universität wien

DIPLOMARBEIT

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„Untersuchung der zytotoxischen Wirkung des Schimmelpilzmetaboliten

KR6-Synchromycin X“

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AIM OF WORK:

In December 2007, a rare fungal species of the genus Chrysosporium was rediscovered in the context of mycological researches. Extracts of this fungus were screened by Romer Labs Division Holding GMBH in Tulln for antibiotic effectiveness. In a further follow with the Department of Pharmacology and Toxicology at the University of Vienna, more than 300 extracts were tested for cytotoxic activity; some of them showed an antibiotic effect. Nearly 20 percent of these extracts were cytotoxic. KR6-SX is an isolated substance from one of these cytotoxic acting extracts.

As part of this work, the cytotoxic effect of KR6-SX in various cancer cell lines and non-cancer cell line was examined. Certain inhibitory concentrations were determined and tested whether the cells are able to recover after toxin contact. All examinations are designed to give first hints to maintain if KR6-SX could represent potential development candidates for a new chemotherapeutic agent against cancer.

INTRODUCTION:

Cell division and cell proliferation are essential processes in all living organisms. Many factors such as growth factors, growth inhibitors and cytokines regulate embryonic, organ development and other essential physiological processes as haematopoiesis and wound healing. The abnormal hyperplastic or neoplastic multiplication of normally non-dividing adult cells *in vivo* also plays an important role in atherosclerosis, tumorgenesis and other diseases. On the other side, apoptosis which is programmed cell death causes the balance between cell proliferation and cell death which is important in determining the cell number in tissues. Apoptosis is an energy dependent cell death and happens normally during development and aging process that occurs due to the activation of a group of cysteine proteases called "caspases" and a complex cascade of events that are involved in the mechanism of apoptosis (Mehmet and Hughes, 2005).

Too little or too much apoptosis leads to many human disorders such as neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer. A wide variety of physiological or pathological stimuli can trigger apoptosis. However, not all cells will die from the same stimulus; e.g. irradiation or drugs used for cancer chemotherapy results in DNA damage in some cells, which can lead to apoptotic death through a *p53*-dependent pathway (Elmore, 2007).

In some cases, the type of stimulus and/or the degree of stimulus determines if cells die by apoptosis or die by another alternative way to apoptosis which is called necrosis. For example at low doses of injurious stimuli such as heat, radiation, hypoxia or cytotoxic anticancer drugs can induce apoptosis but at higher doses of same stimuli can result in necrosis. Necrosis is a toxic process where the cell is a passive victim and follows an energy-independent mode of death.

Table 1 shows the difference in morphological changes that occur between apoptosis and necrosis (Elmore, 2007).

Apoptosis	Necrosis
Single cells or small clusters of cells	Often contiguous cells
Cell shrinkage and convolution, pyknosis and karyorrhexis	Cell swelling karyolysis, pyknosis, and karyorrhexis
Intact cell membrane	Disrupted cell membrane
Cytoplasm retained in apoptotic bodies	Cytoplasm released
No inflammation	Inflammation usually present

Table 1: Comparison of morphological features of apoptosis and necrosis

a) Mechanism of apoptosis:

The mechanism of apoptosis is highly sophisticated. Apoptosis has two main apoptotic pathways, the extrinisic or death receptor pathway and intrinsic or mitochondrial pathway. The two pathways are linked together as the molecules which are produced by one pathway may control the other pathway. There is an extra pathway that occurs by perform-granzyme dependent killing of the cell. In this pathway there are two types of granzyme: granzyme A and granzyme B. The granzyme A pathway is a caspase independent cell death through signal stranded DNA damage while the granzyme B pathway is a caspase dependent cell death that converge with the intrinsic and extrinsic pathways in the same terminal or execution pathway, These pathways activating caspase 3 and lead to DNA fragmentation, degradation of cytoskeletal and nuclear protein with formation of apoptotic bodies. Finally, there is expression of ligands for phagocytic cell receptors that engulf them (Martinvalet et al, 2005).

Caspase:

Caspases are inactive proenzymes which are present in cells. They are activated by other procaspases or can activate themselves. This activation leads to proteolytic cascade in which one caspase can activate other caspases and this amplifies the apoptotic signaling pathway that leads to rapid cell death.

Caspases are able to cleave proteins at aspartic acid residues; they have different specificities involving recognition adjacent amino acids.

