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„Anticancer activity potentiation of the cyclohexadepsipeptides Enniatin B and Beauvericin by the protein kinase inhibitors-inhibitors Sorafenib and Sunitinib“

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Table of content

1a. Abstract

Beauvericin (Bea) and Enniatin B (Enn B) are secondary metabolites of the genus *Fusarium,* which are known as contaminants in grain samples[9-12]. Based on *in-vitro* studies, it is clear that Bea and Enn B engage several molecular mechanisms and cellular targets that lead to cell cytotoxicity[11, 13]. However, the exact mechanisms of the cyclodepsipeptides are not yet resolved, it is probed that their strong lipophilic character allows them to incorporate into cell membranes and triggers a cation influx [14-17]. Furthermore, Bea and Enn B induce apoptosis induction via intrinisic pathway[18, 19].

As both remedies are selectively cytotoxic towards cancer cells, the substances are potential candidates for cancer treatment[19]. To enhance the cytotoxic effects, Bea and Enn B were concomitantly administered with the protein kinase inhibitors Sorafenib and Sunitinib. Protein kinase inhibitors are a relatively new, potent group of anticancer therapeutics, which mechanisms of action are widely resolved and are already established for cancer therapy treatment. Viability tests proved depending on the used concentrations and incubation times synergistic, additive and antagonistic effects for the cell lines KB-3-1, Caki 1, Caki 2 and Hep3B. In addition, cell cycle analysis showed an increase in G_0/G_1-phase after single and combinatory treatment with Bea (5 µM) and Sunitinib (1 – 2.5 µM) in Caki 1. Furthermore, the apoptosis assay JC-1 evaluated apoptosis induction in Caki 1 incubated with Bea (5 µM) and in combination with Sunitinib (1 – 2.5 µM). Moreover, both substances revealed anti-angiogenetic and cell migration inhibiting features at sub-toxic concentrations in the scratch assay and tube formation assay. As a fact, co-administration of Bea and Enn B with Sorafenib and Sunitinib resulted in strengthened effects. Concluding, Bea and Enn B, especially in combination with Sorafenib and Sunitinib show a possible new strategy in cancer therapy and should be evaluated in further studies.

1b. Kurzzusammenfassung

Beauvericin (Bea) and Enniatin B (Enn B) sind sekundäre Metabolite der Schimmelpilz Gattung *Fusarium* und kommen als häufige Verunreinigungen in verschiedenen Getreidearten vor[9-12]. Basierend auf *in-vitro* Versuchen wurde gezeigt, dass Bea und Enn B unterschiedliche molekulare Mechanismen und zelluläre Angriffspunkte beeinflussen, die in weiterer Folge zu einer Zytotoxizität führen[11, 13]. Obwohl, der genaue Wirkmechanismus der Cyclodepsipeptide noch nicht geklärt ist, konnte untersucht werden, dass Bea und Enn B durch ihren starken lipophilen Charakter in Zellmembranen eindringen können und dadurch einen Kationen Einstrom hervorrufen. [14-17]. Des Weiteren induzieren Bea und Enn B den intrinischen Apoptose Weg [18, 20].

Da beide Substanzen eine relativ selektive Zytotoxizität gegenüber Krebszellen aufweisen, stellen sie vielversprechende Kandidaten für eine Krebstherapie dar[19]. Um die Effekte von Bea und Enn B zu verstärken wurden sie gemeinsam mit den Proteinkinasehemmern Sorafenib und Sunitinib verabreicht. Proteinkinasehemmer wurden verwendet, da es sich um eine relativ neue und sehr wirkungsvolle Klasse von Krebstherapeutika handelt. In Zytotoxizitätstests konnten je nach Konzentration und Zeitintervall synergistische, additive oder antagonistische Effekte in den Zelllinien KB-3-1, Caki 1, Caki 2 und Hep3B festgestellt werden. Außerdem zeigte eine Zellzyklusanalyse von Caki 1 einen Anstieg der G_0/G_1 Phase, sowohl von 5 µM Bea als Einzelsubstanz, als auch in Kombination mit 1 – 2,5 µM Sunitinib. Anschließend wurde eine JC-1 Färbung durchgeführt, um eine Apoptose Induktion zu evaluieren. In der Zelllinie Caki 1 kam es nach Inkubation mit 5 µM Bea zu einer Erniedrigung des mitochondrialen Potentials. Dieser Effekt konnte durch gleichzeitige Gabe von 1 – 2,5 µM Sunitinib verstärkt werden. Zusätzlich wiesen beide Substanzen im Scratch Assay und im Tubeformation Assay anti-angiogenetische und zellmigrationshemmende Eigenschaften auf, welche in subtoxischen Konzentrationen auch in Kombination mit Sorafenib und Sunitinib bestätigt werden konnten. Abschließend ist zu sagen, dass Bea und Enn B, insbesondere kombiniert mit Sorafenib und Sunitinib eine potentielle zukünftige Therapieform für verschiedene Krebsarten darstellen könnte. Die vorliegenden Ergebnisse sollten in weiteren Versuchen untermauert werden.

2. Introduction

2.1. Definition of Cancer

Growth and division are the most monitoring processes in the life of a cell. Multi-cellular organisms are based on various cellular response mechanisms that enable a controlled equilibrium between cell growth, differentiation and death. Disturbances in this sensitive balance are mostly prevented by several cellular control mechanisms. However, on rare occasions some of these delicate processes are not working properly. These dysfunctions can lead to uncontrolled cell growth and are often followed by an uncontrolled spreading of these cells into diverse tissues, which is one of the main characteristics of cancer[21, 22].

Merriam Webster Encyclopedia defines cancer as following:

1: *a malignant tumor of potentially unlimited growth that expands locally by invasion and systemically by metastasis*

2: *an abnormal state marked by a cancer [23]*

2.2. Epidemiology of Cancer

Cancer is, following cardiovascular diseases, the second most common deadly disease in industrial countries. In Austria around 38.000 people are diagnosed with malignant neoplasms every year. Men are slightly more often affected than women (Statistic Austria). Approximately 13 million new cancer cases occurred worldwide in 2008. Figure 1 demonstrates the incidence rate of all cancer for both sexes and all ages worldwide in 2008. Lung, breast, colorectal and stomach cancer are most prevalent, as they account for 40% of all diagnosed cancer incidences worldwide. As result of the steadily increasing proportion of elderly people there will occur roughly about 16 million new cases of cancer by the year 2020 [24-26]. Although it is not possible to predict whether a person is going to develop a neoplasm, there are certain

risk factors, which induce cancer formation. Some of these risk factors are avoidable,

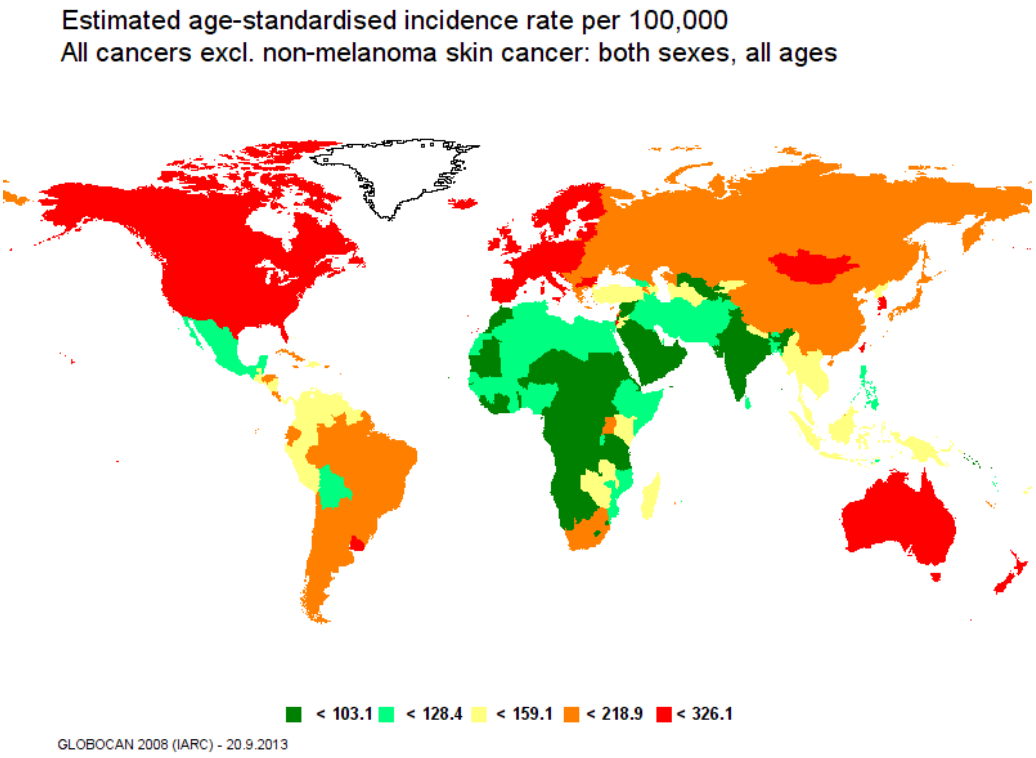

Figure 1: Epidemiology of cancer [7]

including tobacco consumption, excessive alcohol consumption, food that contains high amounts of fat, and massive exposure to UV-light. However, there are several other triggers that raise the hazard of developing cancer that cannot be evaded: Exposure to ionizing radiation, exposure to certain chemicals, infections with viruses or bacteria, intake of certain hormones, inherited genetic preposition, and the leading cause: age [27-29]. As stated above, the factors that lead to malignant neoplasms are multifunctional and though the understanding of tumor development increased significantly in the last decades, cancer is and most likely will remain a crucial threat for public health concern [7].

2.3 Principals of Cancer Development

The development of cancer is a multistep process. As a fact, abnormal proliferation can arise in any kind of cell within the body, therefore, there are beyond 100 different types of cancer, which all exhibit distinct substantial features and as a result, all respond differently to treatment. Thus, it is indispensable to create a classification: at first, cancers are divided into benign and malignant tumors. Further, both benign and malignant tumors are classified according to the type of cell from which they emerge. The three main groups of cancer are carcinomas, sarcomas and leukemias. Carcinomas are the malignancies of epithelial cells and occur most often. As figure 2 shows cancer development is a multistep process, which involves mutation and selection of cells leading to abnormal proliferation, survival, metastasis and invasion. The process starts with the tumor-initiation, which results of a genetic alteration and leads to increased proliferation of a cell. Following, this single cell proliferates and outgrowths a population of clonally derived tumor cells. The ensuing step of tumor progression describes further mutation within the cells. Some of these mutations lead to advantages, such as more rapid growth. Hence, descendants of cells bearing such beneficial features will become dominant and arise within the tumor population. This process is also called clonal selection and continuous throughout cancer development, establishing more rapid growth and increasing malignancy. [30]

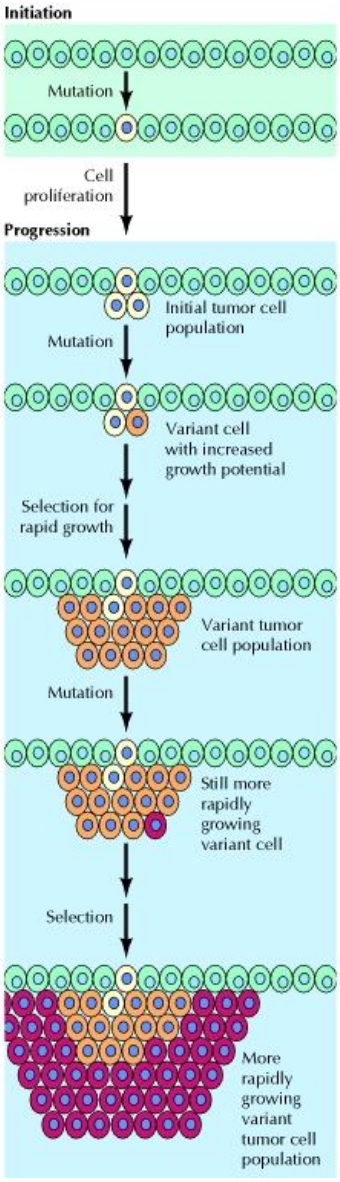

Figure 2: Illustration of the multistep process of cancer development [3]

2.4 Hallmarks of Cancer

Although, cancer and carcinogenesis display an enormous complexity „The Hallmarks of Cancer“ and „The Hallmarks of Cancer: The Next Generation“, reviews written by F. Hanahan and R. Weinberg suggest a relatively simplified overview of the six capabilities that are shared by human tumors and are acquired during their multistep pathogenesis. The comprised capabilities of most or perhaps all types of human

tumors are portrayed in figure 3 and include: **sustaining proliferative signaling, evading growth suppressors, resisting cell death (apoptosis), enabling replicative immortality, sustaining angiogenesis** and **activating tissue invasion** and **metastasis**. During the last decade, two emerging hallmark capabilities have been added to this list: **the reprogramming of energy metabolism** and **evading immune destruction**. In addition, constitution and signaling interactions of tumor microenvironment are crucial for the development of cancer phenotypes, although, because not relevant for this thesis, there are not going to be discussed[31, 32].

2.4.1 Sustaining Proliferative Signaling

The most fundamental characteristic of cancer cells includes their capability to sustain a chronic proliferating condition. In normal cells the production and release of growth factors and therefore the entry and progression in/ into the cell growth and division cycle is strictly controlled. In contrast to healthy cells, tumorous cells do not show a dependence on exogenous growth factors to maintain a stage of growth. Thus, it is suggested that cancer cells are able to generate autocrine growth stimulation. Consequently, dysfunction of critical mechanisms that maintain homeostasis of various cell types within the tissue is disrupted. Numerous types of cancer cells display deregulated signal transducing cell surface receptors. These receptors carry tyrosine kinase activity and take a main part in signal transduction and cellular activity. Hence, another option of exogenous growth factor independence may derive from the constitutive activation of downstream components in certain signaling pathways. [31, 32]

2.4.2 Evading Growth Suppressors

In normal tissue specific signals that inhibit growth/proliferation and induce entrance into a differentiated, post mitotic state, guarantee homeostasis. This post mitotic state is typically connected with the acquisition of quiescent traits. The specific signals are usually received by transmembrane cell surface receptors that lead onwards into intracellular signaling pathways. In general, there are two ways to develop insensitivity to growth inhibitory signals: On the one hand, the deficiency of signal transduction receptors, which results in a lack of initiation of growth-inhibitory pathways. On the

other hand, alterations of tumor suppressor genes lead to the dysfunction of intracellular pathways and eventually to uncontrolled cell proliferation. [32]

2.4.3 Resisting Cell Death

Apoptosis or programmed cell death declines a series of biochemical events that result in disruption of cellular membranes, breakdown of nuclear and cytoplasmic skeletons, chromosomal degradation and fragmentation of the nucleus. In the end phagocytes, engulf the remaining cell fragments until they disappear completely. Frequently, aberrant cells accumulate mutant tumor suppressor genes and do not respond to any repair mechanisms. In this case, apoptosis is induced. However, in most cancer cell lines pro-apoptotic genes are affected by irreversible mutations or anti-apoptotic proteins are overexpressed, leading to evasion of apoptosis. [32], [21]

2.4.4 Enabling Replicative Immortality

Normal human cells lose their ability to proliferate after 50-70 divisions. This phenomenon is also called Hayflick limit and underlies the natural shortening of the telomeres during the cell cycle. Interestingly, this is caused by the DNA polymerase, which shows an inability to replicate the 3´ends of chromosomal DNA. Thus, if the telomeres of a chromosome become too short apoptosis is induced. However, some white blood cells and cells from the stem and germ do not undergo this process, since these cell types are capable of producing telomerase, an enzyme that adds hexanucleotide repeats onto the telomeric DNA. In fact, most cancer cell lines feature an overexpression of telomerase and therefore achieve an immortal status. [32], [22]

2.4.5 Sustaining Angiogenesis

A sufficient supply of nutrients and oxygen provided by the vascular system is crucial for the survival and function of any cell. Angiogenesis is a physiological process and describes the formation of new blood vessels out of pre-existing vessels during wound healing, development and growth. Accordingly, by altering gene transcription tumors achieve the capability of neovascularisation. Thus, there is evidence that many tumors

increase the expression of VEGFs and FGFs or downregulate angiogenetic inhibitors. [32]

2.4.6 Activating Tissue Invasion and Metastasis

A key characteristic that distinguish a benign tumor from a malignant is the ability to invade tissue and to colonize new terrains in the body. As a fact, benign tumors often pose only little risk to their host. In particular, this is explained by cell-adhesion molecules that keep them localized in the originated tissue. Thus, enhanced prospects for surgical resection are achieved. The ability of metastasis and tissue invasion allows the tumor to escape from adverse conditions, such as insufficient nutrient supply, which is provided in the originated tissue. However, malignant tumors are capable of spreading new colonies on distant sites and therefore are able to avoid the affliction of limited space, which is provoked by continuous growth. Although, the process of metastasis is not entirely understood, it generally relies on altered expression of cell adhesion molecules, such as cadherins and integrines. [32]

2.4.7 Emerging Hallmarks

2.4.7.1 Reprogramming of Energy Metabolism

As stated above, uncontrolled cell proliferation represents a main hallmark of neoplastic disease. However, it is indispensable to provide an adequate amount of fuel for this process. Correspondingly, adjustments of the energy metabolism are introduced. Otto Warburg first discovered anomalous characteristics of the cancer cell energy metabolism. As a fact, cancer cells reprogram their glucose metabolism under aerobe conditions. Thus, their energy production enters into a state that is referred to as „aerobic glycolysis“. However, cancer cells have to compensate an about 18-fold lower efficiency of energy production. While normal cells primarily generate ATP for energy via the mitochondrial oxidative phosphorylation (OXPHOS), in various cancer cells glycolysis is enhanced and OXPHOS capacity is reduced. As OXPHOS is much more efficient, to balance or even increase the lack of ATP production, glucose transporters especially GLUT-1 are up regulated and the import of glucose into the cytoplasm is profoundly enhanced. Further, it was demonstrated that gylcolytic fueling is related to the activation of oncogenes and to mutant tumor suppressors. [32]

2.4.7.2 Evading Immune Destruction

The role of the immune system in resisting or destruction of upcoming tumors, late-stage neoplasia and micrometastases is still not entirely resolved. It has been observed that cancer cells disable components of the immune system, such as Cytotoxic T-Lymphocytes and Natural Killer cells. [32]

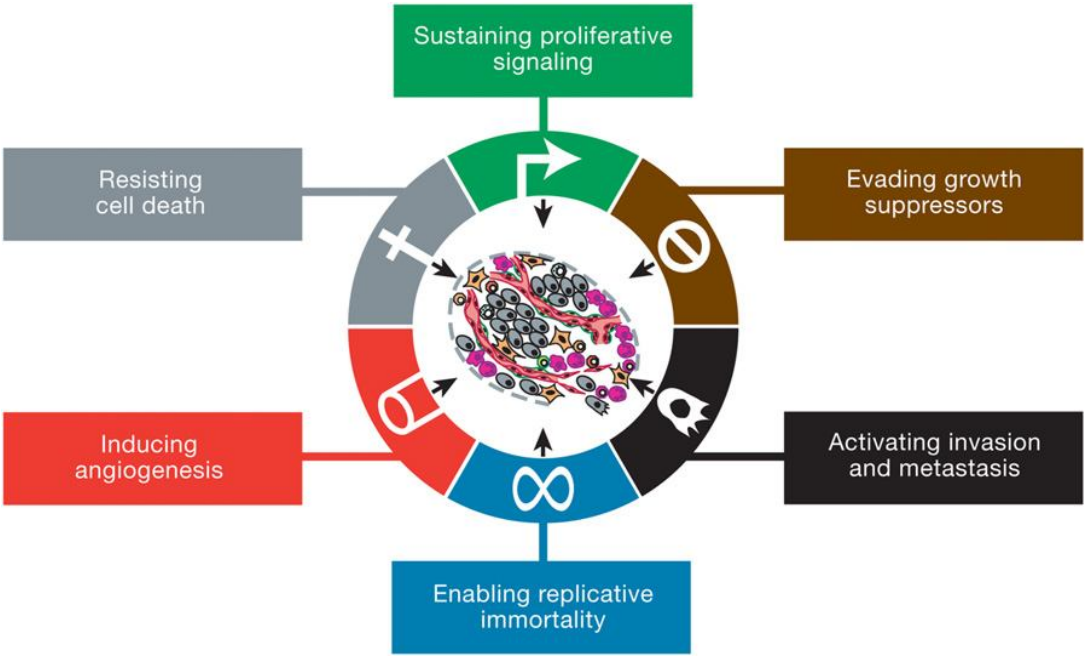

Figure 3: Illustration of D. Hanahan´s and R. Weinberg´s six hallmarks of cancer[18]

2.5 Cell Cycle

The cell cycle constitutes an ordered set of events taking place in the cell, culminating in cell growth and the division into two daughter cells. The proliferation of cells in the body is dictated by mitogenic growth factors that are received from surroundings. Yet, other proteins such as transforming growth factor-β (TGF-β) can halt the proliferation and force the cell to enter a post-mitotic differentiated state. These different signals are received by various cell surface receptors that lead signals into the cytoplasm and the nucleus. In the nucleus the so-called “Cell Cycle Clock” which describes a netting of

interacting proteins, processes and integrates the mixture of different signals and decides whether a cell proliferates or becomes quiescent.

