C camera and presented as relative values to that of control cells from at least three independent experiments. (B) Scratch assays were used to investigate the effect of Enn B and/or Sora treatment at the indicated concentrations on VEGF-induced endothelial cell migration. Open wound area was measured using the Image J program. (C)

HUVEC cells were plated on fibronectin-covered (10 µg/ml) plates. After overnight starvation Enn B and Sora were added at the indicated concentrations for 2 h. Cells were then stimulated with VEGF (50 ng/ml) for 4 min and the extend of VEGFR-2 was determined using Western blot analyses. Statistical significances were evaluated using one-way ANOVA followed by Bonferroni posttest. Asterisks indicate significant differences in protein expression (* between VEGF-R2 and # between pVEGF-R2). $p < 0.05$; $p < 0.01$ and $p < 0.001$ were designated with one, two or three asterisks, respectively.

Figure 7. *In vivo* anticancer activity of Enn B in combination with Sora

KB-3-1 xenografts were grown in SCID mice and treated with Enn B (5 mg/kg bw; i.p.) and/or Sora (25 mg/kg bw; p.o.) for the indicated days. (A) Body weight was measured at the indicated time points and expressed as change of the ratio between the initial value and the treatment value. Effect of therapy is shown as (B) tumor volume and (C) tumor weight determined after scarification at day 14. (D) The percentage of mitotic and apoptotic/necrotic cells was evaluated in H&E-stained tumor sections. Representative photomicrographs (40× objective) of H&E- and Ki-67-stained sections of controls and

tumors treated with the combined setting are shown (scale bars = 50 μm). Statistical significances were calculated using (B) two-way ANOVA followed by Bonferroni posttest and (C, D) one-way ANOVA followed by Dunnett posttest (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

Table 1A
Anticancer activity of Sora and Enn B monotreatment after 72 h

Cell line	Sorafenib (μM)	Enniatin B (μM)
	Mean IC ₅₀ ^a ± SD	Mean IC ₅₀ ^a ± SD
Hep3B	2.84 ± 0.03	3.38 ± 0.11
Caki-2	9.84 ± 0.17	>10
KB-3-1	5.50 ± 1.04	3.72 ± 0.04
C4-I	8.43 ± 1.68	6.42 ± 2.62
CaSki	8.62 ± 1.62	3.38 ± 0.14
HTB-31	3.55 ± 0.38	1.74 ± 0.23

^aIC₅₀ values were calculated from whole dose response curves. Values given are means ± SD from at least two independent experiments performed in triplicates.

Table 1B
Potentialiation of single drug efficacy by Enn B/ Sora combination

IC ₅₀ of Enn B	Hep3B	KB-3-1	C4-I	CaSki	HTB-31
	-fold decrease ^a	-fold decrease ^a	-fold decrease ^a	-fold decrease ^a	-fold decrease ^a
+ 0.5 µM Sora	1.2	1.1	0.80	0.98	0.9
+ 1 µM Sora	1.1	1.1	1.11	1.31	1.1
+ 2.5 µM Sora	2.3	2.3	2.11	1.67	2
+ 5 µM Sora	-	13.7	3.12	1.72	-
+ 10 µM Sora	-	-	-	11.92	-

^aFold decrease of Enn B IC₅₀ values was calculated by dividing IC₅₀ of Enn B alone by IC₅₀ values of Enn B combined with the indicated Sora concentrations.