Once caspase 8 is activated, the apoptotic cascade begins and it becomes irreversible. There are 10 major caspases that have been identified and are classified into initiators (caspase 2, 8, 9, 10), effectors or executioners (caspase 3, 6, 7), and inflammatory caspases (caspase 1, 4, 5). The other caspases, as caspase 11, are involved to regulate apoptosis in septic shock. Caspase 12 mediates endoplasmic specific apoptosis. Caspase 13 is suggested to be a bovine gene. Caspase 14 is present in embryonic tissues but not in adult tissues (Rai et al, 2005).

b) The extrinsic pathway:

It is the activation of a death receptor by binding of its specific ligand. Death receptors consist of extracellular and intercellular regions. The extracellular region consists of cystein-rich domains (CRDs), which are required for ligand binding. Intracellular region contains a death domain. This death domain plays a critical role in transmitting the death signal from the cell surface to the intracellular signaling pathway, including tumor necrosis factor receptor (TNF-R1), Fas-ligand, death receptor (DR), (Apo-3/TRAMP), DR4 (TRAIL-R1), DR5 (TRAIL-R2/TRICK2) and DR6. All these domains share a common structure for stimulating protein-protein interactions towards either apoptosis or inflammation (Brouckaert et al, 2005).

c) Perforin/ granzyme pathway:

T cell mediated cytotoxicity is a variant of type IV hypersensitivity, where sensitized CD8+cells kill antigen-bearing cells. These cytotoxic T lymphocytes (CTLS) are able to kill target cells, tumor cells and virus infected cells. Through a new pathway that involves secretion of transmembrane pore forming molecule perforin with a subsequent release of cytoplasmin granules through the pore into the target cell, the most important component in these granules are proteases granzyme A and granzyme B. Granzyme B acts either by activation of procaspase 10 or directly through execution phase of apoptosis. Granzyme A activates DNAse NM23-H1, a tumor suppressor gene product. DNAse has an important role in immune surveillance to prevent cancer through the induction of tumor cell apoptosis. Granzyme A causes cleavage of the nucleosome assembly protein (SET) complex which is important for DNA integrity, and leads to apoptosis (Elmore, 2007).

d) The Intrinisic pathway:

This pathway (Fig. 1) produces intracellular signals that act directly on mitochondria within the cell. Absence of certain growth factors or hormones can lead to apoptosis; on the other hand exposure to other stimuli as heat, hypoxia or toxins also leads to apoptosis. All of these stimuli cause opening in the inner mitochondrial permeability transition (MPT) pore. This leads to release of two main groups of normally sequestrated pro-apoptotic proteins into the cytosol. The first group is cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi; these proteins active pro-caspase 9, forming an apoptosome with subsequent apoptosis (Hill et al, 2004).

The second group of proapoptotic proteins is endonuclease G and caspase activated DNAse (CAD). These proteins are released from mitochondria during the late stage in apoptosis. Apoptosis inducing factor (AIF) translocates to the nucleus causing DNA fragmentation and chromatin condensation. Endonuclease G also translocates to the nucleus where it cleaves the nuclear chromatin to produce oligonucleosomal DNA fragments. CAD is subsequently released from mitochondria and translocates to the nucleus where it leads to more advanced chromatin condensation after cleavage by caspase 3 (Lie et al, 2001).