As figure 4 demonstrates, the cell cycle separates into the mitotic phase which takes only a very short period of time compared to the much longer interphase that is divided into G_1 (first gap), S (Synthesis) and G_2 (second gap) phase. These three phases are all marked by cell growth, which is enabled through the production of proteins and cytoplasmic organelles. All phases of the cell cycle are regulated mainly by proteins and follow a strict order. Furthermore, a cell is able to retreat in the non-growing, quiescent G_0 state, where no proliferation takes place. The entrance in this phase is usually encouraged by the absence of mitogenic stimuli or by growth-inhibitory factors provided by surrounding medium. Thus, the withdrawal from the cell cycle is reversible and mitogenic stimuli may induce proliferation on later occasion. [21, 30, 33]

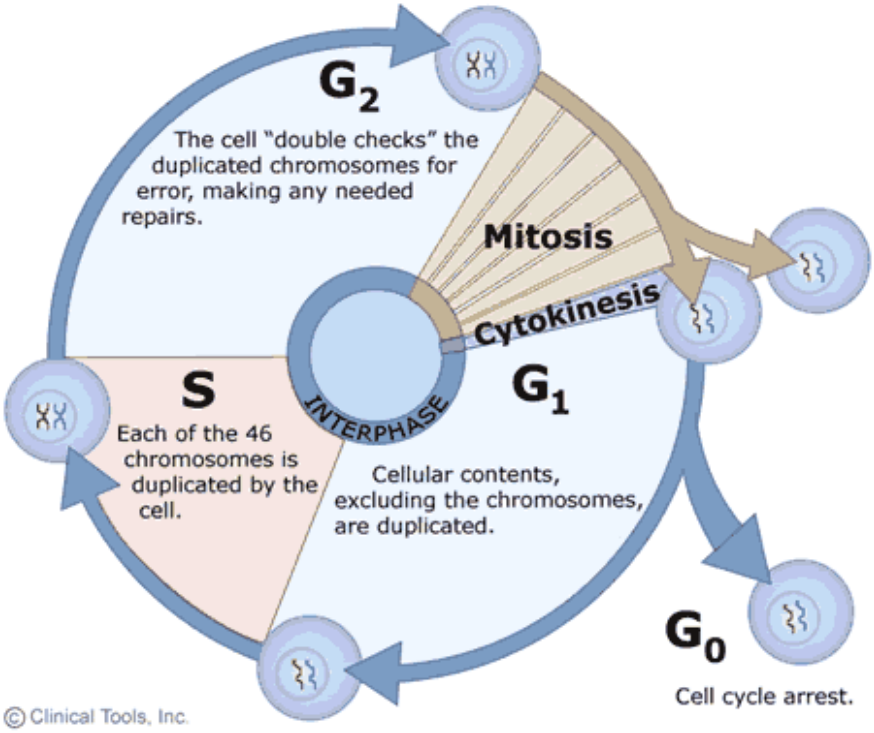

Figure 4: The stages of the cell division cycle[6]

2.5.1 Interphase

In the illustration above, the stages of the cell cycle are pictured. The cell cycle consists of four separated phases. In the **G_1-stage,** which stands for "GAP 1" is the first phase in the interphase and follows the previous mitotic phase. The G_1-phase describes the period between emerge of a new daughter cell and the following onset of DNA synthesis. During this state cells display a high biosynthetic activity with intense cell

growth and protein translation. Especially, enzymes that are responsible for DNA replication in the following S-phase are produced. The G_1-phase is under control of the p53 tumor suppressor gene. This gene is able to activate DNA repair in case of damage, can hold the cell cycle at the G_1/S-regulation checkpoint and therefore arrest cell growth. Moreover, it can also initiate apoptosis in case of irreparable DNA damage. Having passed the G_1-phase a cell is fit to enter the **S-phase**. The S stand for synthesis (or synthetic phase), which describes the task of this stage: The replication of DNA in the nucleus. An accurate DNA duplication is indispensable to prevent genetic abnormalities, which often result in diseases or cell death. Normally, mammalian cells require 6 to 8 hours duplicating their DNA, though the actual length of the S-phase differs greatly depending on the cell type. For instance, lymphocytes and embryonic cells divide in much shorter time than other cell types. The operating S-phase checkpoint slows or stops the DNA duplication in case of DNA damage. [21, 22, 30] The ensuing **G_2-phase** is the second gap phase where the cell continues growing. Prior to entering into mitosis, cells spend over 3 to 5 hours in this gap phase. The G_2-checkpoint mechanism ensures that cells do not entrance mitosis unless the replication of DNA is fully completed. Alterations and especially inaction of the regulation checkpoints in the interphase and mitosis (more information in chapter “Mitosis”) can induce the formation of cancer. During tumor development, different combinations and mutations in the genome are recombined in order to achieve the greatest proliferating advantages. [21, 22, 30]

2.5.2 Mitosis:

The Mitosis or M phase includes and describes the nuclear division or karyogenesis and the cell division (cytokinesis). It constitutes a highly complex and fast process. As the illustration shows it is divided into prophase, prometaphase, metaphase, anaphase and telophase. During mitosis, the pairs of chromatids become alignment and condense. Following, they attach to microtubuli fibers and are pulled to the opposite sides of the cell. As a last step, the cell divides (cytokinesis) and two daughter cells emerge. At the onset of the **prophase** the bundled coil of DNA located in the nucleus is tightly condensed into chromosomes. Moreover, the already duplicated sister chromosomes are bound together by the centromere, a cohesin protein complex. The centrosome, which serves as the main microtubuli organizing center, is replicated by

the nucleus before the mitosis starts. Two centrosomes, nucleate microtubuli and form the mitotic spindle by polymerisation of soluble tubulin. Subsequently, in the **prometaphase** the nuclear membrane disintegrates and kinetochores are formed at the centromere of each chromosome. The kinetochore constitutes an attachment for microtubuli at the sister chromatids. In the **metaphase,** the centrosomes start pulling the chromatids towards the cell´s ends. Because of the movement a high longitudinal pressure, which is caused by opposing kinetochores is exerted upon the chromatids. Thus, centrosomes form the so-called spindle equator, which describes an imaginary line between the centrosome poles. The ensuing **anaphase** delineates the phase of movement. The proteins, which hold the sister chromatids together, are cleaved and separated to daughter chromosomes. By shortening the kinetochore microtubules, the chromatids are pulled apart towards to the respective poles. At the end of the anaphase, the kinetochore microtubules degrade. In this stage, a last regulation checkpoint that efficiently blocks the anaphase is located. At this checkpoint, mitosis is halted, until all chromatids stick properly to the mitotic spindle. In the **telophase,** the cell continuous elongation and the chromatids are surrounded by nuclear membrane. Eventually cytokinesis starts. [21, 22, 30]

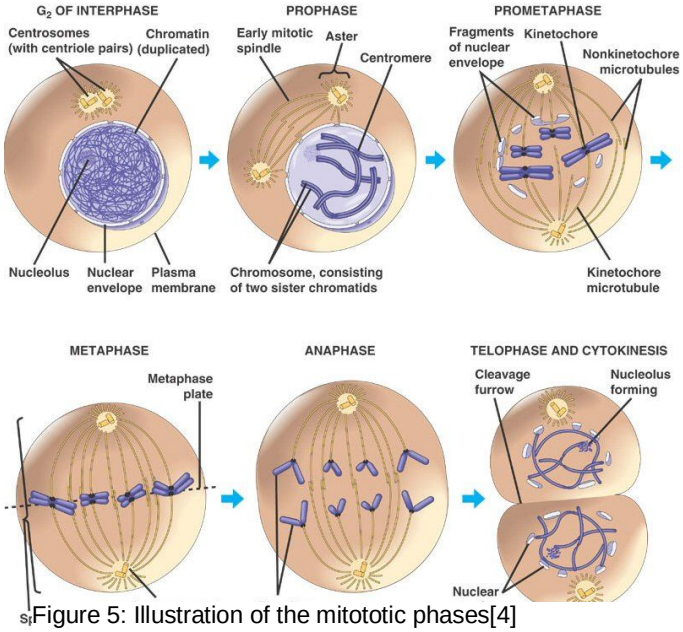

Figure 5: Illustration of the mitotic phases[4]

2.5.3 Regulation of the Cell Cycle

The regulation of cell cycle is crucial for cell survival. Several checkpoints prevent uncontrolled cell division und are responsible for detection and repair of genetic damage. The “Cell Cycle Clock” [21] , which has already been mentioned above is a control system in the nucleus that processes, integrates and filters various signals from the surroundings of the cells. On the one hand, there are mitogenic signals that induce the entrance into cell cycle. On the other hand, there are signals that prohibit further

proliferation, for example TGF-β. The Cell Cycle Clock filters all these signals down to the binary decision whether a cell should divide or not. In general, the cell cycle is regulated by cyclin-dependent kinases (CDK) which are bound to their regulatory proteins, the so-called cyclins, Both proteins are shown in figure 6. These cyclin-CDK complexes are active kinases phosphorylating different substrates and therefore serve as “on or off switch”. The cyclin-CDK complexes function as transmitters that convey signals from the “Cell Cycle Clock” to various responder molecules, which actually carry out the received orders. The members of the CDK subfamily are serine/threonine-kinases. The cyclins constitute a distinct family of proteins. The family contains similar amino acid residues, which are responsible for binding and activation of the CDKs. Until the first regulation boundary in the G_1-phase, CDK 4 and 6 associate with cyclin D1, D2 and D3. Subsequent to the checkpoint in the late G_1-phase, E-type cyclins bind to CDK 2. Following, the phosphorylation of certain substrates triggers the entrance into the S-phase. Ensuing, cyclin A takes over the position of the E- cyclins and facilitates the ongoing events in the S-phase. In the later S-Phase, cyclin A forms a complex with CDK 1, which is also called CDC2. Subsequently, in the G_2-phase, cyclin A is replaced by cyclin B. This final complex triggers many events in the following prophase, metaphase, anaphase and telophase. [21, 22, 30, 33]

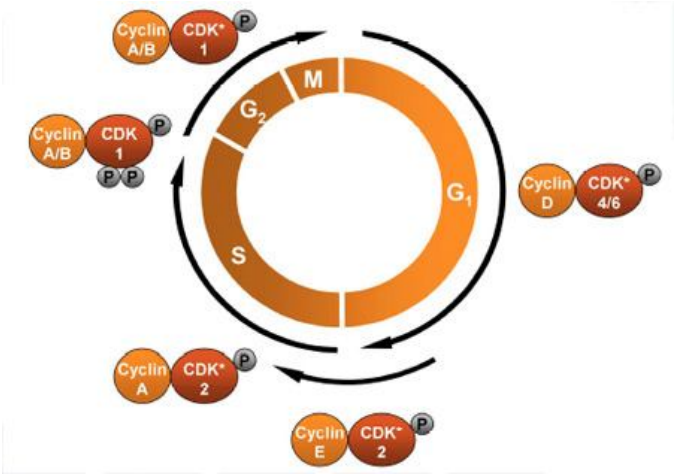

Figure 6: Cyclins and Cyclin dependent Kinases[4]

2.6 Apoptosis

Apoptosis is the process of programmed cell death. In contrast to necrosis, which describes a form of cell death that is induced by acute cellular injury, apoptosis generally constitutes advantages, for example the differentiation of fingers and toes in embryonic development.[34] Moreover, programmed cell death plays a central role in normal tissue physiology. For instance, epithelial cells in the small intestine are continually eliminated and renewed every 4 or 5 days. During blood cell development,

most of the erythrocyte progenitor cells are eliminated by apoptosis as routine process in erythropoiesis. Furthermore, cell death is essential to establish normal development and to ensure a well-performing maturing cycle. In every multicellular organism apoptosis is a crucial process to maintain health by balancing cell death and proliferation.[34] Alterations in this complex and highly defined program can lead to diseases such as diabetes mellitus, AIDS, Parkinson`s disease and cancer. [35] Additionally, apoptosis is involved in carcinogenesis. The establishment of a progressive transformation from healthy human cells to malignant cancer derivatives is a complex multi-step process. It acquires dynamic modifications of the genome and the overriding of intracellular checkpoints in the cell cycle. Thus, a throughout knowledge of apoptotic signaling pathways and different ways of evading apoptosis is crucial to invent new cancer therapies and to improve effectiveness of the treatment, due to related resistances. Apoptosis is initiated in response to stress triggered by outer or inner signals. For instance, heat, radiation, nutrient deprivation, hypoxia, viral infection and increased intracellular calcium concentration. Cell death is morphologically characterized by cell shrinkage, blebbing of plasma membrane, the loss of maintenance of organelle integrity. These changes are pictured in figure 7. Furthermore, the nucleus collapses into a compact structure known as pyknosis and the DNA is cleaved into small fragments. The ingestion of the cell rests, also called apoptotic bodies by neighboring tissue cells and itinerant macrophages follows. Prior to apoptosis the regulatory proteins that initiate the apoptotic pathways are activated. [36-38]

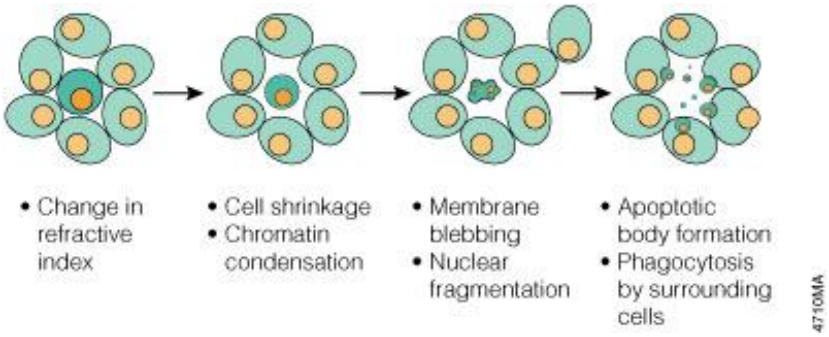

Figure 7: morphological changes during apoptosis[5]

Apoptosis is classified into two death-signaling pathways: the **death receptor mediated (extrinsic)** or **mitochondria-mediated (intrinsic) pathway**. However, these two types of pathways are not exclusive and it has been demonstrated that certain downstream molecules can influence the other pathway. Furthermore, it is suggested that many apoptotic effectors feature also other important cellular functions, which are not related to apoptosis. For example, caspase 2 a highly conserved enzyme that plays a certain role in apoptosis through releasing proapoptotic proteins from the

mitochondria, also exhibit functions in DNA repair, cell cycle and tumor suppression. [21, 22, 30]

2.6.1 Mitochondria-mediated (Intrinsic) Pathway

The intrinsic pathway is triggered by signals originating from within the cell. This pathway is often activated in response to signals resulting from DNA damage, loss of cell-survival factors, or other types of severe cell stress. Normally, pro-apoptotic proteins are released from the mitochondria to activate caspase proteases and trigger apoptosis after activation by p53. The pro-apoptotic protein Bcl-2 is located in the outer mitochondrial membrane and is a member of a complex and large family that controls the stream of cytochrome c and other proteins that are released by the mitochondrion. All together twenty-four Bcl-2 family members are known and encoded by the human genome. Six of these members comprise anti-apoptotic features, the rest acts pro-apoptotic. Additionally, cytochrome c takes a major part in the intrinisic apoptotic program. It resides in the space between outer and inner mitochondrial membrane and transfers electrons as part of the oxidative phosphorylation. Various triggers that initiate apoptosis lead to a depolarisation of the outer mitochondrial membrane. Due to the loss of voltage gradient, cytochrome c is released to the cytosol and consecutively activates a cascade of events that finally result in programmed cell death. Bcl-2 acts as an anti-apoptotic agent and tries to close the channels through which cytochrome c enters the cytosol. In contrast, other members of the Bcl-2 family such as Bax, Bad and Bid, open theses cannels. Interestingly, Bax seems to be encoded by a gene that is activated by p53. The levels of pro- and anti-apoptotic proteins within each channel decide whether cytochrome c remains in the mitochondrion or is spilled out. Eventually, this results in either cell survival or death. Later on, Bax and Bad assemble at the outer surface of the mitochondrion and cause the fragmentation of the ATP-generating machinery. As apoptosis proceeds, more pro-apoptotic proteins are released to the cytosol and the mitochondrion collapses totally. Once cytochrome c is released from the mitochondrion, it associates with Apaf-1 protein and forms a structure that is known as apoptosome. The apoptosome activates procaspase-9 and converts it to the active caspase-9. Following, the protease caspase-9 cleaves and activates procaspases -3, -6 and -7. The executioner caspases -3, -6 and -7 induce the cleavage of “death substrates”. Due to their specialized roles,

caspases are divided into two functional groups: the initiator caspases that trigger the onset of programmed cell death; and the executioner caspases, which are located downstream and actually destroy cell components. Simultaneously, Smac/DIABLO, another protein is spilled out of the mitochondria together with cytochrome c and inactivates certain anti-apoptotic proteins also termed IAPs (inhibitors of apoptosis). The IAPs normally block the caspase action by binding them directly and inhibiting their proteolytic activity. The cascade of caspase cleavage results in the activation of “death substrates”, which finally causes the typical morphological transformations of the apoptotic cells. [21]

2.6.2 Extrinisic or Receptor-mediated Pathway

The extrinsic or receptor-mediated pathway is initiated from outside the cell and activates pro-apoptotic cell receptors. They are often referred to as death receptors indicating their ability to start the apoptotic program. Cross-linkings of death receptors either with their natural ligand or an agonistic antibody are found on the surface of cell membranes and induce the activation of caspase-8 and -3. The last mentioned cleaves target proteins and thereby leads to apoptosis. Death receptors are part of the tumor necrosis factor (TNF) superfamily including TNF, CD95 (Fas), and the TNF-related apoptosis inducing ligand (TRAIL). These different types share a similar structure, containing an extracellular domain to engage the ligands and an intracellular death domain that is responsible for transmitting the death signal. The intracellular death domain is activated by the binding of ligands consequently, leading to the induction of intracellular signaling pathways. The activation of death receptors often results in receptor clustering and in the intracellular formation of the death-inducing signaling complex (DISC). Following, the DISC triggers self-cleavage of caspases and their conversion into active proteases. The initiator caspases-8 and -10 induce the downstream executioner caspases-3, -6 and -7 finally, activating the extrinsic and/or intrinsic pathway. Moreover, caspase-3 cleaves and activates a Bcl-2-related protein, which migrates to the mitochondrial channel and amplifies the pro-apoptotic signals by recruiting mechanisms of the intrinsic program. The connection of the intrinsic and extrinsic pathway is shown in the illustration below [21].

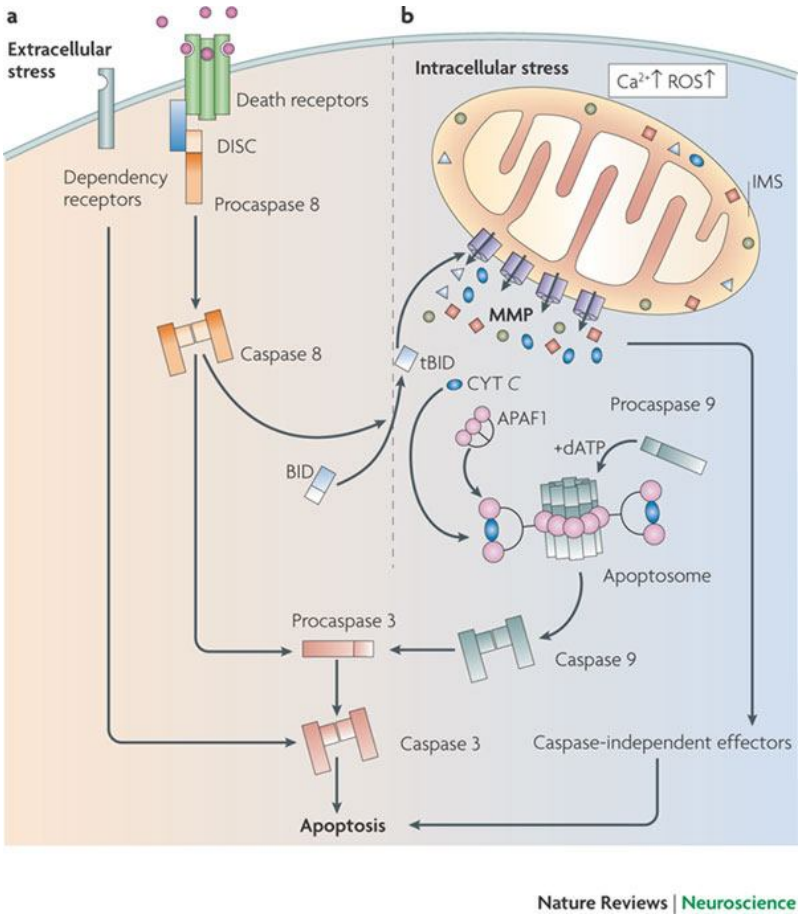

Figure 8: Illustration of the intrinsic and extrinsic pathway[39]

2.6.3 ER-stress Mediated Apoptosis

Disturbances in normal function of the endoplasmic reticulum (ER) lead to unfolded protein response (UPR), which describes an intracellular signaling pathway that is aimed for compensation of damage. As demonstrated in figure 9, UPR can eventually trigger apoptosis, in case ER dysfunctions are severe or chronically. The accumulation of unfolded proteins in the ER represents cellular stress and is induced by multiple stimuli such as hypoxia, oxidative injury, hypoglycemia, high fat diet and viral infections[35]. The ER constitutes a complex membranous network, which is found in all eukaryotic cells and is required to fulfill essential functions within the cell. For instance, it is responsible for the intracellular calcium homeostasis, protein secretion and lipid biosynthesis. Furthermore, the ER consists of the highest calcium concentrations. This is necessary for the Ca^{2+}-dependent chaperones as well as for post-translational modifications of secretory proteins, especially in the process of protein folding and transport in secretory pathways. Moreover, the ER contains an

oxidizing environment that is required for the formation of disulfide bonds in synthesized proteins. Disruptions in the cellular redox system, triggered by the above-mentioned stimuli, interfere with disulfide bonding. As they are located in the lumen of the ER they are leading to protein unfolding or misfolding. [35, 40, 41] Consequently, the global protein synthesis is down regulated by inhibiting RNA translation. [35] In addition, the folding capacity is enhanced by selective expression of chaperone proteins. Moreover, degradation of misfolded proteins is increased by a process called ER-associated protein degradation (ERAD). [41] The UPR is activated by three transmembrane stress sensors: the pancreatic ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). As a first step a pro-surviving signaling mediated by PERK is promoted. Hence, posterior it promotes cell death. In more detail, there are certain components of UPR, which are tightly associated to initiation of ER stress-induced apoptosis.

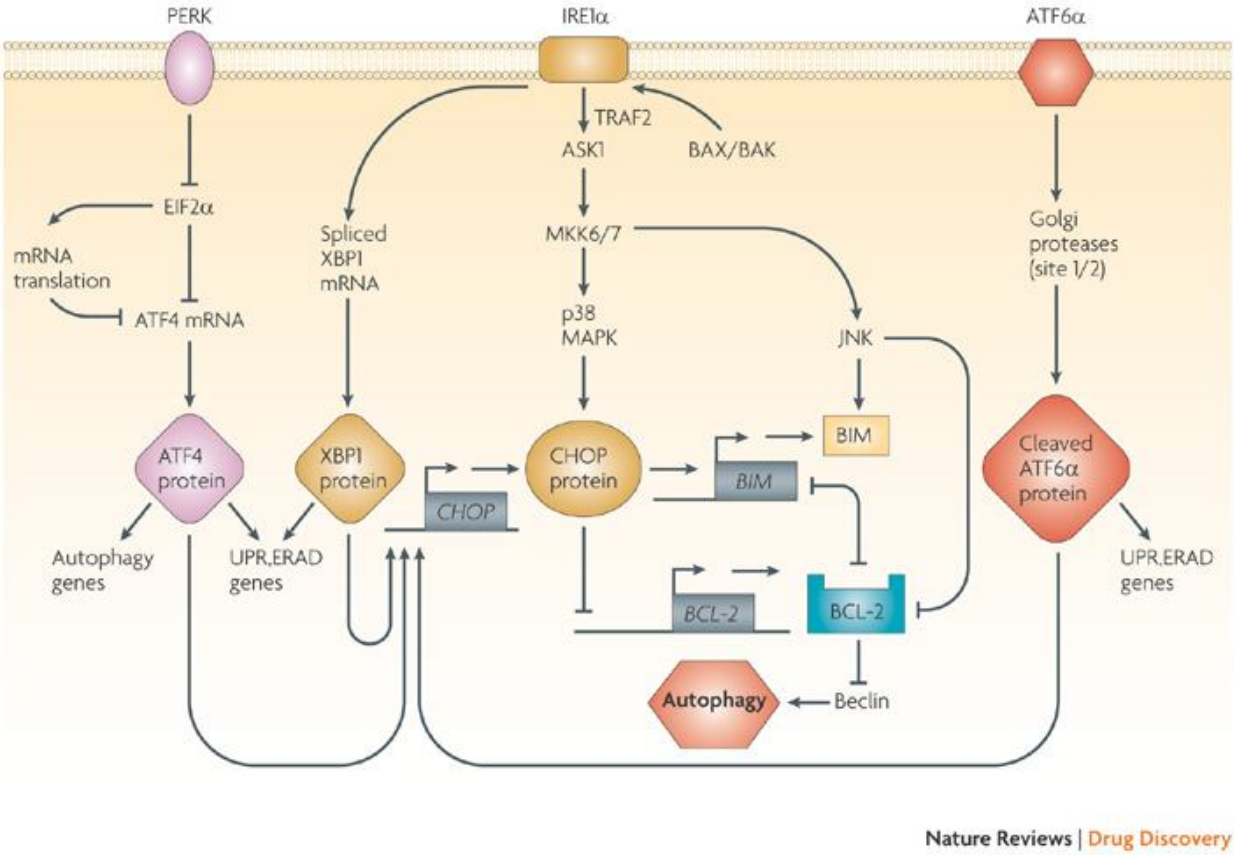

Figure 9: ER Stress pathway and its relation to the cell death machinery [44]

For instance, C/homologousprotein (CHOP), which is also called growth arrest- and DNA damage-inducible gene 153, alters the balance of pro-survival and pro-apoptotic Bcl-2 family members and consequently promotes the mitochondrial apoptotic pathway. [42] Interestingly, it has been demonstrated that CHOP leads to up-regulation

of TRAIL receptors and thus stimulates the extrinsic apoptotic pathway. Apart from CHOP, active IRE1 recruits the TNF receptor-associated factor 2 (TRAF2). Following, TRAF2 induces downstream activation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinases (p38 MAPK). Both kinases lead to activation of the pro-apoptotic protein Bim, while blocking the pro-survival protein Bcl-2. Thus, the intrinsic apoptotic pathway is induced. As a result, ER mediated cell death is associated with diseases including brain ischemia, diabetes, neurodegenerative diseases and cancer. [35, 42]

More precisely, protective UPR proteins are found up-regulated in cancer cells, presuming a connection to hypoxic environments. In this regard, it has been reported that ischemic regions in tumors, which emerge in consequence to the rapid growth that outstrip their vascular supply, may induce ER stress, leading to a protective UPR. Following this approach, agents that inhibit key signaling UPR participants could prevent adaption of tumors to hostile environments and therefore define a new type of cancer therapy. [35, 41]

2.7 Cytoplasmic Signaling

Cell growth and division is coordinated by the actions of a huge amount of distinct proteins. *In-vitro* examinations in 1981 showed that cells cultured in medium with reduced levels of serum-associated growth factors enter reversibly into the quiescent, non-growing G0 phase. If these cells, after days of starvation, are introduced to fresh medium with abundant amounts of growth factors, they rapidly start to replicate their DNA and initiate cell division. Although, this experiment gave no hint about how changes in cell behavior are related to molecular variances, it posed a large question. Today we know that signaling pathways reach from the surface of a cell into the nucleus, where expression of various genes is induced or increased. Growth factors are able to elicit multiple cellular responses, which can be explained by the fact that these specific receptors are able to activate specific combinations of downstream signaling pathways. Each signaling pathway is responsible for certain molecular changes that result in different cell behavior. [21, 43]

2.7.1 Protein Kinases

Protein kinases are enzymes that can transfer a phosphate group of Adenosintriphosphate (ATP) to a protein in a cell. A subclass of protein kinases are the tyrosine kinases, in this case the amino acid tyrosine is the molecule where the phosphate group is attached. As well, other amino acids that contain hydroxyl groups, such as serine or threonine are able to perform this task. Tyrosine kinases work as an “on and off switch” for various cellular functions. The phosphorylation of proteins by kinases is an important mechanism for signal transduction and the regulation of cell activity. Besides, tyrosine kinases are responsible for the signal transmission from the cell membrane to the nucleus. In the nucleus, cell cycle and transcription factors are controlled by the downstream substrates of tyrosine kinases. Tyrosine kinases are involved in mitogenesis, the induction of mitosis in a cell. [21, 44]

2.7.2 EGF- Receptor

The epidermal growth factor signaling pathway, shown in figure 10 is one of the best-understood and important pathways regulating growth, survival, differentiation and proliferation of eukaryotic cells. [21, 43, 45-48] The epidermal growth factor receptor (EGFR) also referred to as erbB1/HER-1 is a member of a superfamily of receptors that all contain an intrinsic tyrosine kinase activity. The other members of this family are erbB2/HER-2, erbB3/HER-3 and erbB4/HER-4. All family members are embedded in the cytoplasmic membrane and share a similar structure, consisting of an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain that is responsible for the initiation of signaling pathways.[43, 48] EGFR is widely expressed on various cell types, but especially on epithelial and mesenchymal cells. EGFR is related to biological responses, such as mitogenesis, apoptosis, enhanced cell motility, protein secretion, differentiation and dedifferentiation. The unstimulated EGFR presents a monomer. Through stimulation by a ligand, for instance EGF (epidermal growth factor) or TGF-α (transforming growth factor α) the receptor undergoes dimerization, which results in auto-phosphorylation of the cytoplasmic regions. In consequence of this phosphorylation, specific docking sites for proteins that contain a Src homology 2 and phosphotyrosine-binding domains are provided. Following, these certain proteins bind to the specific tyrosine residues and intracellular signaling via several pathways is initiated. [43, 46, 48]

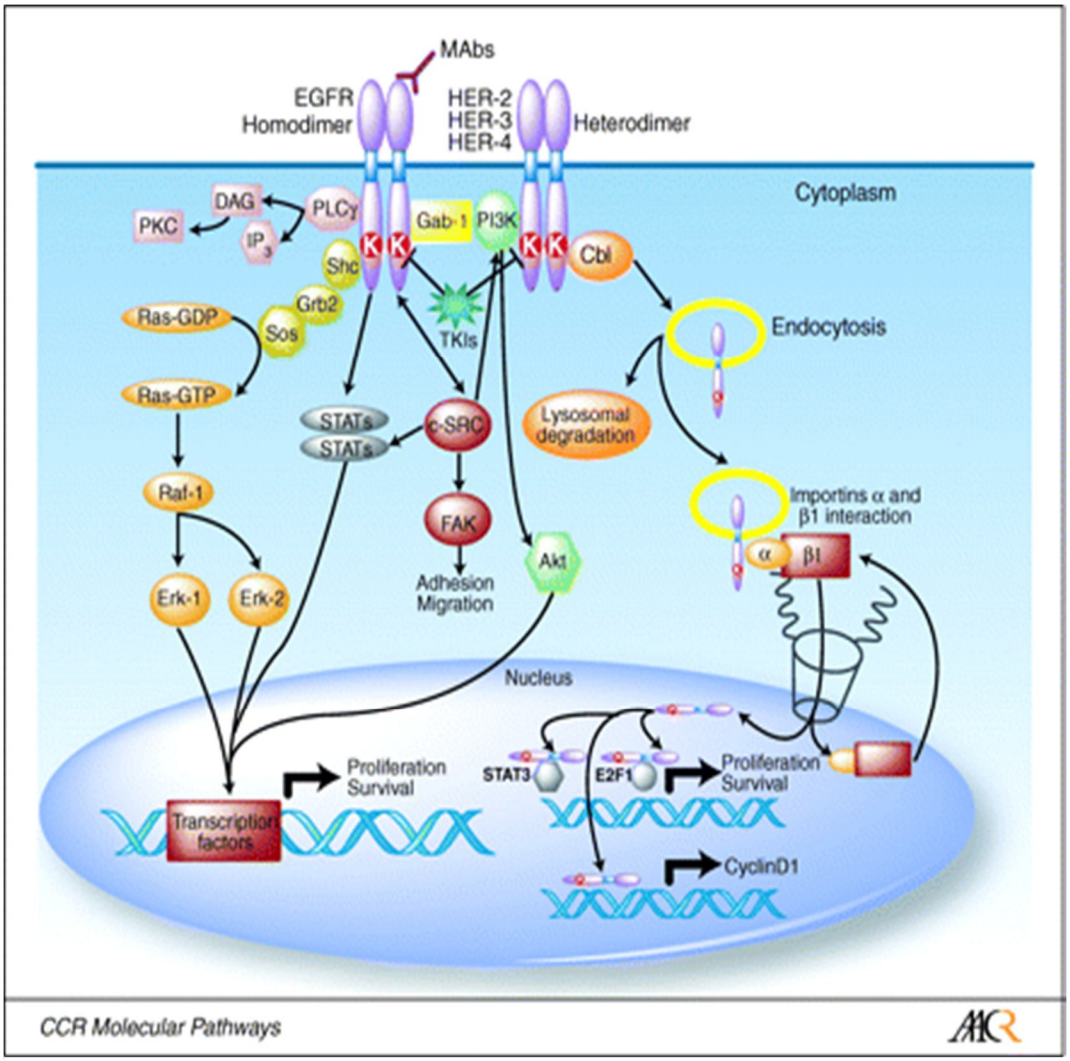

Figure 10: Illustration of the epidermal growth factor receptor and its signaling pathways[61]

2.7.2.1 MAPK Pathway

The MAPK pathway is named after the mitogen-activated protein kinase and initiated by the small G-protein Ras, which is phosphorylated and activated by the intracellular side of a tyrosine kinase receptor (e.g.: EGF- receptor). Consequently, the activation of the Raf kinase follows. The Raf kinase phosphorylates substrate proteins at their serine or threonine residues. Prior to its activation, Raf is found in the cytosol. Afterwards it is, as well as Ras, tethered to the plasma membrane. Thereafter, Raf activates MEK, another kinase that has dual phosphorylation features and therefore is able to activate not only serine and threonine residues but also tyrosine residues. Thus, it is able to phosphorylate Erk 1 and Erk 2. Erk 1 and 2 are **e**xtracellular signal-**r**egulated **k**inases that, when activated phosphorylate many substrates, which regulate various cellular processes. In addition, Erk 1 and 2 are able to translocate to the

nucleus and to induce the phosphorylation of transcription factors. For instance, eIF4E a translation factor, is activated by a substrate, which is phosphorylated by Erk1/2. [21, 49, 50]

2.7.2.2 Akt/PKB Pathway

Another important pathway that is triggered by the Ras kinase is the Akt/Protein kinase B (PKB) cascade. The Akt/PKB pathway starts with the cleavage of the membrane component phosphatidylinositol (PI). IP3 is one of the products that emerge from the cleavage. Since it is purely hydrophilic it can be detached from the plasma membrane, as a result it diffuses into the cytoplasm and thereby serves as intracellular second messenger. Because membrane-associated phospholipids exhibit an amphiphilic structure, a second hydrophobic molecule is produced: Diacylglycerol (DAG). DAG is able to activate the protein kinase C (PKC), a key signaling kinase, which regulates various cellular functions. IP is modified by several kinases, each phosphorylating another particular hydroxyl group of the inositol moiety. For instance the Phosphatidylinositol 3-kinase (PI3K) attaches a phosphate group to the 3´hydroxyl group of the inositol functional group of the membrane-ingrained PI. Prior to phosphorylation by PI3K, PI is phosphorylated to phophatidyl-inositol-(4,5)diphosphate (PIP2) by PI kinases. PI3K converts PIP2 into phosphatidylinositol (3´,4´,5´)-triphosphate (PIP3). GTP-activated Ras binds PI3K and therefore amplifies its functional activity. Thus, PI3K is able to operate as direct downstream effector of Ras. The attachment of PI3K to Ras causes that PI3K translocates to the membrane and becomes closely associated with PI. PI3K plays a central role in the activation of many signaling pathways. PIP3 seems to show the most interesting features of all the mentioned phospholipids. Most of the cytosolic proteins that dock to PIP3 contain a pleckstring homology (PH) domain. Akt/protein kinase B is probably the most important protein containig a PH region. Akt/PKB is a serine-threonine kinase, which is activated by the association with PIP3 and its following phosphorylation. Akt/ PBK further phosphorylates several substrate resulting in three major biological effects: 1. Reducing the change of the activation of apoptosis and therefore leading to enhanced cell survival; 2.The stimulation of cell proliferation; 3. Stimulation of cell growth. [21, 48, 50]

2.7.2.3 Phospholipase Cγ

Thirteen kinds of mammalian phospholipase C are classified into six isotypes. One example is the phospholipase Cγ, which directly interacts with activated EGFR by binding to its juxtamembrane region. Thereafter, the phopholipase Cγ hydrolyses phosphatidylinositol-4,5-diphosphate and creates inositol-1,3,5-triphosphate, which takes an important role in calcium release. Furthermore it releases 1,2-diacylglycerol, a cofactor of in protein kinase C activation. In turn, protein kinase C activation can result in MAPK and c-Jun NH2- terminal kinase activation. [48]

2.7.2.4 Signal Transducers and Activator of Transcription Pathway

Signal transducers and activators of transcription (STAT) interact with their SH2 homology domains, dimerize and translocate into the nucleus. There they drive the expression of certain targets. Interestingly, STAT3 is often found constitutively activated in numerous tumor-derived cell lines and is related to oncogenesis and tumor progression. The Src kinase, another signal transducer activates the focal adhesion kinase, PI3K and STAT proteins and is critical for the regulation of cell proliferation, migration, adhesion and angiogenesis. [48]

2.8 Angiogenesis

Angiogenesis is a physiological process and describes the formation of new blood vessels out of pre-existing vessels. Under physiological conditions, angiogenesis takes place during the process of wound healing, in the female reproduction organs, as well as in pregnancy and embryonic development. Angiogenesis takes a major role in various human diseases like malignant tumors, rheumatoid arthritis, retinal and coronial neovascularization and various skin diseases. Thus, Judah Folkman, an American scientist originated the concept of angiogenetic therapy. His idea based on the destruction of tumors by cutting off their blood supply. [51, 52] Thenceforth, researchers gained a lot of essential information about biological mechanism of angiogenic vessel growth and numerous regulatory molecules. As a fact, certain biological signals, including the vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGFs) activate endothelial surface receptors on pre-existing

blood vessels and initiate the invasion of the surrounding matrix and the formation of sprouts towards the angiogenic stimulus.[51]

2.8.1 Vascular Endothelial Growth Factors and their Receptors

VEGF is a 45 kDa protein, which consists of two subunits and belongs to the most substantial mediators of angiogenesis. It is an endothelial cell-specific mitogen and does not induce cell proliferation in any other type of cell. In the human body five different isoforms of VEGF are found, each of them containing a distinct number of amino acids (VEGF121, 145, 165, and 206). These isoforms emerge because of alternative splicing of the mRNA, implying eight exons. VEGF 121 and VEGF 165 are the only soluble isoforms; the second mentioned is also the most powerful stimulator of endothelial cell proliferation. [51] Further, the injection of VEGF receptor antibody (VEGFR-1-IgG chimera) in newborn mice causes death due to liver and renal failure, precipitated by increased apoptotic endothelial cell death in these organs. However, elderly animals survive such a treatment without visible effects. Moreover, VEGF exhibits importance in human diseases. As a fact, high levels of VEGF correspond with a low response to conventional chemotherapy and to a poor survival rate. Besides, VEGF is a valuable marker for tumor angiogenesis in breast cancer. Based on this knowledge, several different strategies to inhibit VEGF function and therefore limit tumor angiogenesis emerged. [51, 52]

2.9 Tyrosin Kinase Inhibitors

2.9.1 Sunitinib

Sunitinib (Sutent®, Pfizer Inc.) is an oral administered multi-targeted receptor tyrosine kinase inhibitor. Sunitinib is approved by the FDA for the treatment of renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor. The drug inhibits all isoforms for the vascular platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGFR). Both receptors play a role in angiogenesis and cell migration. The inhibition of those two targets leads to a reduced blood supply of the tumor, therefore to cell death and ultimately to tumor shrinkage. Sunitinib also inhibits KIT (Cluster of Differentation 117), which is a Mast/stem cell growth factor

receptor and is therefore used to treat KIT positive gastrointestinal stromal cell tumors. About 85-90% of imatinib-resistant, gastrointestinal stromal tumors are implicated with a gain of function mutation in KIT. Clusters of differentiation are molecular markers on the cell surface, as recognized by specific antibodies, used to identify the cell type, the stage of differentiation and the activity of the cell. CD 117 is used to identify certain types of hematopoietic blood progenitors in the bone marrow. Altered forms of CD 117 are associated to the occurrence of various cancer types. Sunitinib has a significant profile of side effects. The most common are yellowing of the skin (because of the yellow color of sunitinib and its metabolites), hand-foot syndrome, hypertension, anorexia, nausea and diarrhea. The drug is dosed in cycles of 50mg daily for four weeks followed by a two weeks pause in which the patient can recover from the sunitnib related toxicities. In approximately 50% of the treated patients, a dose reduction to 25/37.5 mg is required due to adverse effects. In addition, the percentage of patients, who discontinue the intake of sunitinib is relatively high with 19%. There are interactions with ketoconazole, which result in a 51% increase in sunitinib´s AUC. Rifampicin causes a 46% increase of the AUC of sunitinib, which is primarily metabolized by CYP3A4 generating an active metabolite. During sun light exposure, the most stable Z-isoform (cis-configuration) of the double bond between the indoline and the pyrrole rings is converted to the E-isoform (trans-configuration). Over some time in dark, the E isoform reconverts to the Z- Isomer, which is 100-fold stronger active against the VEGFR. [44, 53, 54]

2.9.2 Sorafenib

Sorafenib (Nexavar®, Bayer Pharma AG) is a drug approved for the treatment of advanced renal cell carcinoma and unresectable hepatocellular carcinoma. The drug is a multiple kinase inhibitor targeting C-RAF, BRAF and mutant BRAF and receptor cell surface kinases as KIT, FLT-3, RET, VEGFR-1, VEGFR-2, VEGFR-3 and PDGFR-β. Several of these kinases stand in correlation to tumor cell signaling, angiogenesis and apoptosis. A double blind, placebo-controlled trial in patients with unresectable hepatocellular carcinoma, resulted in an improvement in overall survival. Sorafenib is known to cause a wide range of side effects and toxicities. The incidence of cardiac ischemia/infarction and of hypertension was noted to be increased. The inhibition of the VEGF receptors seems to be associated with negative effects particularly with

function of the cardiovascular and renal systems, wound healing and tissue repair. Sorafenib is an oral administered remedy, typically dosed at 400 mg without food, twice a day. When taken with a high fat meal, the bioavailability decreases intensively. In blood, sorafenib is highly bound to plasma proteins. Sorafenib is primary excreted unchanged in the feces, 15-20% are eliminated in urine as glucuroniated metabolites and 5% of the medication is metabolized through oxidative metabolism. The major metabolism pathways are CYP3A4 catalyst oxidations and UGT1A9 controlled glucuronidation. Sorafenib interacts with CYP2C19, CYP2D6 and CYP3A4 as a competitive inhibitor. Those enzymes are particularly responsible for the metabolisation of many remedies. However, when sorafenib was concomitantly administered with midazolam (CYP3A4 substrate), omeprazole (CYP2C19 substrate) or dextromethorphan (CYP2D6 substrate) there was no significant increase in the area under the curve. A clinical drug interaction does occur when sorafenib is co-dosed with substrates of UDP-glucuronosyltransferases. Irinotecan, a topoisomerase 1 inhibitor, has an active metabolite SN-38 that is conjugated by UGT1A1. When irinotecan and sorafenib are given concomitantly, there is a 26- 42% increase in the AUC of irinotecan. [44, 55]

2.10 Cyclodepsipeptides

Cyclodepsipeptides are cyclic peptides where at least one amino acid is replaced by an alpha-hydroxy acid, which forms an ester bond. The cyclic structure of these substances is suggested to be indispensable for their biological features because linear homologues have shown no action.[11] Members of this class exhibit interesting biological activities and some have already been tested in clinical trials in continuous cancer therapy in combination with other cytotoxic drugs. [9] The spectrum of biological activities depends on the number of atoms the cyclodepsipeptides are built of. For example, there are cyclooctadepsipeptides that have potent antihelmintic properties. Other cyclodepsipeptides appeared to have cytoprotective activity against HIV-1 infection, growth inhibitory effects towards cancer cells and antimalarial and

antimycobacterial activity. A feature that all cyclodepsipeptides have in common is their ionophoric property. [9]

2.10.1 Enniatin and Beauvericin

Enniatin B (Enn B) and Beauvericin (Bea) are secondary metabolites of the genus *Fusarium,* which are known as contaminants of cereals. In particular, maize grains are infected by *Fusarium* species.[10, 56, 57] The metabolites belong together with Moniliformin and Fusaproliferin to the most frequent contaminants in various cereals such as maize, rice, barley, wheat, oat and rye.[9] Due to their late discovery and their increasing importance in the last years the metabolites mentioned above are called „emerging mycotoxines“. Enn B was first isolated from *Fusarium oxysporum* in 1947 and BEA in 1969 from *Beauveria bassiana*. The secondary metabolites found in these species were highly concentrated ranging in an amount of microgramm/ kilogramm [56, 58, 59]. Thus, cyclodepsipeptides can enter the food chain through contaminated cereals. The bioavailability is about 50% and therefore it is considerable that cytotoxic concentrations of these secondary metabolites are applied with our daily nutrition.[60] [13]

2.10.2 Chemical Structure

Bea and Enn B are cyclodepsipeptides that contain three alpha-amino acids with one variable lipophilic group and three d-2-hydroxyvalerian acids, that form an alternating ring of 18 components. In contrast to enniatins, which contain aliphatic moieties, Bea consists of aromatic side chains. The three nitrogen atoms, which are part of the alpha-amino acids are methylated. The molecular structures of Bea and Enn B are shown in Figure 12 and 13. Apart from Enn B a lot of similar structured Enniatins have been found and are demonstrated in Table 1.[14] However, this thesis focuses solely on Enn B and Bea, which already showed significant effects in various *in vitro* studies. [9]

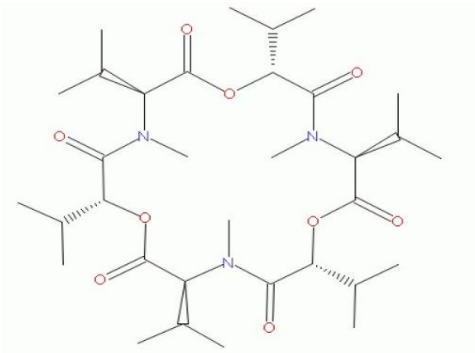

Figure 12: Stucture of Enniatin B[4]

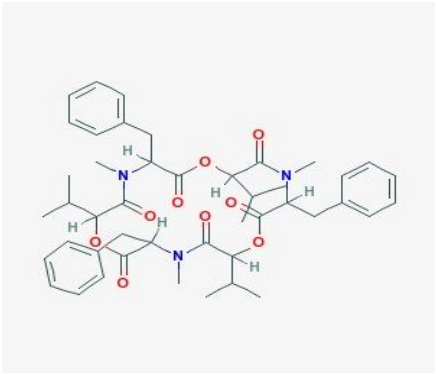

Figure 12: Stucture of Beauvericin[3]

Substance	M1	M2	M3
Enniatin A	sec-butyl	sec-butyl	sec-butyl
Enniatin A1	isopropyl	sec-butyl	sec-butyl
Enniatin A2	sec-butyl	isobutyl	sec-butyl
Enniatin B	isopropyl	isopropyl	isopropyl
Enniatin B1	isopropyl	isopropyl	sec-butyl
Ennatin B4	isopropyl	isobutyl	isopropyl
Beauvericin	phenylmethyl	phenylmethyl	phenylmethyl

Table 1: Table of different Enniatins and Beauvericin; M= moiety [9]

2.10.3 Characteristics

Bea- and Enn-mediated cytotoxicity towards mammalian and cancer cell lines is not entirely understood, due to their late discovery and the fact that several cellular targets and molecular mechanisms are involved. Their strong lipophilic character allows them

to incorporate into cell membranes and to build cation specific pores. Thus, it is probed that Bea and Enn selectively direct a flux of cations - especially calcium - into the cell. This increase of calcium levels in the cell appears to be at least partly responsible for the cytotoxicity in many cell lines [15]. Related to their ionophoric properties Bea and Enn B show phytotoxic, insecticidal, antibiotic, anthelmintic, antiviral, anti-inflammatory and fungicide properties as well [16, 61, 62].