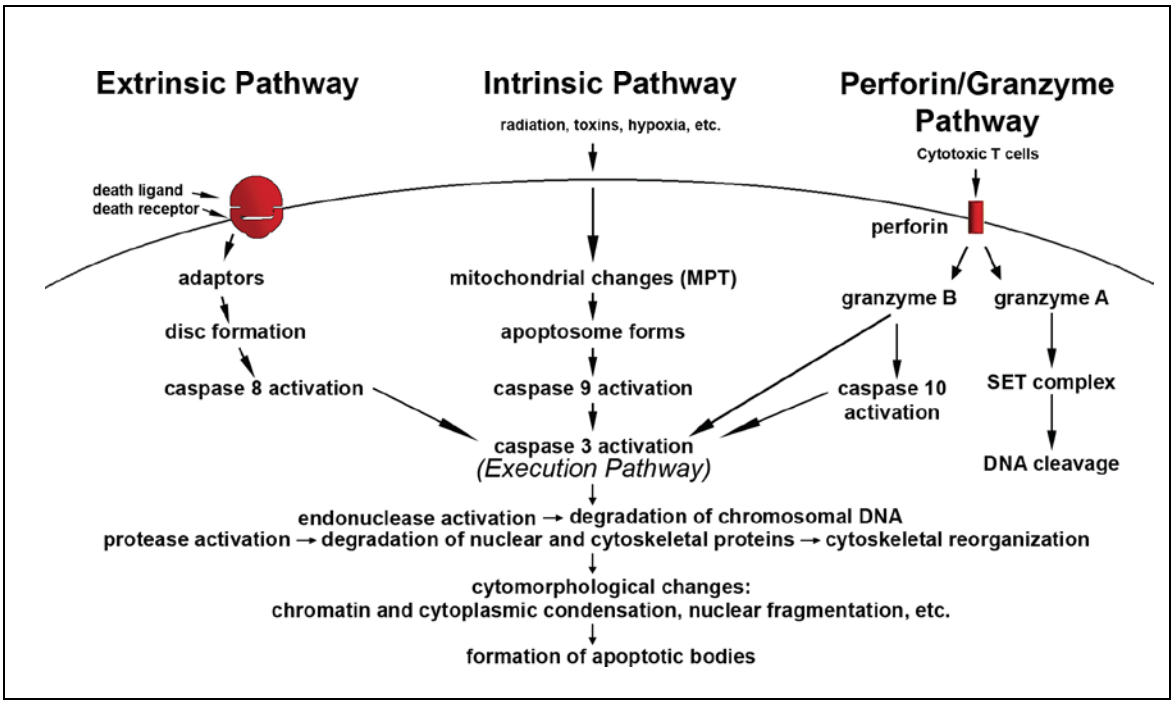

Figure 1: Mechanism of apoptosis showing three different pathways (Elmore, 2007).

Control of these apoptotic mitochondrial events occurs by members of the Bcl-2 family proteins which in turn is regulated by tumor suppressor protein p53 (Lie et al, 2001).

e) Bcl-2 family:

The Bcl-2 family (Fig. 2) consists of twenty various proteins called Bcl-2 homology domains (BH domains). They are structurally divided into pro-apoptotic domains which are multi-domains, e.g. Bax and Bak, and BH3 domain only proteins, e.g. Bad, BID and CI-I or anti-apoptotic proteins including Bcl-2 proper, Bcl-xL, and Bcl-w (Shangary et al, 2005)

Both types of proteins, anti-apoptotic and pro-apoptotic, regulate mitochondria dependent apoptosis. Keeping homeostasis in normal tissues needs a balance between the anti-apoptic and pro-apoptic Bcl2 family proteins.

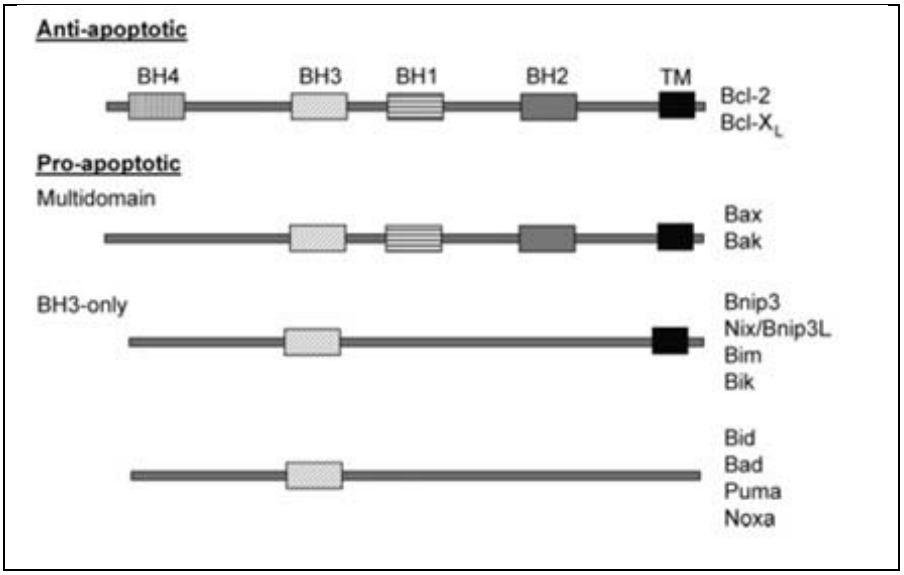

Figure 2: Domain structure of Bcl-2 family proteins. Bcl-2 homology (BH) and transmembrane (TM) domains are indicated (Gustaffson and Gottlieb, 2007)

When anti-apoptic Bcl-2 proteins are over-expressed, inhibition of apoptosis occurs. This is stimulated by various apoptosis stimuli including chemotherapy drugs, ɣ-radiation, FasL and TNF-α. When the cells are stressed, Bax and Bak transfer from the cytoplasm to the mitochondrial outer membrane and oligomerization occurs.