Healthy human cells including normal fibroblasts and HUVEC cells (human umbilical vein endothelial cells) are relatively insensible to the effects of the *Fusarium* metabolites [19]. In nanomolar concentrations the substances slightly but significantly stimulate cell proliferation. At higher concentrations, but in a low micromolar range, antiproliferative effects against human cancer cell types, including “cell cycle arrest” and a reduction in DNA synthesis, were demonstrated [19]. Moreover, *in-vitro* tests showed that in submicromolar concentrations *Fusarium* metabolites deplete the mitochondrial transmembrane potential, uncouple oxidative phosphorylation and lead to mitochondrial swelling and a decreased calcium retention capacity. These mitochondrial effects are related to the potassium-ionophoric feature of the secondary metabolites [9, 63]. Additionally, the mycotoxins initiate programmed cell death via the internal mitochondrial pathway. So far, in lung carcinoma cells (A549) it was shown that the cyclic depsipetides lead to an increase of proapoptotic proteins such as Bax, Bak and p-Bad while the amount of antiapoptotic Bcl-2 was decreased. As a result, cytochrome c is released and caspase 3 is activated consequently, leading to apoptotic cell death [18, 20]. The morphologic characteristics of apoptosis include cell blebbing, cell shrinkage, nuclear fragmentation and chromatin condensation [19]. In addition, the metabolites activate several cellular regulators and signaling pathways, including NF-κB, MAPK and p53 [59]. Moreover, increased intracellular calcium concentrations trigger the activation of thrombocytes, the reduction of motility of sperms and decrease the contraction force in the terminal ileum [64]. Furthermore, cell culture tests showed an inhibition of cell motility and angiogenesis [65]. Although, there are no detailed studies on the consequences of chronic and bolus exposure to the *Fusarium* metabolites so far, Bea and Enn B exhibit interesting pharmacological qualities as they are particularly cytotoxic to cancer cells and are not mutagenic [65, 66]. Furthermore, Beauvericin was found the most potent inhibitor of the cholesterol acetyl transferase of microbial origin. This enzyme is correlated with atherosclerosis, a wide spread disease in modern countries [67]. In traditional Chinese Medicine Bea is commonly

used for this indication [68]. A mixture of Enniatin A, B and C is approved as the antibiotic remedy Fusafungin, Locabiosol®. This antibiotic is particulary used against gram-positive bacteria, Haemophilus influenzae and Legionella pneumophila [69, 70].

2.10.4 Impact of ABC Transporters on Enn- and Bea-mediated cytotoxicity

Interactions with the ATP-binding cassette (ABC) transporters influence the bioavailability of xenobiotics and pharmaceuticals. Therefore, such interactions are an important aspect in the research of new cytostatics. ATP-binding cassettes are transmembrane proteins that are responsible for the translocation of various substances across membranes [71]. In former studies it was investigated that ATP-binding cassette transporters influence Enniatins- (Enns) and Bea-induced cytotoxicity [19, 72]. The overexpression of breast cancer resistance protein (ABCG2) in short time exposure assays slightly but significantly reduced the cytotoxic effects of Bea but not of the Enns. Multidrug resistance-associated-protein-1 (ABCC1) and P-glycoprotein (ABCB1) overexpression had no protective effect under the same conditions. In long-term exposure assay the overexpression of ABCG2 and ABCB1 lead to a protective effect against Enn´s cytotoxicity. The effect of Bea was reduced to a lesser extent. Both substances, especially Bea, potently inhibit the ABCG2- and ABCB1-mediated efflux of specific fluorescent substrates. Moreover, the interaction with ABCG2 and ABCB1 was proofed by using ATPase and photoaffinity-labelling assays [71]. Interestingly, a 2 years selection of KB-3-1 cells against the two cyclohexadepsipeptides led only to marginal resistance development. However, the selected sublines displayed upregulation of multidrug resistance proteins and consequently, were cross-resistant to other chemotherapeutics [71].

3. Aim of this thesis

This thesis is intended to complement the existing data on Bea and Enn B, whose research increased substantially in recent years. Bea and Enn B exhibit interesting pharmacological features, which were collected by means of *in-vitro* and very few *in-vivo* tests [73]. This diploma thesis focused on the synergistic effects of Bea and Enn B with the protein kinase inhibitors Sorafenib and Sunitinib. In several cell lines the cytotoxic, anti-angiogenetic and migration inhibitory effects were examined. Moreover, Western Blot and cell cycle analysis were performed to investigate the molecular mechanisms of Bea´s and Enn B´s cytotoxicity.

The following specific objectives were determined for this thesis:

- Create dose-response curves for Bea and Enn B as single drug and in combination with Sorafenib/ Sunitinib, determine and compare the IC_{50} values

- Examine the molecular mechanism of Bea and Enn B as single agent and co-administered with Sorafenib/Sunitinib with Western Blot analysis

- Explore Bea´s and Enn B´s impact when combined with Sorafenib/ Sunitinnib on apoptosis and the cell cycle

- Obtain inhibitory effects on cell migration and angiogenesis of Bea and Enn B as single agent and concomitantly applied with Sorafenib and Sunitinib

4. Materials and Methods

4.1 Drugs and Chemicals

Enzo Life Siences supplied the ionophore antibiotic drug **Enniatin B**. **Beauvericin** was provided by Sigma-Aldrich GmbH. Stock solutions (1mg/1ml) were frequently prepared in DMSO and stored at +4°C, as BEA and Enn B are not readily soluble in water. The kinase inhibitors **Sunitinib Sorafenib** were used for synergistic trials. All drugs were applied in concentrations from 0.5µM to 10µM.

4.2 Cell Lines

The human umbilical vein endothelial cells **HUVEC** (generously donated by Thomas Mohr, Medical University of Vienna), which were grown in endothelial basal medium (EBM)-2 (Lonza, MD, USA) supplemented according to the instructions of the manufacturer, and the following adherent growing epithelial cancer cell lines were used in this study: The epidermal cervix carcinoma derived cell line **KB-3-1** (generously donated by Dr. Shen, Bethesda, MD) was cultivated in RPMI 1640. (Roswell Park Memorial Institute) The epidermal hepatocyte carcinoma derived cell line **Hep3B** was grown in EMEM (Eagle´s Minimum Essential Medium). The epithelial kidney carcinoma cell lines **Caki 1** and **Caki 2** (obtained from the American Type Culture Collection, Manassas VA) were cultivated in Mc Coy´s Medium 5A. All culture Media weresupplemented with 10% fetal calf serum.

4.3 Cell Culture

Cell culture refers to the transfer of cells from their natural environment to controlled artificial conditions. When primary cultures are put into the next culture vessel they become a cell line. All above mentioned cell lines are adherent and the cells have to be detached, using the protease trypsin and a Ca^{2+}-chelating agent, such as EDTA. Following, the cells are subdivided, which is also referred to as passaging. In contrast

to normal cells, cancer cells are immortal and continue growing even if the surface of the vessel is already covered. Normal cells stop growing after building one thin layer of cells that just touch each other. This process is also referred to as contact inhibition. Furthermore, cancer cells can normally grow on simpler culture conditions than normal cells. [31, 74, 75]

4.3 MTT Assay (Viability Assay)

Background:

The MTT assay is an *in-vitro* nonradioactive cell proliferation and viability assay, which allows an indirect determination of the cytotoxicity of different drugs, applied as single agent or in combination to the cells. The colorimetric assay, shown in figure 14, which is based on the enzymatic reduction of tetrazolium salt 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (MTT). Within the living cell, the tetrazolium salt is metabolized to formazan, which gives an intense orange color. This reduction requires active and functional mitochondrial reductase enzymes; therefore, the MTT assay provides an excellent discriminating factor between living and dead cells. Dead cells remain uncolored because mitochondria are inactivated a few minutes after cell death[76, 77]. In this study the EZ4U Kit, developed by BIOMEDICA, was used. It is a less time consuming and simplified alternative to the classical MTT assay. Instead of insoluble reduction products, soluble ones are added, which do not kill the cells.

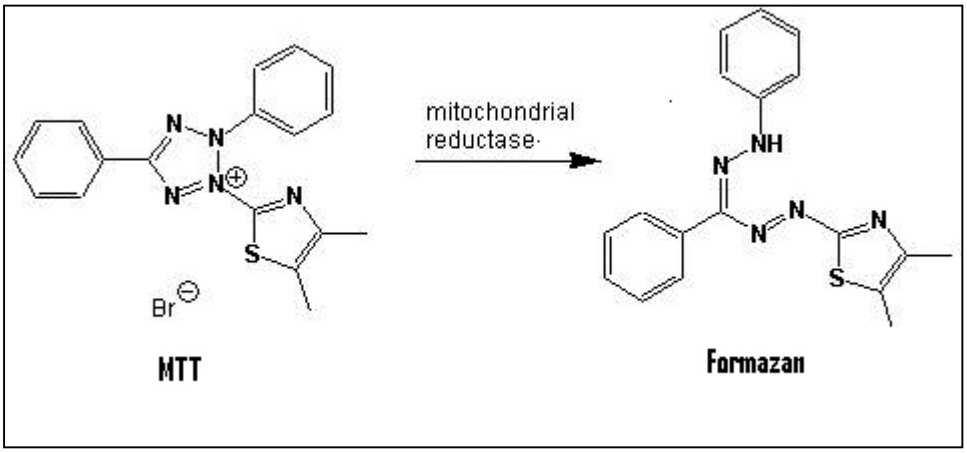

Figure 13: Mechanism of the mitochondrial reductase[4]

Procedure:

The cells were trypsinised and counted after washing with fresh medium. Following, the cell lines were seeded in 96-well microtiter plates at a density of 2-4 x10^4 cells per well, depending on the proliferation time of each cell line. 100µl/well of the respective cell culture medium containing 10% FCS was applied and the cells were allowed to recover for 24h. Then 100µl drug solution (2x concentrated), which was diluted in medium with 10% FCS was added in ascending concentrations in triplicates to the plate. For modulator studies, 50 µl (4x concentrated) drug solution and 50 µl modulator solution were applied. Afterwards, cells were exposed for 72 h, incubating at 37°C, 5% CO_2.The medium was replaced with 100 µl activated EZ4U solution, which was diluted in 10% FCS cell culture medium (following the manufacturer´s recommendation). Three wells only containing 100µl reactant solution were used as blank control. Due to diverse metabolic capacity of each cell line, the plates were incubated for 1 to 3 hours to yield a significant increase in color intensity. For the spectrophotometric measurement at 450 nm (620 nm as a reference), a microplate reader was used. With the obtained data dose-response curves were calculated using the Graph Pad Prism software.

4.4 Protein Analysis

4.4.1 Protein Extraction

Cells were seeded in 6-well-plates at a density of 3x10^5 cells/ml (2ml/well in 10% FCS cell culture medium) and allowed to recover for 24h prior drug treatment. The drug solutions were diluted in medium with 10% FCS. After incubation, the cells were collected by scraping into medium and were centrifuged at 1200 rpm for 5 minutes. After resuspension with 1ml PBS and centrifugation, the cell pellet was lysed in 30-50µl lysisbuffer on ice for approximately 15-20 minutes. To improve the lysis cells were treated for another 5 minutes with ultrasound in a sonicator. Another centrifugation step for 15 minutes at 14.000 rpm at 4°C followed. The supernatant is the protein lysate, which was stored at -80°C. The pellet, which contains nuclei and dense cell particles, was discharged. The protein concentrations were determined by using the Pierce BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, USA) At first

the solution was bright green. Then, there was a protein-dependent reduction of Cu^{2+} to Cu^{+} in alkaline medium. BCA forms with 2 molecules of bicinchonic acid a purple water-soluble complex. The more protein is present, the faster and stronger, the color change takes place. After 2 hours of incubation at 37 ° C, the plate was measured with a spectrophotometer at 620 nm. Finally the protein concentration could be calculated by a calibration line.

Recipes:

Lysis buffer:
500µl lysis buffer (see Western blot analysis)
10µl/ml phenylmethanesulfonyl fluoride (PMSF) - serine protease inhibitor, Roche
25µl/ml Complete (protease inhibitor cocktail tablets, Roche)

4.4.2 Western Blot Analysis

Background:

Western blotting is an immunochemical method for specific protein detection and quantification. It is the standard procedure for detecting specific proteins in prepared cell lysates. Via specific antibodies, a visualization of significant proteins is possible. [78]

The investigation of protein levels is generally used to get a better insight into molecular changes of cells after drug treatment. The proteins in a given heterogenic protein lysate are separated by electric current using SDS-Page (sodium dodecyl sulfate polyacrylamide gel electrophoresis), an electrophoretic method, which fractionates proteins by size. The SDS-Page consists of two different polyacrylamide gels, a collecting and a separating one. The samples are loaded into the collecting gel; there

the components become stacked into a very thin and sharp layer. Subsequently the probes enter the separating gel and are fractionated by molecular weight. Both gels comprise denaturating SDS buffer, an anionic detergent that binds to the proteins, unfolds them and imparts a negative charge. The inherit charge of the proteins is masked, which results in an equal charge-to-mass ratio for all proteins. An equal charge-to-mass ratio is indispensable to achieve the unique migration of all different proteins in an installed electric field towards the positively charged anode. Another essential condition for this movement is the destruction of secondary and tertiary structures, which is provided by the denaturing characteristics of SDS. For this study, a discontinuous buffer system, which employs different buffers for the gels, is used. [79]Tris-HCl buffer with pH 6.8 is applied to the collecting gel. The separating gel contains Tris-HCl buffer with a pH value of 8.8. Due to electric current the glycine ions, provided by the electrode buffer, enter the collecting gel and get zwitterionic character. In contrast to chloride ions, which are negatively charged and very small, the glycin ions are decelerated in their mobility. Caused by their much higher mobility the chloride ions from the gel represent the leading front. The intermediate proteins are compressed into a sharp band creating the focused narrow zone at the beginning of the separating gel. In the separating gel, another buffer is applied and therefore the pH value increases rapidly. Thus, the glycine ions become negatively charged and their mobility towards the positive electrode is enhanced. However, due to higher acrylamide concentrations in the separating gel, the smaller pores of the gel decrease the mobility of the proteins, which are now fractionated by size. [80-83] This effect is pictured in figure 15.

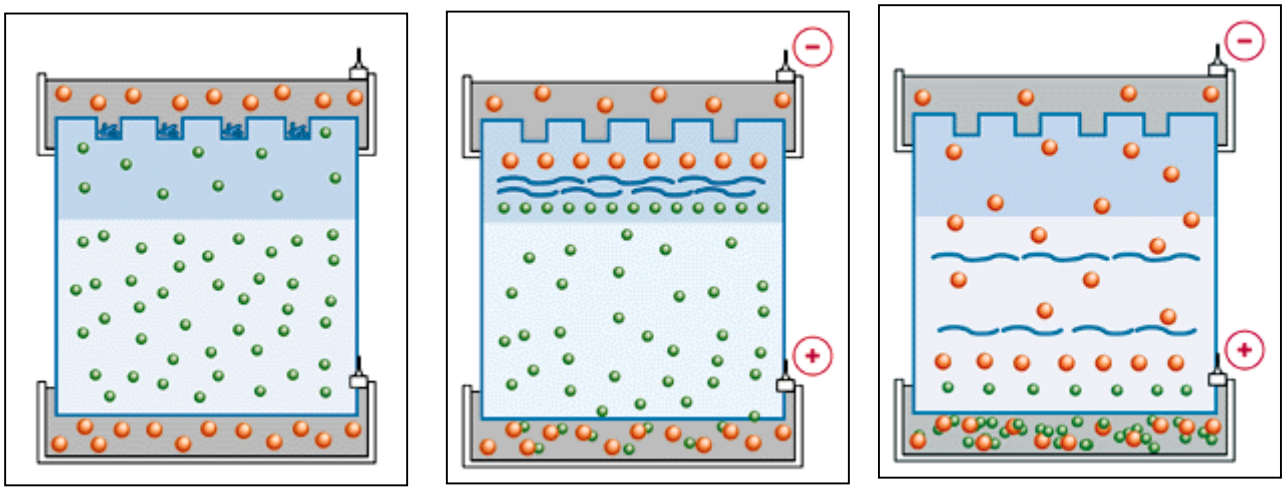

Figure 14: At first the probes are loaded onto the collecting gel that contains chloride ions (green) and zwitterionic glycine (red). Due to the pH value and electric current, the chloride ions start to migrate in front of the glycine molecules. Between these two two charged compounds, intermediate layer of proteins is formed and a thin and sharp band is created. Subsequently the proteins pass over to the separating gel and are fractionated by size.[8]

To enable antibody detection the separated proteins have to be transferrend onto a solid membrane, this is demonstrated in figure 16. In this study, polyvinylidene fluoride (PVDF) membrane and the semi-dry blotting technique were used. The separation principle of electrophoretic method is the division by the electric charge. The negatively charged proteins are forced to migrate onto the membrane, where the proteins stay mainly bound through hydrophobic interaction. Two stacks of filter paper, which are soaked with anode buffer and methanol (above the membrane) or cathode buffer (below the membrane), are used as ion-reservoirs and maintain the electric potential between the poles. The methanol added to the anode buffer, dissolves the SDS bonds of proteins, and therefore provides a more efficient binding to the membrane. To avoid nonspecific background binding by unbound components the membrane is washed with TBST (tris-buffered saline with Tween). Secondly, the membrane is placed into a BSA (Bovine Serum Albumin) and non-fat dry milk solution, which supplies an overage of proteins that occupy all polypeptide-free areas on the membrane. Thus, antibodies are not able to attach unspecifically to the membrane. To finally detect and visualize the proteins of interest the membrane is incubated with a solution, comprising a primary antibody that is directed against a certain epitope of the targeted protein. After incubation overnight at 4°C, the primary antibody solution is removed and secondary antibody, which binds to species-specific regions of the first antibody, is added. When it forms an antigen-antibody complex, the secondary antibody depicts a reporter enzyme, which starts to produce luminescence in the presence of a chemoluminescent agent by cleaving it. For the detection, a sensitive photographic film is uttered on the membrane for a certain time [84-86].

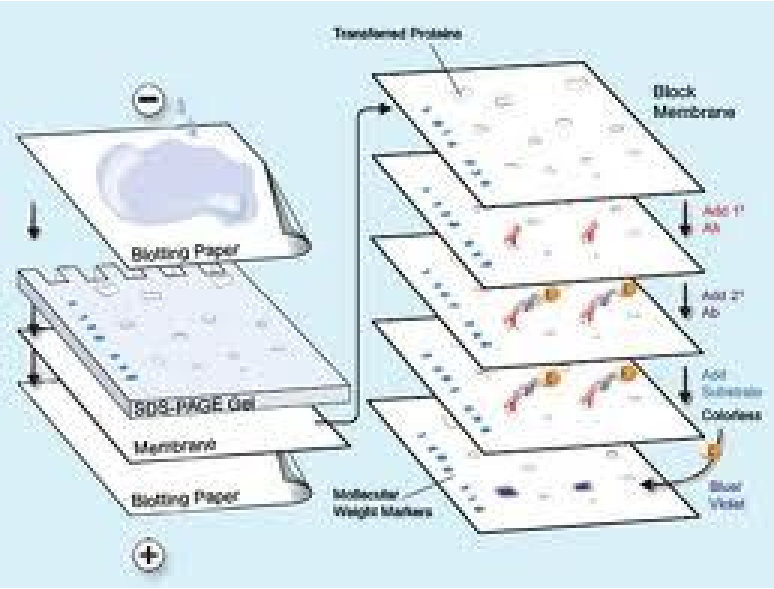

Figure 15: steps during the western blotting procedure[78]

Procedure

25µg of the protein lysates were diluted in lysisbuffer and 4x Loading Buffer was added to each sample. Then they were loaded to the prepared gels. For the SDS-Page 10% separating gel and 4.5% collecting gel were used. The electrophoresis was conducted with 90V, until the proteins reach the end of the Western Blot chamber. Afterwards, the gels were blotted using semi-dry blotting technique onto a PVDP (polyvinylidene difluoride membrane) with 0.08 mA for 45 minutes. Then, the proteins were controlled with Ponceau staining solution (0.1 % Ponceau S in 5 % acetic acid). After a 3-times 10 minutes washing step with TBST, the membrane was blocked with milk solution at least for one hour, prior to overnight incubation at 4°C with the primary antibody. In this study antibodies against Akt, pAkt, Erk, pErk, Bax, p38, pp38and β-Actin were used. Afterwards, the membranes were washed three times with TBST and incubated for 1 hour with HRP-coupled (Horseradish peroxidase) anti-rabbit or anti-mouse antibodies (1:10 000 dilution in 1% BSA). Once again the membranes were washed three times with TBST in each case for 10 minutes. Finally, the proteins were detected using Santa Cruz detection Kit, a chemoluminescent agent, following the manufacturer´s instructions.

Antibody	animal	company
Akt	Rabbit	Cell Signalling
pAkt	Rabbit	Cell Signalling
β-Aktin	Mouse	Sigma Biosciences
Bax	Rabbit	Cell Signalling
Cyclin D1	Mouse	Santa Cruz
Cyclin E1	Rabbit	Santa Cruz
Erk 1/2	Rabbit	Cell Signalling
p38	Rabbit	Cell Signalling
pp38	Rabbit	Cell Signalling

Table 2: used antibodies in this thesis

Recipes:

Tris- HCl 1.5 M, pH= 8.8 18.2g Tris Σ 100ml ddH_2O, pH= 8.8	**Tris- HCl 0.5 M, pH= 6.8** 3g Tris Σ 50ml ddH_2O, pH=6.8
Lysis buffer: 50mM Tris-HCl, pH= 7.6 300mM NaCl 0.5% Triton X-100 Σ 500ml ddH_2O	**10x TBS:** 120g Tris 90g NaCl Σ 1l dd H_2O, pH= 7.6
Bjerrumbuffer with Methanol: 5.82g Tris 2.93g Glycine 200ml Methanol Σ 1l ddH_2O	**10x Laemmli-Electrophoresis buffer:** 30g Tris 144g Glycine 10g SDS Σ1l dd H_2O
Bjerrumbuffer with SDS: 5.82g Tris 2.93g Glycine 0.375g SDS Σ 1l ddH_2O	**4x Sample loading buffer:** 4 ml 10% Glycine 0.92 g SDS 2.5 ml 1M Tris-HCl (pH=6.8) 2 ml 2-Mercaptoethanol
SDS-Page-10% Separating Gel: 3.65 ml dd H_20 1.875 ml Acrylamide 1.875 ml 1.5 M Tris-HCl, pH=8.8 5 µl 10% APS 5 µl TEMED 75 µl 20% SDS	**SDS-Page- Collecting Gel:** 1.65 ml dd H_20 0.281 ml Acrylamide 0.625 ml 1.5 M Tris-HCl, pH=6.8 12.5 µl 10% APS 5 µl TEMED 25 µl 20% SDS
1x TBST: 100ml 10xTBST 900ml ddH_2O 1ml Tween 20 (Biorad)	**10x TBS:** 120g Tris 90g NaCl Σ 1l dd H_2O, pH= 7.6

Table 3: recipes for western blot analysis

4.5 Flow Cytometry

The flow cytometry is a laser-based method, which is used for sorting and counting of cells. Moreover, there is a specialized type of flow cytometry. The fluorescent activated cell sorting (FACS) provides a cell sorting based upon their distinct fluorescent characteristics. The cells are marked with different fluorescent dyes giving a specific light scattering. Prepared single-cell-suspensions are soaked in by a capillary and are aligned in the flow cell. The flow cell guarantees a single cell passing through the sensing laser. The following measuring and detection system transform the fluorescence signals into electric signals. [87]

4.5.1 JC-1 Staining

Background

JC-1(*5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanin iodide*) is a lipophilic cationic fluorescent dye, which is often used for flow cytometry and fluorescent microscopy. JC-1 can selectively enter into mitochondria, where it reversibly changes color from green (529nm) to red (590nm) as the membrane potential increases. The mitochondrial membrane potential constitutes a significant indicator of cell health. In healthy cells with high mitochondrial potentials JC-1 spontaneously forms intense red J-aggregates. In contrast, apoptotic cells with low mitochondrial membrane potential show green fluorescence, since JC-1 remains in the monomeric form. [88, 89]

Procedure:

The cells were seeded in 6-well-plates in a density of $5x10^5$ cells per well. After a recovery of 24h, the cells were incubated with the testing substances in different concentrations. Thereafter, the media was replaced by JC-1 solution (Sigma-Aldrich 10µg/ 1ml) and a 10 minutes incubation time followed. Finally the JC-1 solution was

removed. Consecutively, the cells were washed and quantified with the flow cytometer. (BD LSR Fortessa®)

4.5.2 PI Staining

Background:

The propidium iodide (PI) flow cytometric assay is a widely applied method used for cell cycle analysis. It is based on the principle that the DNA of mammalian, yeast and bacterial cells can be dyed by various DNA-binding stainings. DNA dyes include besides other: propidium iodide (PI), Hoechst 33342 and 33258 and 4´6´-diamino-2-phenylindole (DAPI). However, PI is applied more often than the other mentioned dyes, as it is stable, economical and constitutes an excellent discrimination factor for cell surviving. As a fact, the entrance of PI into the cell and the nucleus depends upon the permeability of the membrane. Therefore, PI does only stain late apoptotic or necrotic cells, in which the membranes are decreased. Consequently, to create a cell cycle analysis, cells have to be fixed with alcohol or aldehyde to allow the stain to enter the cell. [90-92] The PI assay gives a rapid and precise evaluation of DNA content, as it stoichiometrically intercalates into the double-stranded DNA and RNA and becomes fluorenscent.[91, 93] Consequently, cells in the S-phase, which contain more DNA than cells in the G_1-phase, will proportionally take up more dye and therefore show a higher fluorescence. Additionally, apoptotic cells show, besides many other features, DNA fragmentation and the following loss of nuclear content. [90, 91]

Procedure:

Initially, the cells were seeded in 6-well-microtiterplates depending on the proliferation time of each cell line in a density of 3-5x10^5cells/well. Following a 24h recovery the cells were treated with the substances in desired concentrations and were transferred

into test tubes centrifuged and suspended in 100µl 0.9% NaCl. Afterwards, they were dropped into alcohol. Subsequently, the cells were stored in ice-cold ethanol (70% v/v) for 1 hour at 4°C. Thus, the cells were fixed and PI was able to enter the cells. Such alcohol-fixed cells are stable for several weeks at 4°C. [91] Thereafter, the ethanol was replaced by PBS and 2µl RNAse (10µl/ml) was added. The RNAse was applied to prevent intercalation of PI with the RNA, which would have biased the results. Finally, 5µl PI (1mg/ml in PBS) was added and 30 minutes later the probes were measured with the cytometer at 4°C. [90, 91]

4.6 Tube Formation Assay

The Tube formation assay is a common *in-vitro* model to display the reorganization stage of angiogenesis. In more detail, the assay measures the ability of endothelial cells to form capillary-like structures (tubes). The endothelial cells are plated at subconfluent concentrations with appropriate extracellular matrix support. Thus, the Tube formation assay is usually employed to determine the capability of various compounds to promote or inhibit tube formation. Substances that inhibit tube formation may be useful in cancer, where tumors stimulate blood vessel formation through angiogenesis in order to gain nutrients and oxygen. [94]

4.7 Scratch Assay

The scratch assay is an *in-vitro* method to measure cell migration. The assay is based on creating a scratch in a cell monolayer and capturing pictures right after the scratch and in defined time intervals until cell migration closes the scratch. By comparison of the images, the migration rates of the cells are quantified. The scratch assay is suitable to display the effects of mimic cell migration during wound healing and of cell-cell interactions in cell migration[95].

Procedure:

The cells were seeded in fibronectin-coated-6-well-plates. To establish a scratch assay a 100% confluent cell monolayer is crucial, which was verified microscopically. Just before scratching a nude gap into the monolayer with a pipette, the cells were incubated with subtoxic concentrations of the testing substances. Afterwards, images of the cells, taken right after the scratch and 9 or 15 hours later were compared.

5. Results and Discussion

5.1 Cytotoxic activity of test substances in diverse cell models

The following results were obtained by using MTT-Assay. KB-3-1, Caki 1 and Caki 2 were treated with Bea and Enn B (0.5-10 µM) and Sunitinib (0.5-10 µM) for 24/48/72h. Hep3B was incubated with Bea and Enn B (0.5-10 µM) and Sorafenib (0.5-10 µM) for 24/48/72h. Since Sorafenib is approved for patients with heptacellular carcinoma and advanced renal cell cancer, the hepatocellular carcinoma cell line Hep3B was used. Sunitinib is approved by the FDA for the treatment of renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor, therefore the renal cancer cells Caki 1 and Caki 2 were used. Additionally, the Enn B-sensitive epidermal carcinoma-derived cell line KB-3-1 was used. IC_{50} values were calculated from whole dose response curves. Table 4 summarises the values as means from at least two independent experiments performed in triplicates. Indicated by the calculated IC_{50} values in KB-3-1 and Caki 1 a profound time and dose dependent growth inhibition was observed for Enn B as well as for Bea and Sunitinib monotreatment for 24/48/72h. In contrast, Caki-2 cells responded less sensitive to Bea, Enn B and Sunitinib monotreatment. Only after 72h treatment the cell growth was severly inhibited. As displayed in table a, Hep3B was strongly affected by Bea and to a lesser extent by Enn B after 24/48/72h incubation. Moreover, a profound growth inhibition was observed for Sorafenib treatment after 24/48/72h.

Table 4: Anticancer activity of Beauvericin, Enniatin B, Sorafenib and Sunitinib after 24/48/72 hour treatment.

		Beauvericin	**Enniatin B**	**Sorafenib**	**Sunitinib**
		Mean IC_{50}	**Mean IC_{50}**	**Mean IC_{50}**	**Mean IC_{50}**
KB-3-1	24h	4.59 µM	4.99 µM	-	>10 µM
	48h	4.29 µM	3.87 µM	-	5.88 µM

	72h	3.79 µM	3.22 µM	-	4.32 µM
Caki 1	**24h**	4.11 µM	6.12 µM	-	6.42 µM
	48h	2.31 µM	2.21 µM	-	5.43 µM
	72h	2.48 µM	2.08 µM	-	2.02 µM
Caki 2	**24h**	>10 µM	>10 µM	-	>10 µM
	48h	>10 µM	>10 µM	-	5.27 µM
	72h	4.26 µM	6.11 µM	-	4.55 µM
Hep 3B	**24h**	>10 µM	>10 µM	3.11µM	-
	48h	3.15 µM	8.41 µM	2.56µM	-
	72h	3.47 µM	3.25 µM	2.54µM	-

Table 4: Anticytotoxic activity of Beauvericin, Enniatin B, Sorafenib and Sunitinib after 24/48/72 hour treatment. IC50 values were calculated from whole dose response curves. Values given are means from at least two independent experiments performed in triplic

5.2 Combination Trials with Beauvericin and Enniatin in combination with Sorafenib/Sunitinib

To investigate the impact of Beauvericin and Enniatin B in combination with the potent multikinase inhibitor drugs Sorafenib and Sunitinib, combination trials were performed. Moreover, to evaluate the effects concentration-response curves were generated. Protein kinase inhibitors are a relatively new, potent group of anticancer therapeutics, which mechanisms of action are widely resolved. They are already established for cancer therapy treatment. In Table 5 and 6the potentiation of single drug efficacy by Bea and Enn B in combination with Sunitinib and Sorafenib after 24/48/72h treatment are summarized. The following fold decrease values were calculated by dividing Bea and Enn B IC_{50} of Bea/Enn B alone by IC_{50} of Bea/Enn B in combination. Concomitant administration of Bea and Sunitinib in KB-3-1, Caki 1 and Caki 2 potentiated their cytotoxic effects indicated by an increase in their IC_{50} up to 1.59-fold (10 µM Sun) and 2.69-fold (1 µM Sun). However, as displayed in Table 5 the decrease of IC_{50} in Caki 2

was less intense, this also correlates with the slight effects of the drugs in monotreatment. As a fact, a treatment with Enn B in combination resulted no or only slight potentiation. In Caki 1 and Caki 2 the IC_{50} levels increased 1.21-fold (1 µM Sun) and 1.26-fold (2.5 µM Sun). A concomitant exposure to Enn B and Sunitinib in KB-3-1 showed no increase in IC_{50} values. After combination of Bea with Sorafenib in Hep3B drug efficacy was potentiated as the IC_{50} increased up to 1.90 (2.5 µM Sun). Moreover a concomitant administration of Enn B and Sorafenib lead to a level up to 1.64 (2.5 µM).

Table 5: Potentation of single drug efficacy by Bea/Enn B with Sora/Sun combination after 72h treatment

	KB-3-1	**Caki 1**	**Caki 2**
	-fold decrease	-fold decrease	-fold decrease
IC_{50} of Bea			
+0.5 µM Sun	0.98	1.48	1.06
+1 µM Sun	0.99	2.69	1.09
+2.5 µM Sun	1.09	-	1.05
+5 µM Sun	1.53	-	0.92
10 µM Sun	1.59	-	1.06
IC_{50} of Enn B			
+0.5 µM Sun	0.83	1.15	0.90
+1 µM Sun	0.82	1.21	0.94
+2.5 µM Sun	0.76	0.64	1.26
+5 µM Sun	0.79	0.821	0.642
+10 µM Sun	0.89	1.09	0.82

Table 5: Fold decrease was calculated by dividing IC50 of Bea/Enn B alone by IC50 values

Table 6: Potentation of single drug efficacy by Bea/Enn B with Sora/Sun combination after 72h treatment

	Hep3B		
	-fold decrease		-fold decrease
IC_{50} of Bea		IC_{50} of Enn B	
0.5 µM Sor	1.20	+0.5 µM Sor	1.16
+1 µM Sor	1.20	+1 µM Sor	1.07
+2.5 µM Sor	1.90	+2.5 µM Sor	1.64
+5 µM Sor	-	+5 µM Sor	-
10 µM Sor	-	+10 µM Sor	-

Table 6: Fold decrease was calculated by dividing IC50 of Bea/Enn B alone by IC50 values

Additionally, CI (combination index) values were calculated using CalcuSyn software to evaluate synergistic, additive or antagonistic effects of the combination of Bea/ Enn B with Sunitinib in the cell lines KB-3-1, Caki 1 and Caki 2. The CI values of Bea/ Enn B with Sorafenib in Hep3B were also determined. CI < 0.9, CI 0.9 – 1.1, CI > 1.1 represent synergism, additive effects and antagonism. The CI values mentioned in the text are means of one representative experiment in triplicates. Depending on the combined concentrations and the incubation periods the performed viability tests with KB-3-1, Hep3B Caki 1 and Caki 2 showed synergistic or additive, as well as antagonistic effects. In KB-3-1 strongest effects were discovered with Bea/ Enn B (2.5 – 5 µM) in combination with Sunitinib (0.5 – 2.5 µM) showing a CI value of 0.61. As a fact, Bea and Enn B (10 µM) destroyed all cells completely as single drugs, thus, no combinatory effects could be observed. Interestingly, 48h incubation seems to be more potent than 72h incubation. Caki 1 showed the strongest synergistic effects with a CI of 0.26 after 72h in combination of Bea/ Enn B (1/ 2.5 µM) with Sunitinib (1 µM). The synergistic effects in Caki 2 were slightly lower indicating a CI value of 0.58 after 72h incubation with Bea/Enn B (5 µM) and Sunitinib (1 – 5 µM). Bea and Enn B showed no synergistic effects in Hep3B after 24h. However, after 48/72h incubation additive and

synergistic effects could be found, averaging a CI value of 0.55 after treatment with Bea/ Enn B (5 µM) and Sorafenib (1 µM).

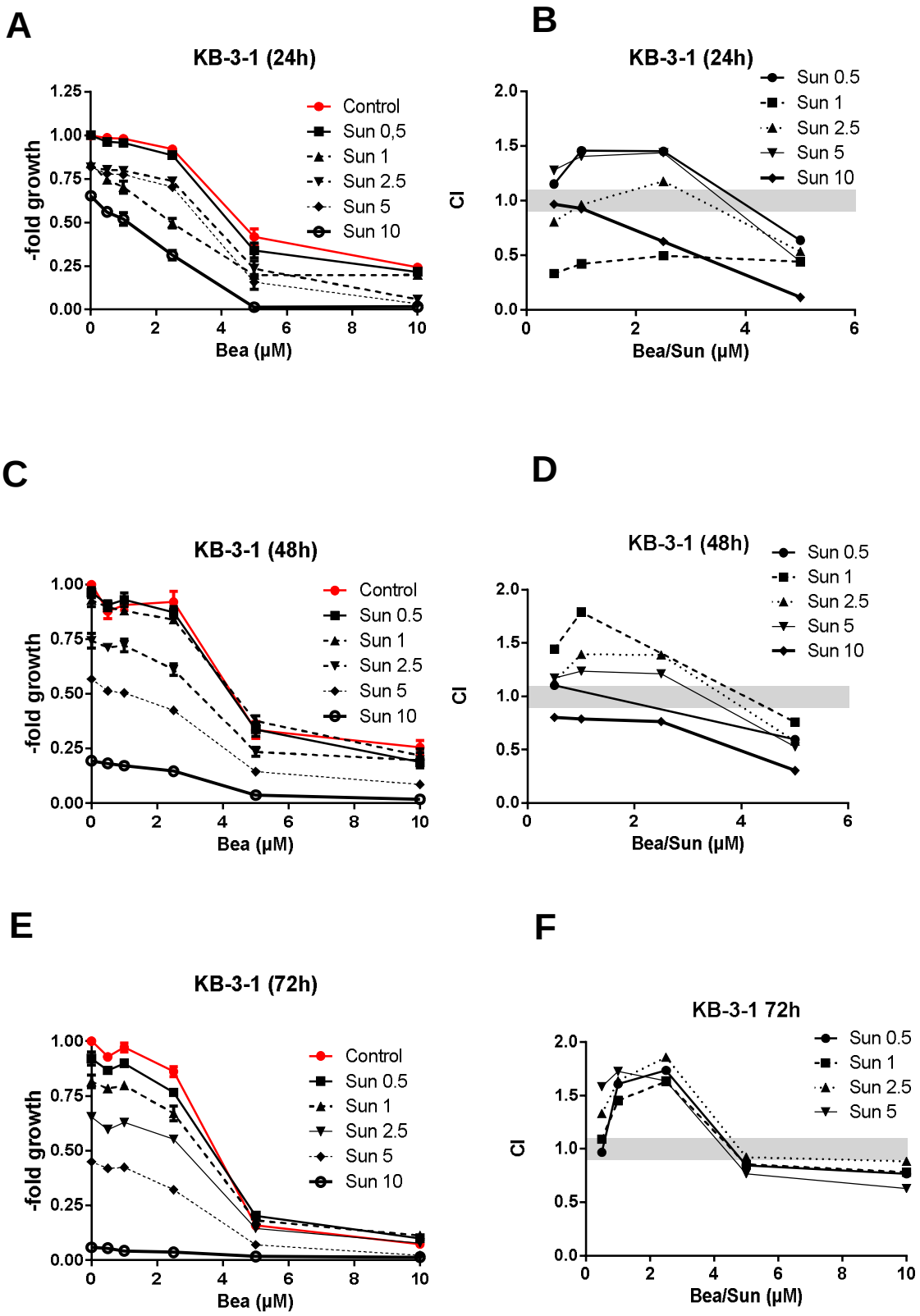

Figure15: Viability tests of KB-3-1 incubated with Bea (0.5, 1, 2.5, 5, 10 µM) in combination with Sunitinib (0.5, 1, 2.5, 5, 10 µM) for 24/48/72h.

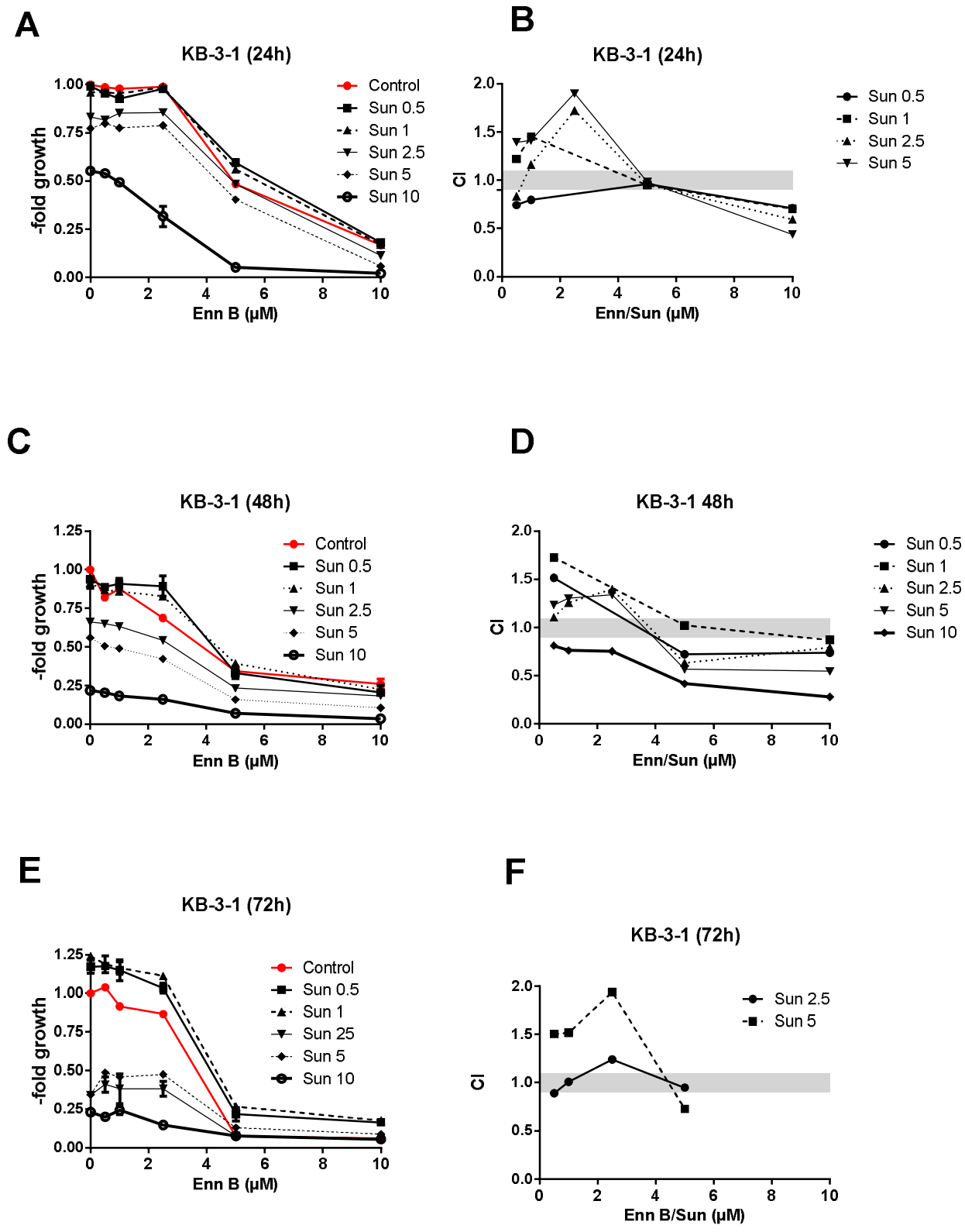

Figure 16: Viability tests of KB-3-1 incubated with Enn B (0.5, 1, 2.5, 5, 10 µM) in combination with Sunitinib (0.5, 1, 2.5, 5, 10 µM) for 24/48/72h.