Oligomerized Bak and Bak insert themselves into the membrane producing cytochrome c. Bcl-2 binds selectively to Bax preventing its insertion into the mitochondrial membrane. Therefore, release of cytochrome c and other apoptosis activating proteins from the mitochondria, control the mitochondrial membrane permeability promotions or suppressions which determine the interaction of pro- and anti-apoptotic Bcl-2 family proteins (Yang, 2005).

f) Bax:

Bax is an agonist of apoptosis and is considered a member of the Bcl-2 family. Bax exerts two different mechanisms: Heterodimerization and activation of the permeability transition pore. These two mechanisms lead to stimulation of cytochrom c and cytosolic caspase. Bax synthesis occurs in neurons and is increased by activation of glucocorticoid receptors and also leads to apoptosis in the dentate granular cell population. There is a theory that recombinant Bax protein, that is added to isolate mitochondria, is used to stimulate the release of cytochrom c and activation of cytosolic caspases (Van Lavker, 2006)

g) p53 and p21 are regulators in apoptosis and cell cycle:

Cellular stress is a term covering a wide range of molecular changes that occur in the cells in response to environmental stress, e.g. extremes of temperature exposure, toxins or mechanical damage. This stress can lead to apoptosis or cell cycle arrest (Fig. 3) (Garetel and Tymer, 2002).

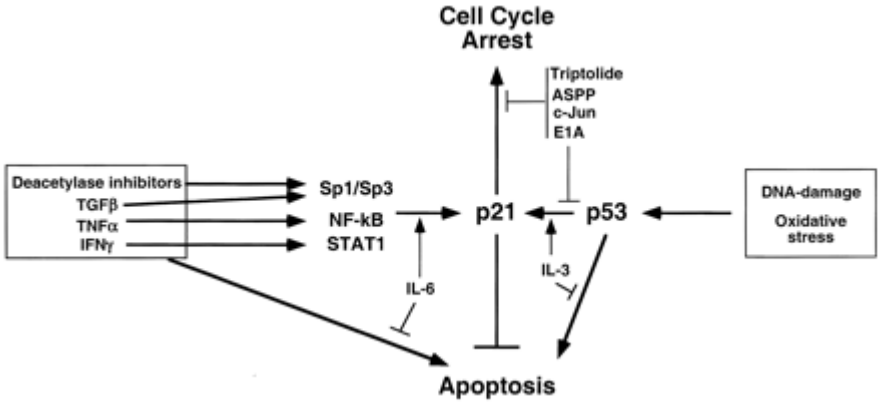

Figure 3: Mechanism of action of p21 in cell cycle arrest (Gartel and Tymer 2002).

h) Mechanism of p21 in apoptosis:

The cyclin dependent kinase inhibitor p21 is activated by p53 and other factors following cellular stress. The activation of p21 might cause cell cycle arrest (Fig. 4). Thus, it may play acrucial role in preventing tumor development. A number of recent studies described that p21 can act also as inhibitor of apoptosis in anumber of systems and this might counteract its tumor suppressive function as a growth inhibitor (Gartel and Tymer 2002).

p21 protein acts as a protective binding protein agent for the cells from genotoxic and other cellular stresses. After DNA damage, p53 stimulates the p21 gene and an increased level of p21 protein occurs, thus protecting the cells from apoptosis by arresting the cell cycle and as well allowing DNA repair. It also acts as an anti-apoptotic component (Schwab, 2008).

The cell cycle arrest allows cells to pause and repair cell nuclear damage, and then to continue cell division. Therefore, p21 can protect cells from stress induced apoptosis and in the same time can act as a tumor suppressor (Gartel and Tyner, 2002).