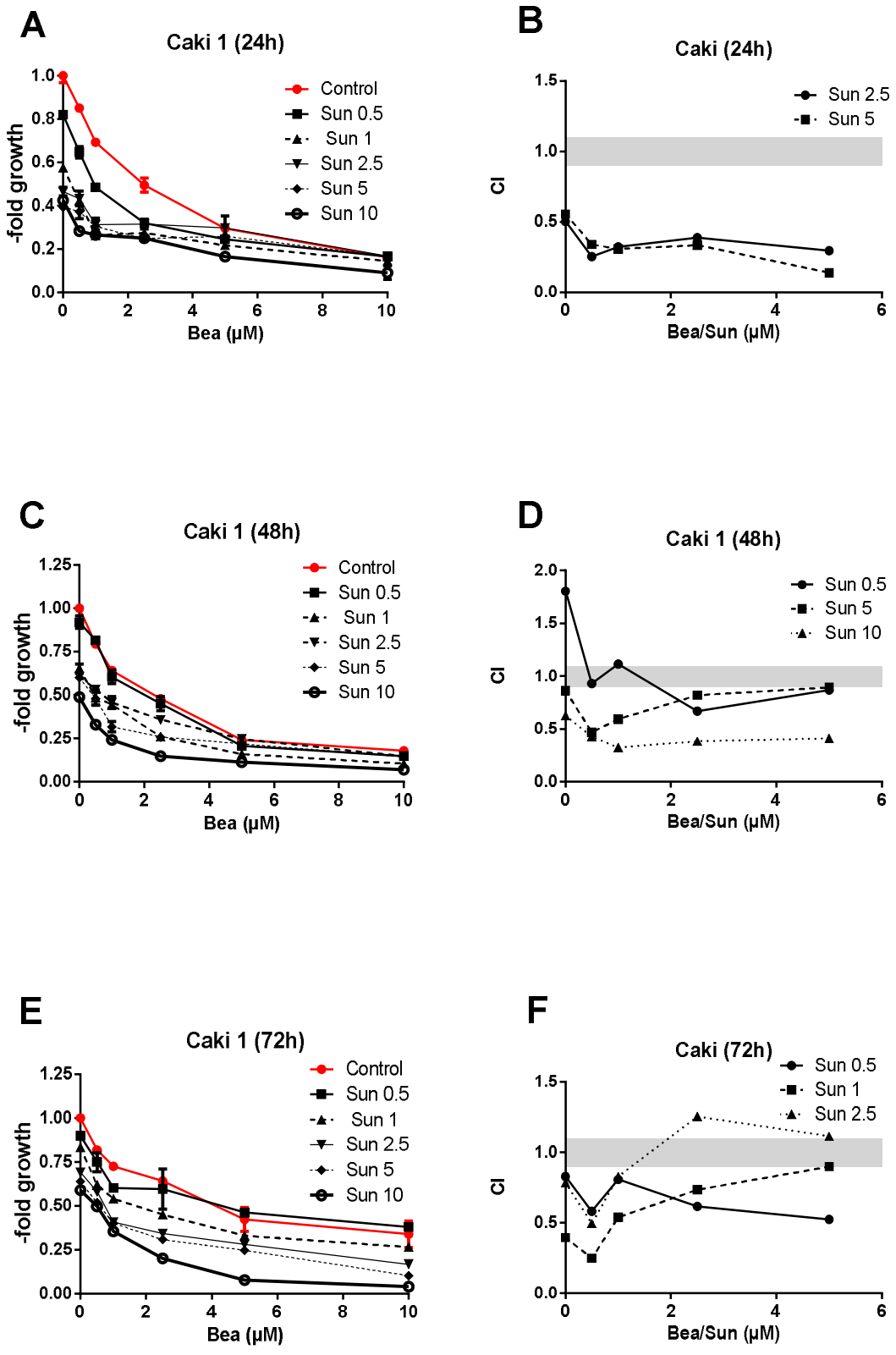

Figure 17: Viability tests of Caki 1 incubated with Bea (0.5, 1, 2.5, 5, 10 µM) in combination with Sunitinib (0.5, 1, 2.5, 5, 10 µM) for 24/48/72h.

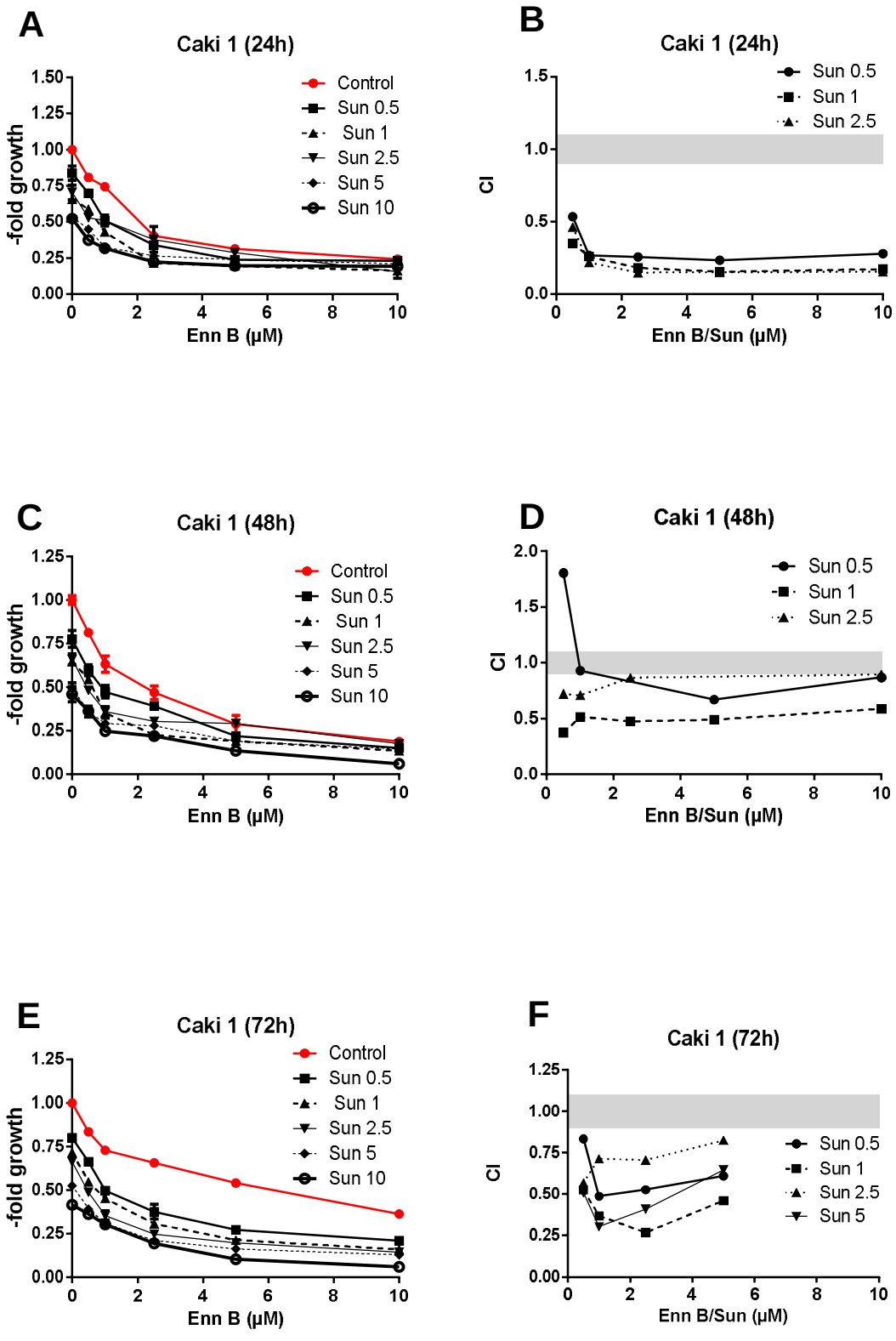

Figure 18: Viability tests of Caki 1 incubated with Enn B (0.5, 1, 2.5, 5, 10 µM) in combination with Sunitinib (0.5, 1, 2.5, 5, 10 µM) for 24/48/72h.

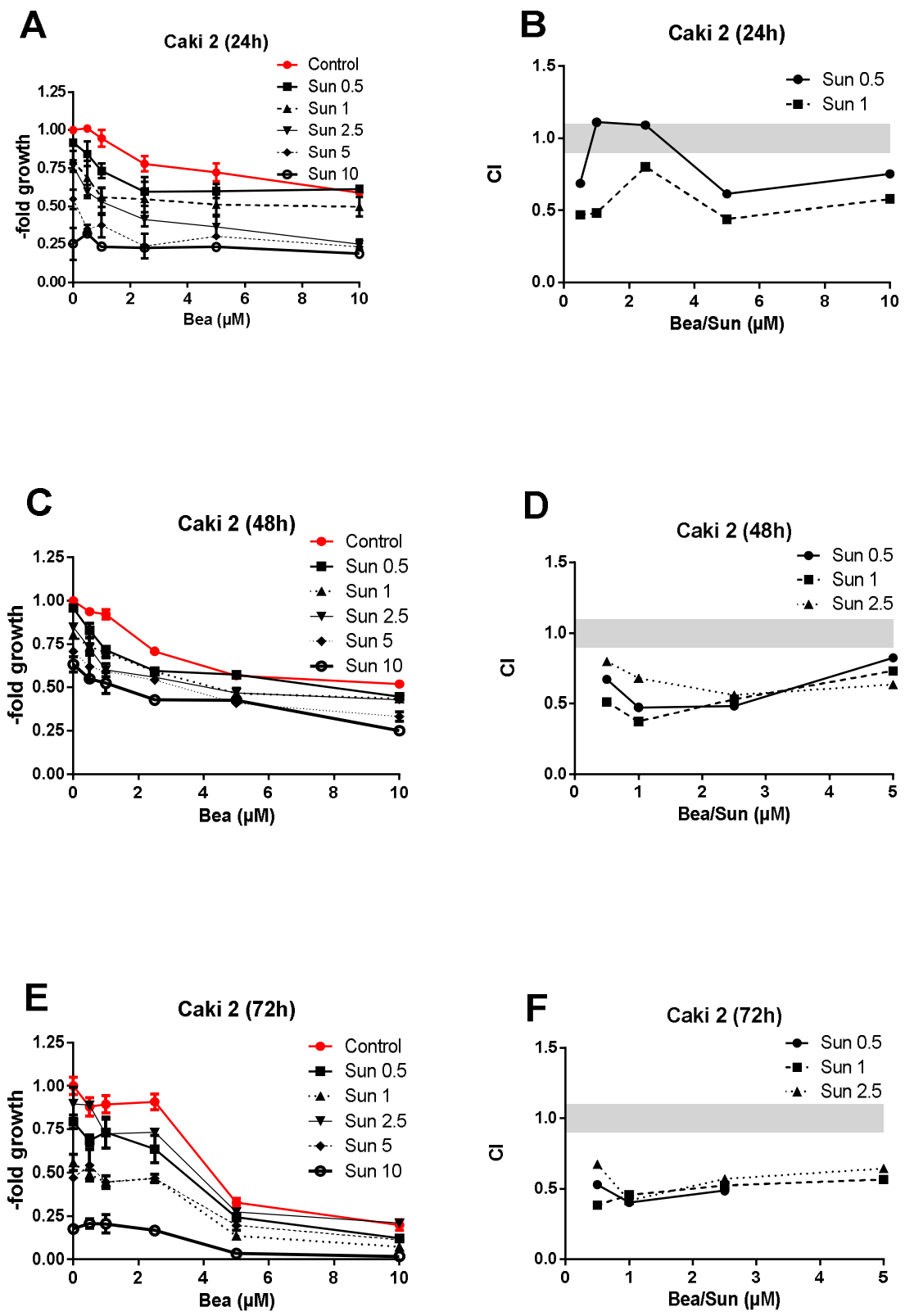

Figure 19: Viability tests of Caki 2 incubated with Bea (0.5, 1, 2.5, 5, 10 µM) in combination with Sunitinib (0.5, 1, 2.5, 5, 10 µM) for 24/48/72h.

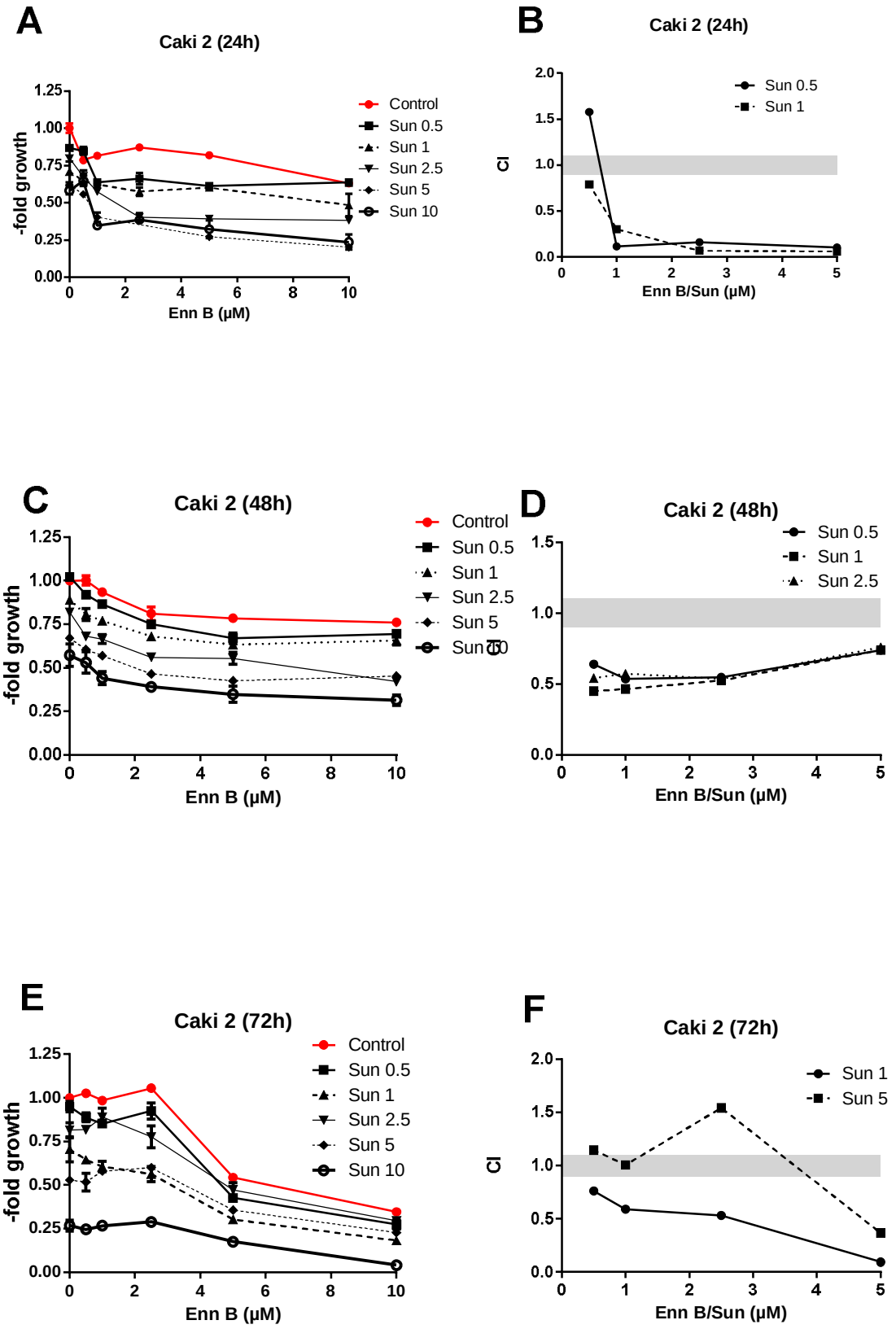

Figure 20: Viability tests of Caki 2 incubated with Enn B (0.5, 1, 2.5, 5, 10 µM) in combination with Sunitinib (0.5, 1, 2.5, 5, 10 µM) for 24/48/72h.

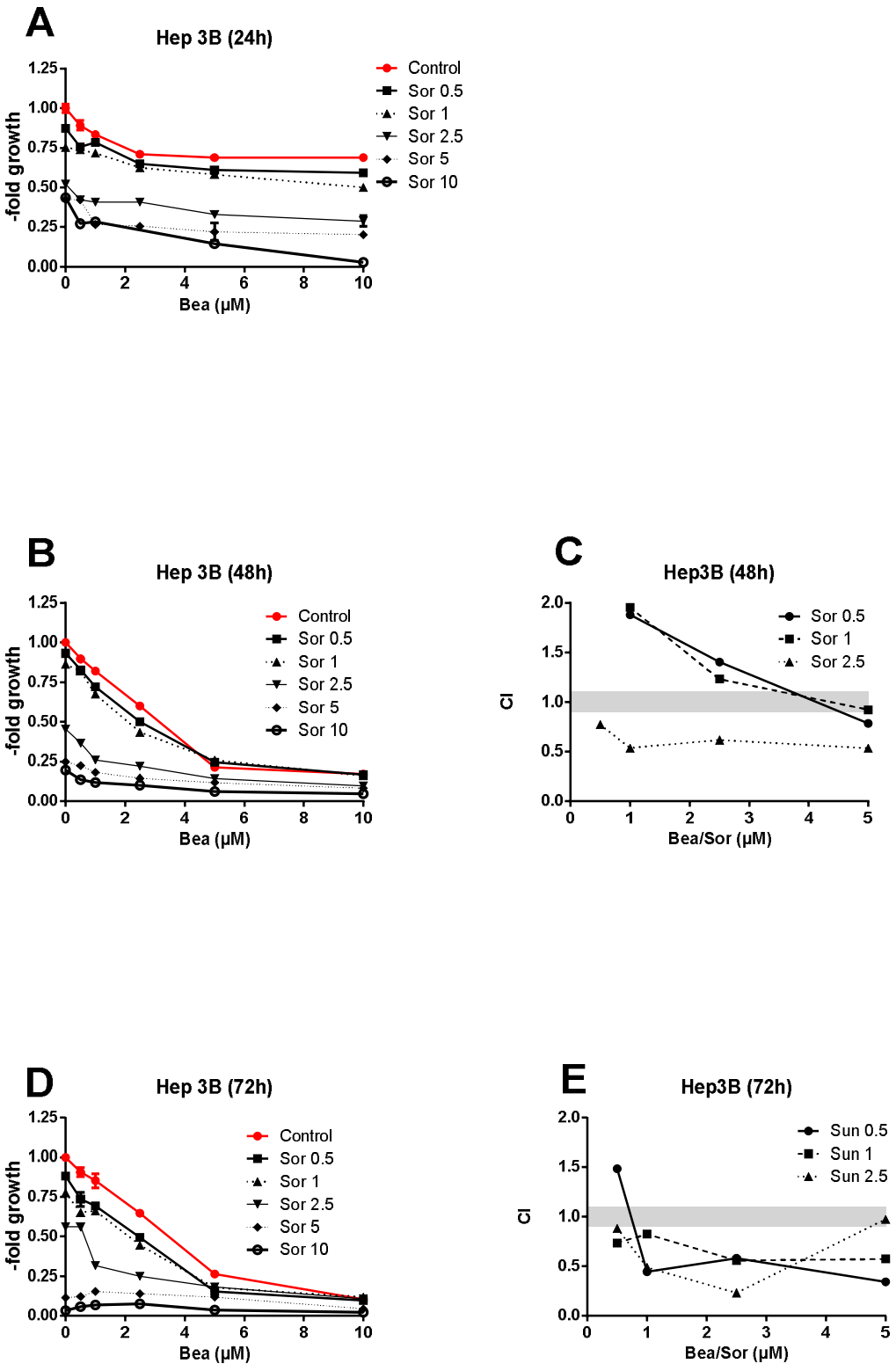

Figure 21: Viability tests of Hep3B incubated with Bea (0.5, 1, 2.5, 5, 10 µM) in combination with Sorafenib (0.5, 1, 2.5, 5, 10 µM) for 24/48/72h and with Enn B (0.5, 1, 2.5, 5, 10 µM) in combination with Sorafenib(0.5, 1, 2.5, 5, 10 µM)

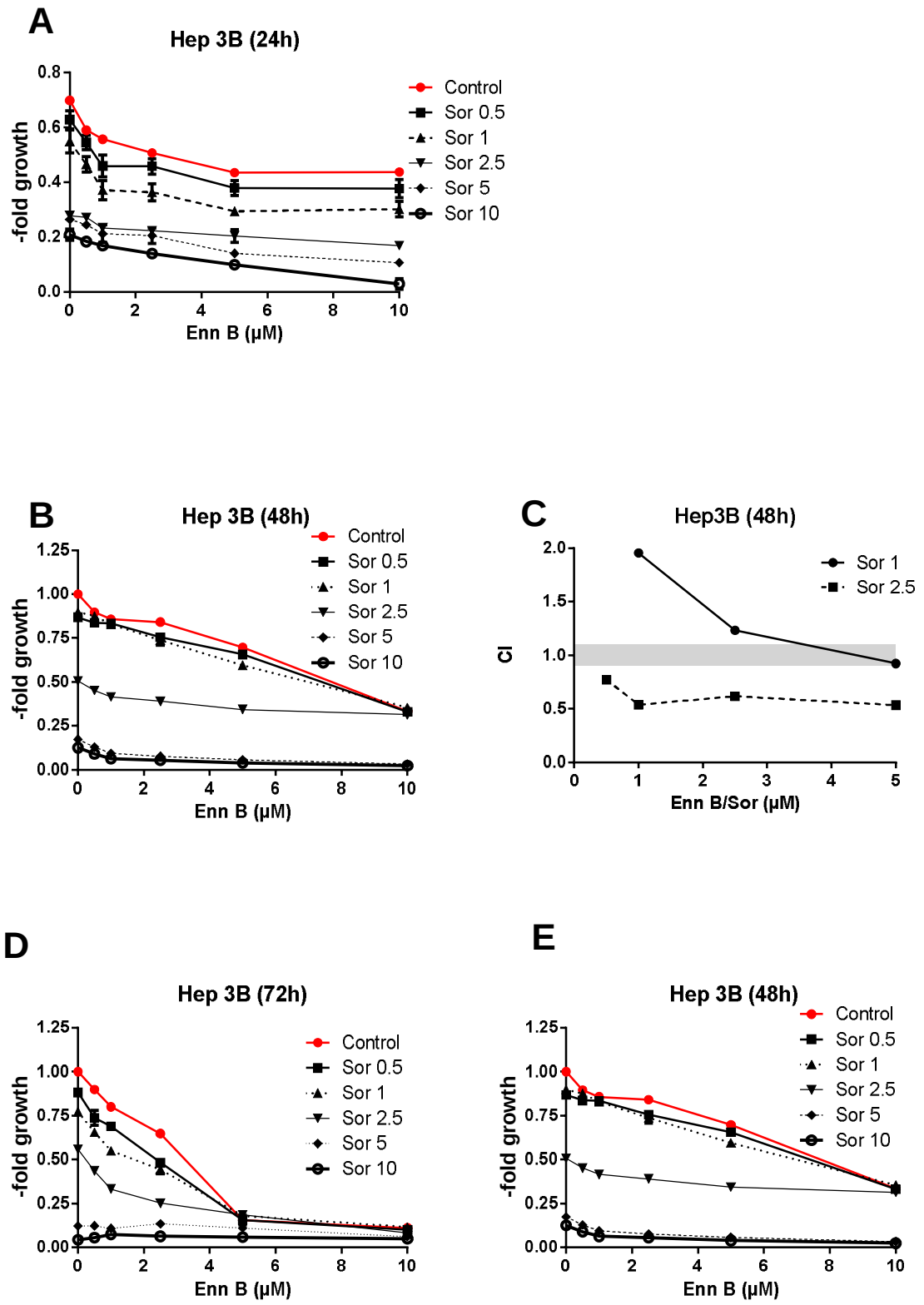

Figure 22: Viability tests of Hep3B incubated with Enn B (0.5, 1, 2.5, 5, 10 µM) in combination with Sorafenib (0.5, 1, 2.5, 5, 10 µM) for 48/72h.

5.2 Western Blot Analysis

To gain insights into the molecular mechanisms underlying the synergistic effects of Bea and Enn B in combination with Sunitinib and Sorafenib in KB-3-1, Caki 1, Caki 2 and Hep3B Western blot analyses were performed. All cells were incubated with the respective drugs for 24h in sub-cytotoxic concentrations as single agent and in combination, in order to determine the differences. Afterwards, the proteins were extracted and the analysis was carried out. KB-3-1 was incubated with Bea and Enn B (1, 2, 3 µM) and Sorafenib (2.5, 5 µM), since these concentrations have already shown strong synergistic effects in viability tests in a former diploma thesis. Caki 1 and Caki 2 were treated with Bea and Enn B (5 µM) in combination with Sunitinib (2.5 µM), as synergistic effects could be demonstrated in viability tests of these concentrations.
Bea and Enn B (1, 2, 3) and Sorafenib (2.5, 5 µM) were concomitantly administered to Hep3B.
Erk1/2, the extracellular-signal-regulated kinase, which is part of the Map-kinase pathway remained constant in all concentrations in all cell lines tested.

However, pErk, which is the active phosphorylated isoform of Erk1/2 and a sign for apoptosis and repair mechanisms was profoundly induced in KB-3-1 when Enn B was administered as single drug. As a result, a single treatment of Bea in KB-3-1 had no considerable effect. A combination of Bea/Enn B with Sorafenib reversed the effect and the band faded and therefore indicated that cytotoxic concentrations had already been reached. In Hep3B pErk was slightly induced in combinations. In Caki 1 pErk was enhanced, especially when Bea and Sunitinib were administered as single drugs. In Caki 2 pErk was induced in all concentrations. This effect was even enhanced in combination.

Additionally, the effects on p38 (p-38 mitogen activated protein kinase) and its active isoform pp38 were investigated. Both kinases are involved in stress response triggered by ultraviolet radiation, osmotic or heat shock and cytocines. Interestingly, in Hep3B pp38 was induced strongest after applying Bea and Enn B as single agents. Also after Enn B administration as single agent in KB-3-1, the most profound effects could be found. However, the combination of Bea and Sorafenib showed more potent effects than applied as single drug. As well in Caki 2, a combination of Bea and Enn B even

increased the inductive effect. In Caki 1 only Sunitinib showed an inductive effect of pp38.

Additionally, Akt and the active isoform pAkt, which are involved in the PI3K/Akt/mTor pathway which leads to cell proliferation and apoptosis was examined.

In KB-3-1 pAkt was slightly enhanced in combinations. As in KB-3-1 pAkt got induced in Hep3B when Bea and Sorafenib were concomitantly administered. As a fact, Enn B enhanced pAkt stronger as single agent than in combination. In Caki Akt was reduced while pAkt was enhanced in all concentrations.

PARP (Poly ADP Ribose Polymerase) is another repair mechanism, which takes place in the nucleus and can also induce apoptosis. Former studies already showed that a mixture of different Enns is able to shift the proportion of PARP towards the active cleaved PARP[19]. Likewise, a former diploma thesis showed that PARP is induced after treatment of KB-3-1 with Bea and Enn B in combination with Sorafenib[96]. Also in Caki 1 after cocomitant administration of Bea, Enn B with Sunitinib cleaved PARP was profoundly induced.

Bax, a member of the Bcl-2 family, which works as co-factor for p53 is slightly reduced in Hep3B when Sorafenib is added as single drug. Further, in Caki 1 Bax is most strongly reduced ,when Enn B was administered as single drug. However, combinations led to a less intense effect. In Caki 2 Bax remained constant in all concentrations.

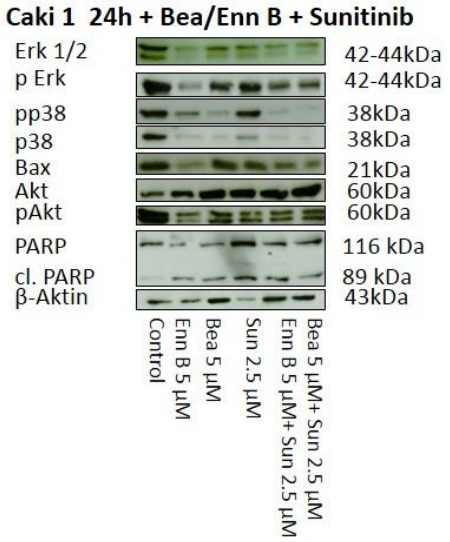

Figure27: Western Blot of Caki 1 with Bea/Enn B in combination with Sunitinib after 24h

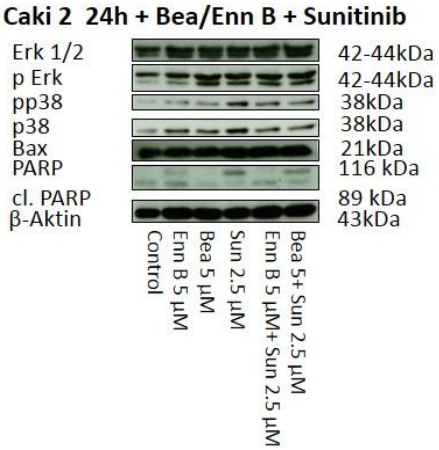

Figure 28: Western Blot of Caki 2 with Bea/Enn B in combination with Sunitinib after 24h

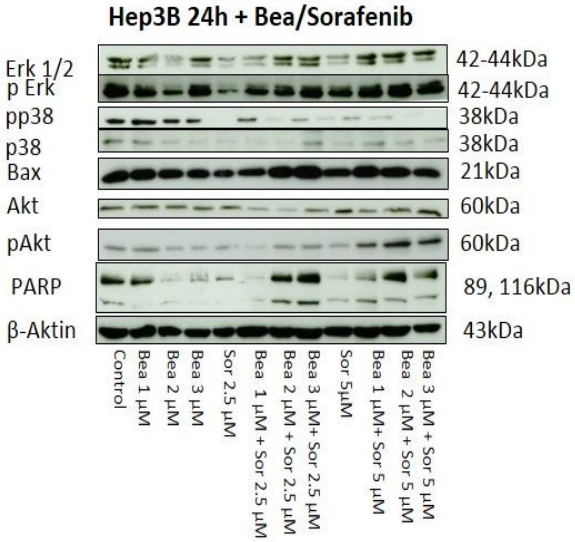

Figure 23: Western Blot of Hep3B with Bea in combination with Sorafenib after 24h

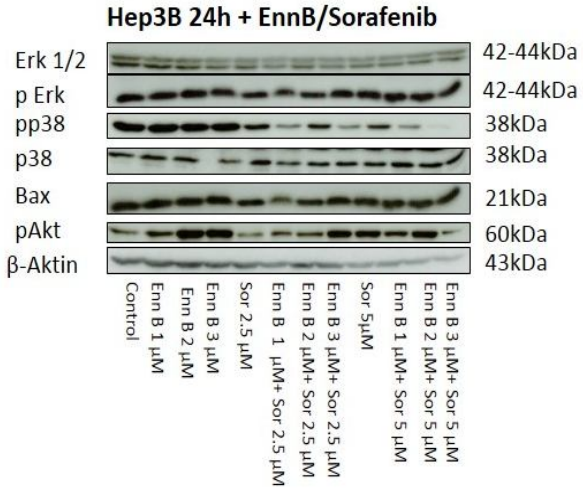

Figure30: Western Blot of Hep3B with Enn B in combination with Sorafenib after 24h; [3]

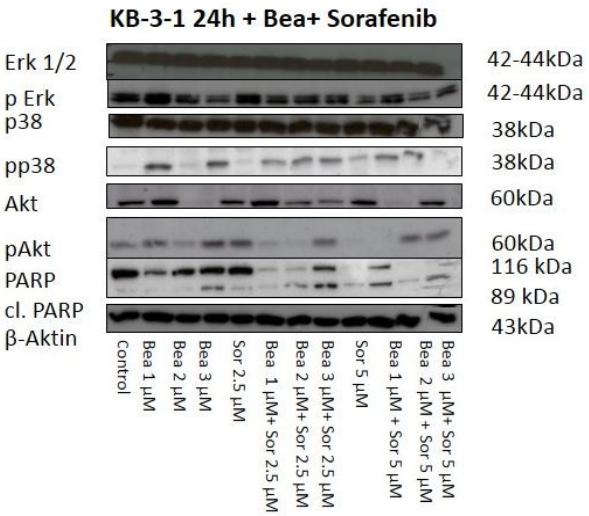

Figure31: Western Blot of KB-3-1 with Bea in combination with Sunitinib after 24h [1]

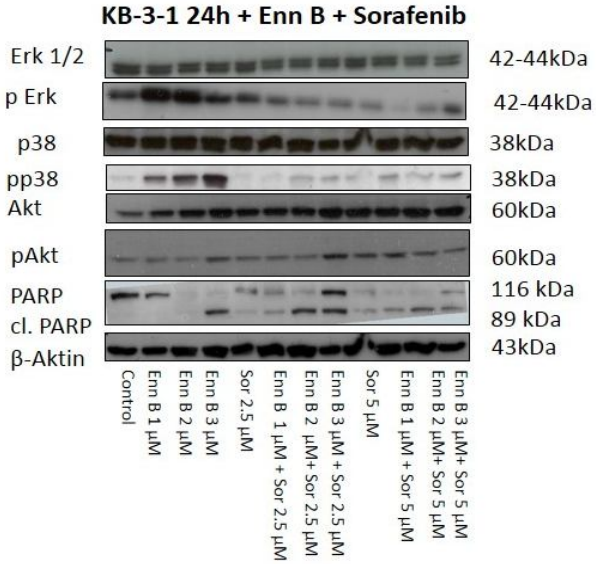

Figure32: Western Blot of KB-3-1 with Enn B in combination with Sunitinib after 24h [2]

5.3 Effect on cell cycle

5.3.1 PI staining

As a next step, the influence of Bea on cell cycle progression was measured with propidium iodide (PI) flow cytometric assay. Therefore, Caki 1 cells were incubated for 24h with Bea (1, 2.5, 5 µM) and Sunitinib (1, 2.5 µM) as single drug and in combination. The results showed an increase of the G_0/G_1 stage from 62.72% in the control after 5 µM Bea treatment. Moreover, Bea (5 µM) concomitantly administered with Sun (1 µM) displayed a slight enhancement of this effect showing 82.46%. Corresponding, the S-phase was reduced to 6.03% (Bea 5 µM), 3.81 (Bea 5 µM + Sun 1 µM) contrasting 19.56% in the control. Bea (1, 2.5 µM) and Sun (1, 2.5 µM) displayed no influence on the cell cycle. A former diploma thesis showed already that Bea has an influence on the cell cycle and shifts the proportion towards the G_0/G_1-phase in KB-3-1. In order to evaluate the effects of Bea onto the cell cycle more experiments in different cell models have to be performed.

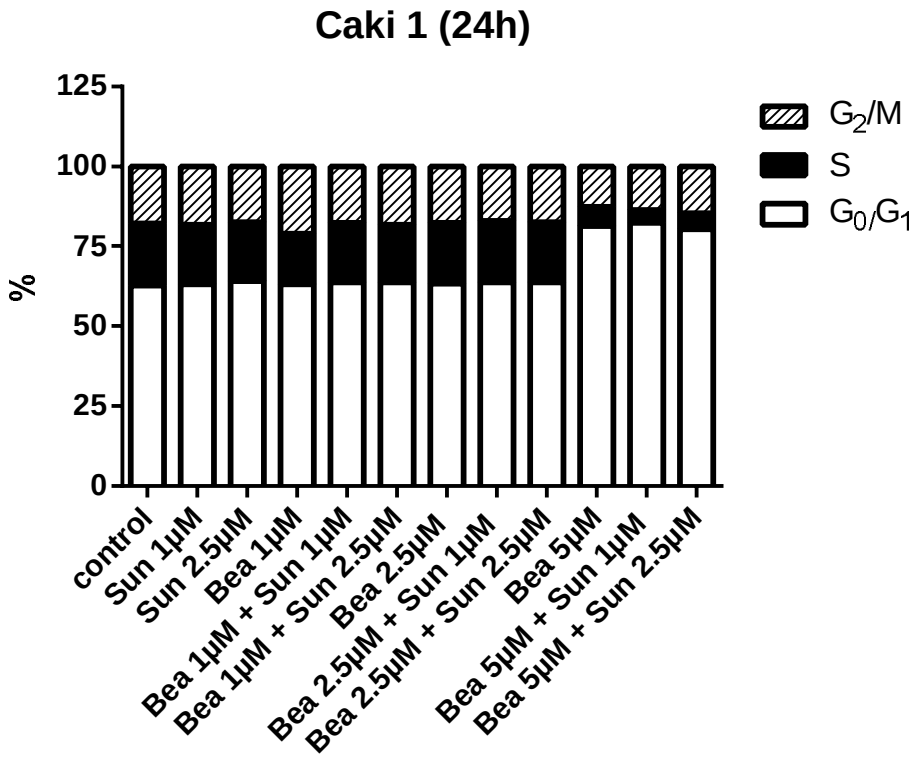

Figure 24: PI- staining of Caki 1 with Bea (1, 2.5, 5 µM) and Sun (1 and 2.5 µM)

5.3.1 JC-1 Staining

To further investigate the impact of Bea and Enn B either as single drug or in combination, apoptosis induction was examined. The JC-1 staining discriminates apoptotic and normal cells due to their mitochondrial membrane potential. As Caki 1 and KB-3-1 displayed an induction of cleaved PARP in the Western blot assay, these cell lines were used to gain more insights. Caki 1 was incubated with Bea (1, 2.5, 5 µM) and Sunitnib (1, 2.5 µM) for 24h. Since a former diploma thesis already showed synergistic effects of Bea with Sorafenib in KB-3-1 cells, Enn B (1, 2.5, 5 µM) and Sorafenib (1, 2.5 µM) were applied for 24h. Bea (5 µM) as single agent and in combination with Sunitinib (2.5 µM) led to a collapsed mitochondrial membrane potential, indicative for apoptotic cell death. Bea (5 µM) applied as single agent displayed a mitochondrial depolarization of 5.93%, compared to 2.87% in the control. Co-administration of Bea and Sunitinib even enhanced the effect leading to 10.44%. Further studies need to evaluate on which molecular processes the apoptosis induced by Bea is based on. Although, the cleavage of PARP was detected, this allows no detailed distinction, since this cleavage is performed only at a late stage of cell death.Still it is known that high concentrations of Bea and Enn B lead mainly to apoptosis through the intrinsic pathway. As a next step, proteins, which are part of the mitochondrial pathway, as the effector caspases 3 and 7, Bcl-2 and cytochrom need to be investigated.

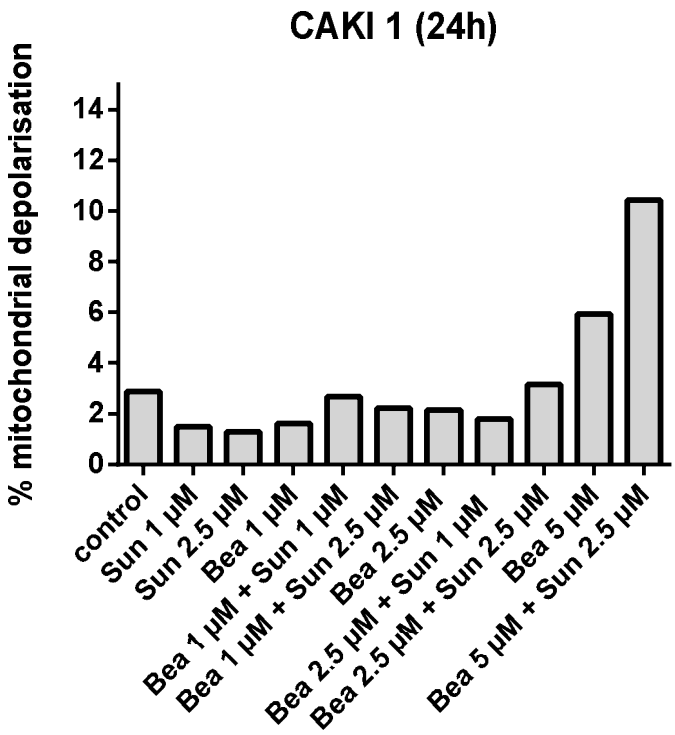

Figure 25: JC-1 staining of CAKI 1, with Bea (1, 2.5, 5 µM) and Sun (1, 2.5 µM)

5.4 Effect on cell migration and angiogenesis

Cell migration and angiogenesis are both controlled by VEGF and FGF, and therefore closely connected in terms of their regulation. Sorafenib and Sunitinib inhibit VEGF and show anti-angiogenetic features. [52] Former studies revealed that Beauvericin exibits anti-angiogenetic effects on prostate and breast cancer cell lines.[65]
In the following scratch and tube formation assays anti-angiogenetic and migration inhibiting features of Bea, Enn B, Sorafenib and Sunitinib were examined.

5.4.1 Scratch assay

The scratch assay is an *in-vitro* model for cell migration. The HUVEC cells (human umbilical vein endothelial cells) were incubated with Bea (2, 2.5, 3, 4 µM), Enn B (2, 2.5, 3, 4 µM), Sorafenib (0.01 µM) and Sunitinib (0.01 µM) as single agents and in combination. The applied sub-cytotoxic concentrations were obtained from outcomes of former viability tests with HUVEC cells. Before performing the scratch, 22 µl VEGF were added to each well. Two wells without VEGF worked as negative control. The cells were pictured right after the scratch (0h) and 15 hours later. After 15 hours the VEGF control showed an open area of 37.68% or was totally overgrown in the two experiments. The open areas of the treated wells and the control were calculated and compared to each other. The results showed a profound inhibition of migration by Bea (3, 4 µM), displaying an open area averaging about 53.37% and 57.24%. In combination with Sorafenib and Sunitinib synergistic effects were found, even increasing the open area up to 83.42% (Bea 3µM + Sor), 84.27% (Bea 4 µM + Sor), 92.06% (Bea 3 µM + Sun) and 93.28% (Bea 4 µM + Sun). Interestingly, Sunitinib seems to have slightly stronger anti-migration features than Sorafenib, displaying an open area of 78.46%, when applied as single agent. Sorafenib revealed an open area of 57.46%, when used as monotreatment. Moreover, Enn B (3, 4 µM) seems to have stronger anti-migration effects than Bea, showing an open area of about 75.00% and 78.47%. A combination of Enn B with Sorafenib or Sunitinib resulted in a slight enhancement of the open area averaging 83.73% (Enn B 3 µM + Sor), 86.90% (Enn B 4 µM + Sor), 88.36% (Enn B 3 µM + Sun) and 92.50% (Enn B 4 µM + Sun). Inhibitory effects on migration ability of Bea were already found in former studies. [65] Interestingly, minor changes in the amino acids of Bea weaken its antiangiogenetic potency. Whereas an introduction of two or three fluorine atomes onto the aromatic structure of the moieties strengthens the cytotoxic effect, but does not change the antimigration abilities. [97] As angiogenesis is crucial for tumor survival and growth and metastasis, it is essential to perform in-vitro tests, like the scratch assay. It is indispensable to predict the response to antiangiogenetic therapies. [98]

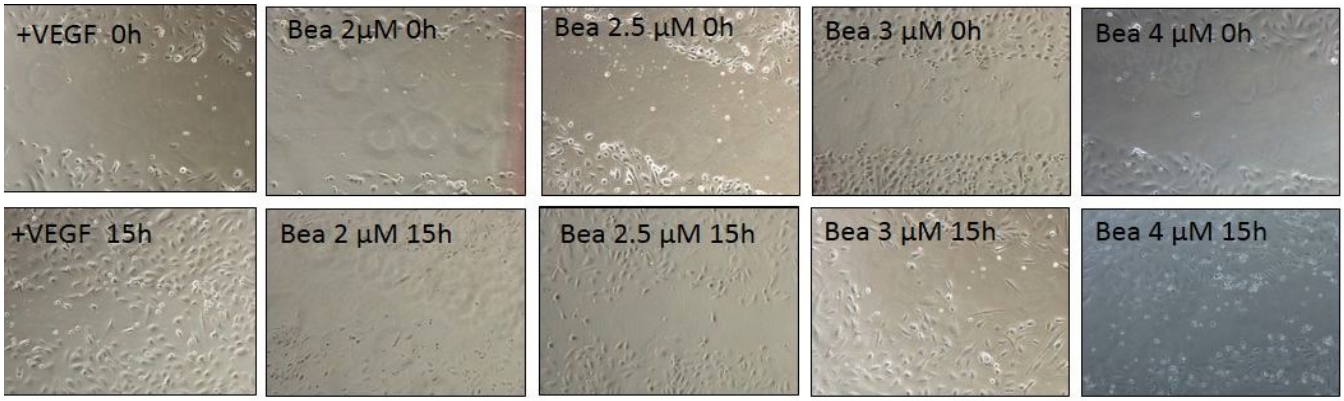

Figure 26:Scratch assay with control, Bea (2, 2.5, 3, 4 µM), 0 and 15 hours later

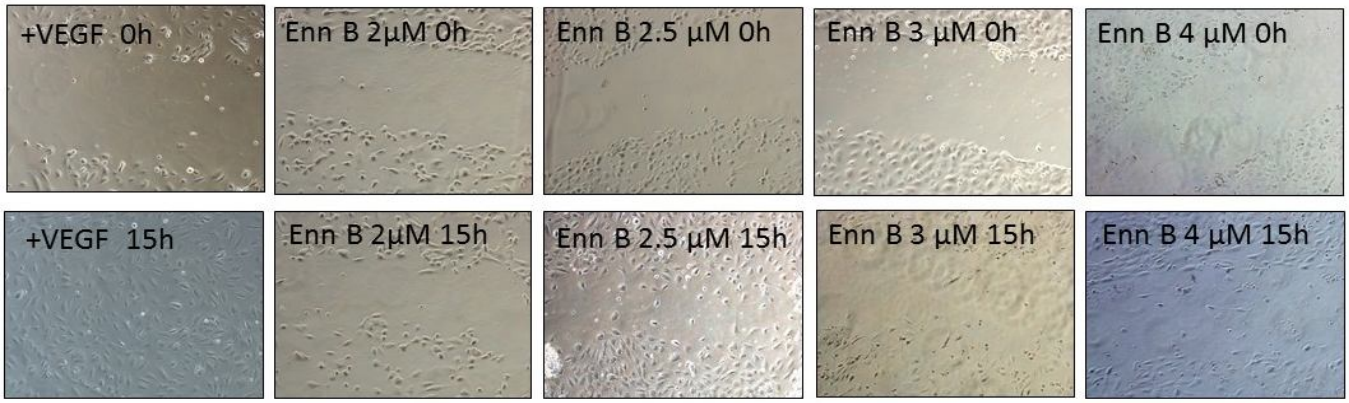

Figure26: Scratch assay with control, Enn B (2, 2.5, 3, 4 µM), 0 and 15h later

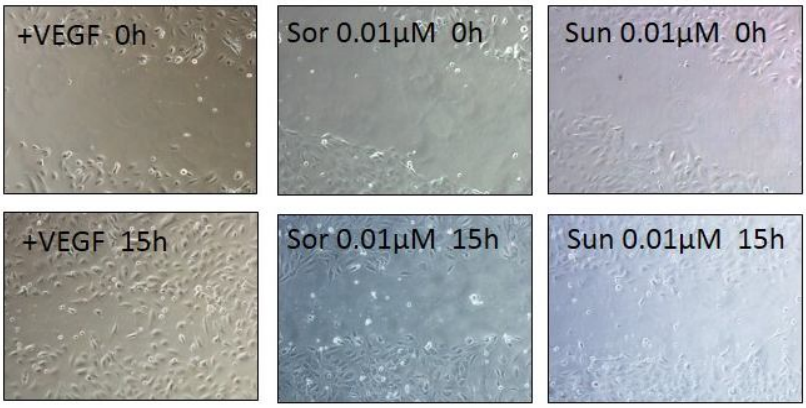

Figure27: scratch assay with control, Sor (0.01 µM) and Sun (0.01 µM), 0 and 15h later

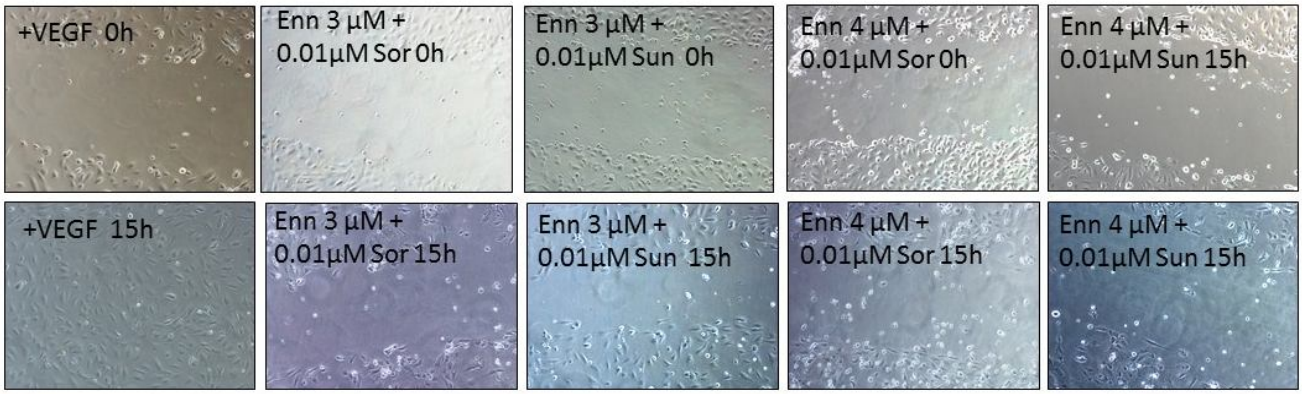

Figure 27: scratch assay with control, Enn B (3, 4 µM) in combination with Sor/Sun (0,01 µM), 0 and 15h later

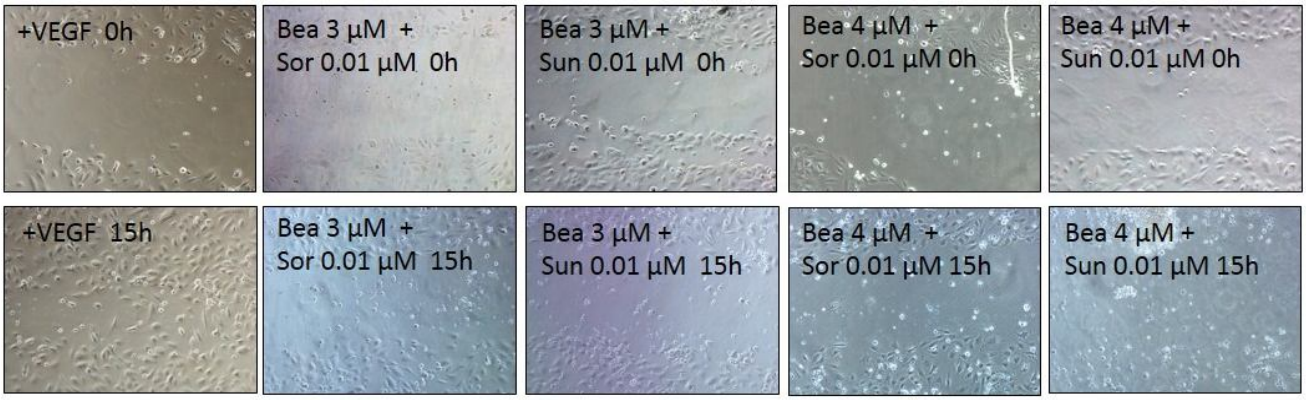

Figure27: scratch assay with control, Bea (3, 4 µM) in combination with Sor/ Sun (0,01 µM), 0 and 15h later

Sratch Assay HUVEC

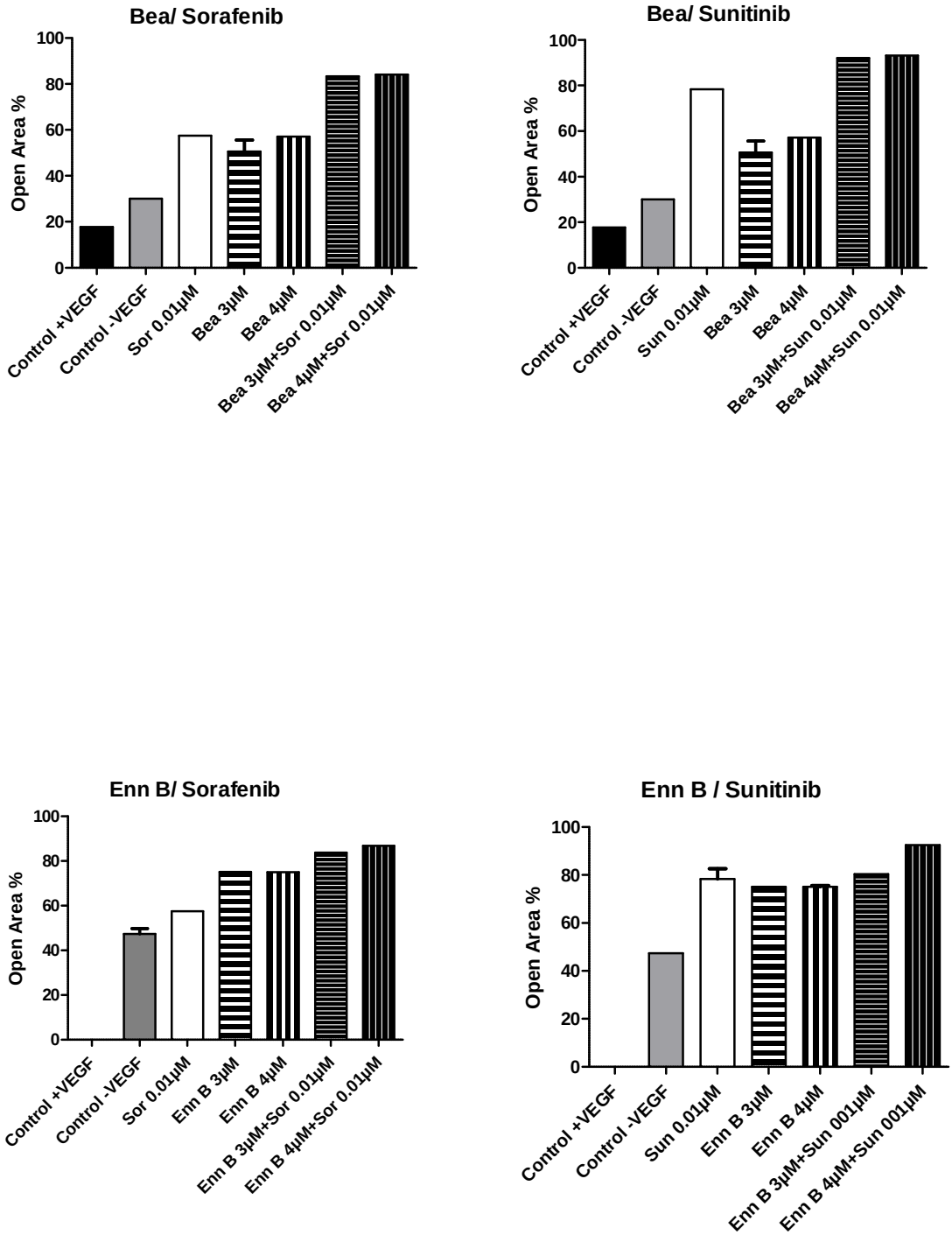

Figure39: Scratch assay of HUVEC, with +VEGF control, -VEGF control, Sor (0.01 µM), Sun (0,01 µM), Bea (3 µM), Bea (4 µM), Enn B (3 µM), Enn B (4 µM) as single agent and in combination

5.4.2 Tube formation assay

The Tube formation assay is a common *in-vitro* model to examine the reorganization stage of angiogenesis. HUVEC cells normally form capillary-like structures and branching networks when cultured in a three dimensional supportive matrix. Thus, it was examined whether Bea, Enn B and Sorafenib in monotreatment and in combination inhibit the tube formation in sub-cytotoxic concentrations. The cells were treated with Bea (2.5 µM), Enn B (2.5 µM) and Sorafenib (0.01 µM). Figure 29 reveals that both substances inhibit this key endothial characteristic. The effect was even enhanced when Bea and Enn B were administered concomitantly with Sorafenib. As a fact, a treatment of Bea as single drug reduceed the tube formation down to 44.03%. Enn B extered even stronger inhibitory effects on cell migration, leading to a reduction to 34.58%. The combination of Bea and Enn B with Sorafenib resulted in a reduction to 26.98 and 21.34% respectively. These outcomes indicate strong anti-migration effects of Bea and especially Enn B and synergistic enhancement with Sorafenib. An in-vitro angiogenesis inhibition may prove to be a promising opportunity for a future anti-cancer therapy.

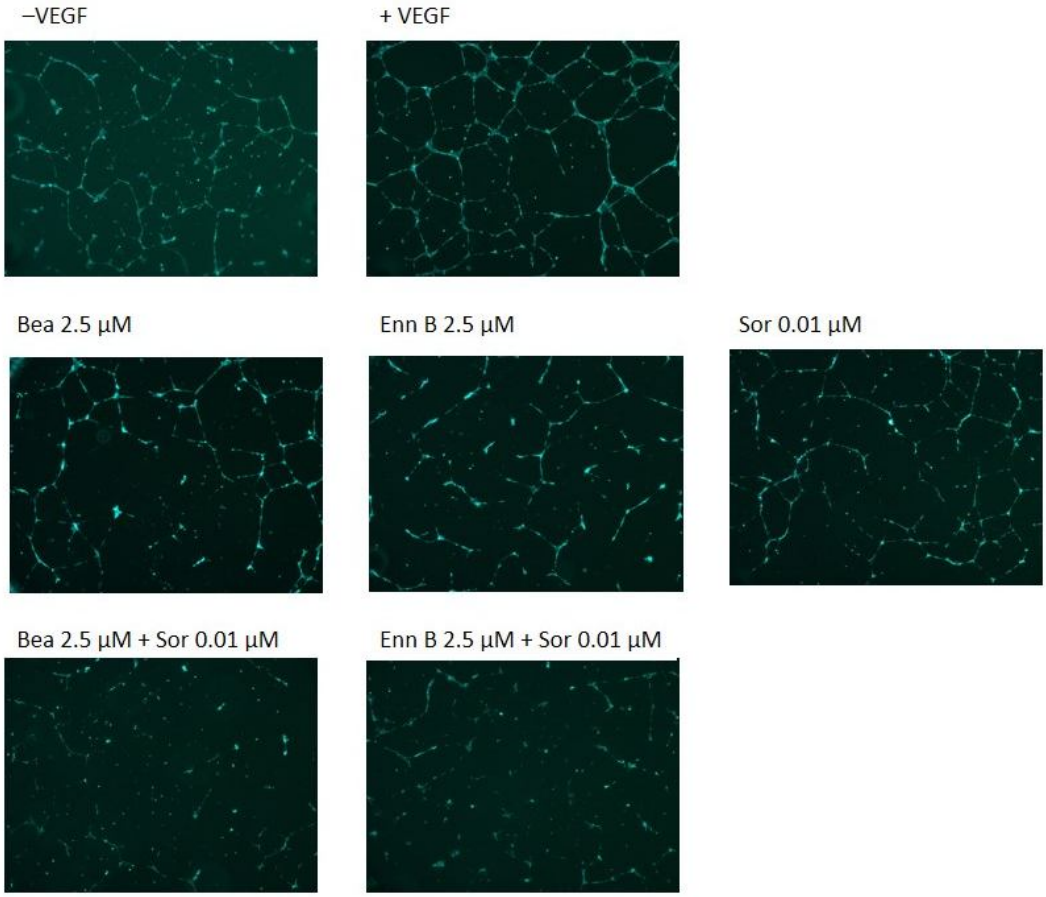

Figure 28: Tube formation assay with HUVEC cells

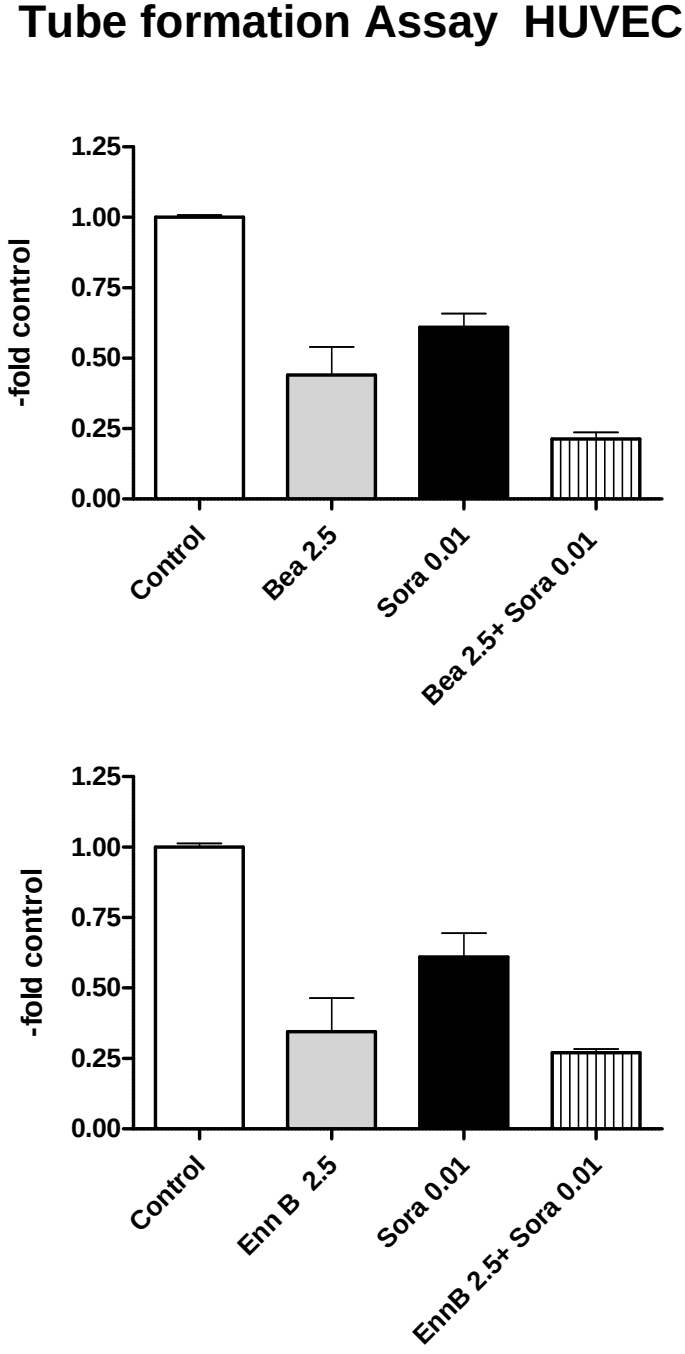

Figure 29: Tube formation assay of HUVEC, with Bea (2.5 µM), Enn B (2.5 µM) and Sorafenib (0.01 µM)

6. Summary

In conclusion, Bea and Enn B show cytotoxic characteristics in all used cell lines in a micromolar range. Bea seems to be more effective than Enn B in the viability assays. Moreover, a combination of Bea and Enn B with the protein kinase inhibitors Sorafenib and Sunitinib showed depending on the used concentrations and incubation periods antagonistic, additive and synergistic effects. To investigate the molecular mechanisms of Bea and Enn B as single agents and in combination Western blot analysis was performed. The results showed an interference with the ERK and p38, as well as the PI3K/Akt/mTor pathway, which are involved in cell proliferation and apoptosis.

Subsequent studies showed that the synergism is accompied by an increase in mitochondrial injury reflected by mitochondrial membrane depolarisation and cleavage of PARP. Interestingly, while Bea monotreatment led to cell cycle arrest in G_0/G_1-phase concomitant drug administration did not alter the observed cell cycle pattern of the monotreatment.

In addition, the JC-1 staining resulted in a collapsed mitchondrial membrane potential after applying Bea (5 µM). This effect was increased in combination with Sunitinib. As a final step the anti-angiogenetic and cell migration inhibiting features of Bea and Enn B as single drugs and in combination were tested. As a result, both substances showed marvellous effects in the scratch assay and the tube formation assay. However, the results indicated that Enn B reveals even stronger inhibitory features. Combination of the substances with Sorafenib and Sunitinib pronounced the inhibitory effect. Finally, it is to say that a combination of Bea and Enn B with Sorafenib and Sunitinib is a promising variant for cancer therapy, which needs to be corroborated in further studies.

5. List of Abbrevations

ABC	ATP-binding-cassette
ATP	Adenosintriphosphate
Bad	Bcl-2-associated death promoter (BAD)
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bea	Beauvericin
Bid	BH3 interacting-domain death agonist
Bim	Bcl-2-like protein 11
BRAF	breast cancer resistance protein
CD95	cluster of Differentation 95
CDC	cycline- CDK-complex
CDK	cycline dependent kinase
CHOP	C/homologous protein
C-RAF	RAF proto-oncogene serine/threonine-protein kinase
DAG	Diacylglycerol
DIABLO	second mitochondria-derived activator of caspases
DISC	death-inducing signaling complex
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor

eIF4E	Eukaryotic translation initiation factor 4E
EMEM	Eagle´s Minimum Essential Medium
Enn B	Enniatin B
ER	Endoplasmatic reticulum
ERAD	Endoplasmic-reticulum-associated protein degradation
Erk 1, 2	extracellular-signal-regulated kinases
Fas	Fas ligand or fas receptor
FGF	Fibroblast growth factors
FLT-3	Fms-like tyrosine kinase 3
Glut-1	Glucose transporter 1
HUVEC	human umbilical vein endothelial cells
IAP	Inhibitor of apoptosis
IP3	Inositol trisphosphate
IRE 1	FGF-1 internal ribosome entry site
JNK	c-Jun N-terminal kinases
KIT	tyrosine-protein kinase Kit
MAPK	Mitogen-activated protein kinases
MEK	Mitogen-activated protein kinase kinase
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide)
OXPHOS	mitochondrial oxidative phosphorylation
P53	tumor suppressor p53

PDGFR	Platelet-derived growth factor receptors
PERK	Eukaryotic translation initiation factor 2-alpha kinase 3
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKB	protein kinase B = Akt
PKC	protein kinase B
Raf	Rapidly Accelerated Fibrosarcoma
Ras	*p21/Ras protein*
RCC	Renal cell carcinoma
RET	*RET* proto-oncogene
RPMI	Roswell Park Memorial Institute medium
Smac	second mitochondria-derived activator of caspases
STAT	Signal Transducer and Activator of Transcription
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
TNF	Tumor necrosis factors
TRAF 1	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
UPR	unfolded protein response
VEGF	Vascular endothelial growth factor

6. Figure Legend

7. Table Legend

8. References

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2. p38, pp38 and β-Aktin were performed by Markus Zlesak
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