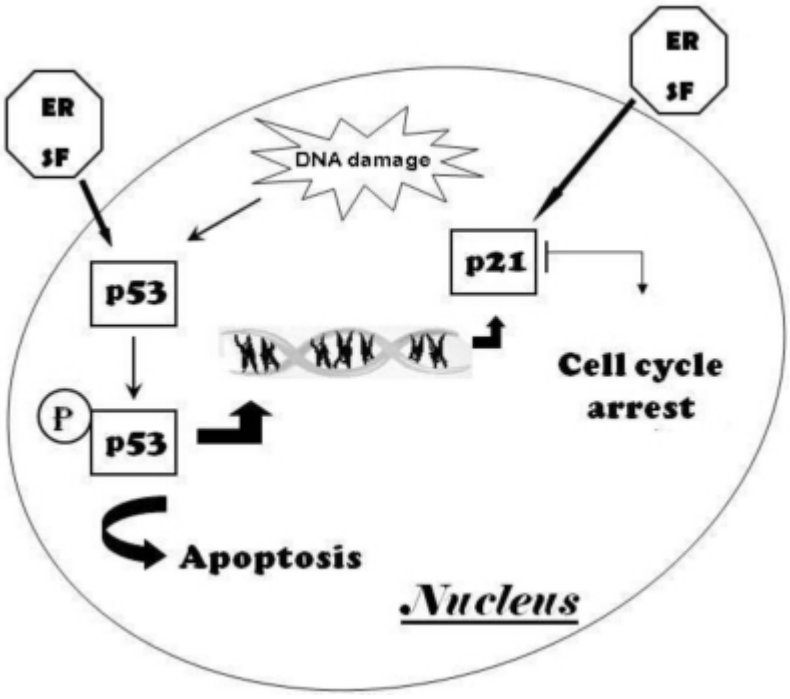

Figure 4: Role of p21 in the cell arrest (Melchini and Traka, 2010)

i) Mechanism of action of p53 in apoptosis:

In 1979 p53 protein was discovered by Arnold Levine, David Lane and William Old. It was believed for more than 10 years that it was an oncogene.

Vogelstein and others described in 1989 that p53 is a tumor suppressor gene and its most important function is destruction of abnormal cells. p53 also participates and controls many functions of the cell cycle proliferation, growth and death. Thus, it was given the name “guardian of the genome”.

There are genes activated by p53 protein, which can be regulated either up or down and act in a process of apoptosis. The up-regulated genes are p53 apoptosis effector, related to PMP (PERP), p53 induced protein with a death domain (PIDD), p53 dependent damage-inducible nuclear protein (p53 DINP1) and p53 apoptosis including protein1 (p53 AIP1). All of these genes can stimulate apoptosis. p53 is a phosphatase and tensin homologue (PTEN). A down regulator gene of the phosphatidylinositol-3-kinase (PI3-kinase) - AKT/PKB - is an example of a gene that stimulates apoptosis. This pathway regulates different cellular processes such as proliferation, growth and apoptosis. The most important function of p53 is binding to DNA causing subsequent trans-activation of the pro-apoptotic genes. However, some cells having p53 mutants are unable to cause p53 dependent apoptosis and these mutants are also unable to induce an effect at the level of mitochondria (Rivera-Malo, 2006).

j) Regulation of p53:

p53 is induced by a number of stress factors, e.g. DNA damage, presence of short single strands of DNA, ionizing radiation, hypoxia, changes in redox conditions, oncogene activation, telomere shortening and ligand binding, resulting in a significant role in up-regulating many genes. p53 is an unstable protein with a half-life of less than 30 minutes, which is controlled by its degradation induced by Mdm2 tumors. Regulating the stability and transcriptional activity of p53 plays a significant role in its post transcriptional modification. The positive or negative ability for p53 to DNA binding have an effect on these changes which can occur affecting other regions of p53 and clear differences in response to different agents (Rivera- Malo, 2006).

k) The p53 network in apoptosis:

The p53 tumor suppressor gene is stimulated by exposure to the cellular stress leading to growth arrest or apoptosis (Fig. 5). The choice between cell growth arrest and apoptosis (cellular responses) is affected by various factors including the type of the cell and stress, and the action of p53 co-activators. p53 induces a wide network of signals that acts through major apoptotic pathways. The extrinisic death receptor pathway initiates the stimulation of a caspase-cascade, and the intrinsic which is the mitochondrial pathway changes the balance in the Bcl-2 family to the pro-apoptotic membranes, stimulating the induction of the apoptosome and afterwards the caspase mediated apoptosis. The reactions of the two apoptotic pathways may be induced through the p53 specific apoptotic target genes and stimulate most of the apoptotic effect. Under specific conditions, p53 can induce apoptosis by a transcription mechanism. Therefore, many mechanisms are controlled by p53 to ensure satisfactory stimulation of apoptosis specialized manner. Thus, an attractive target for cancer therapy is constituted by manipulation of the apoptotic functions of p53 (Haupt, 2003).

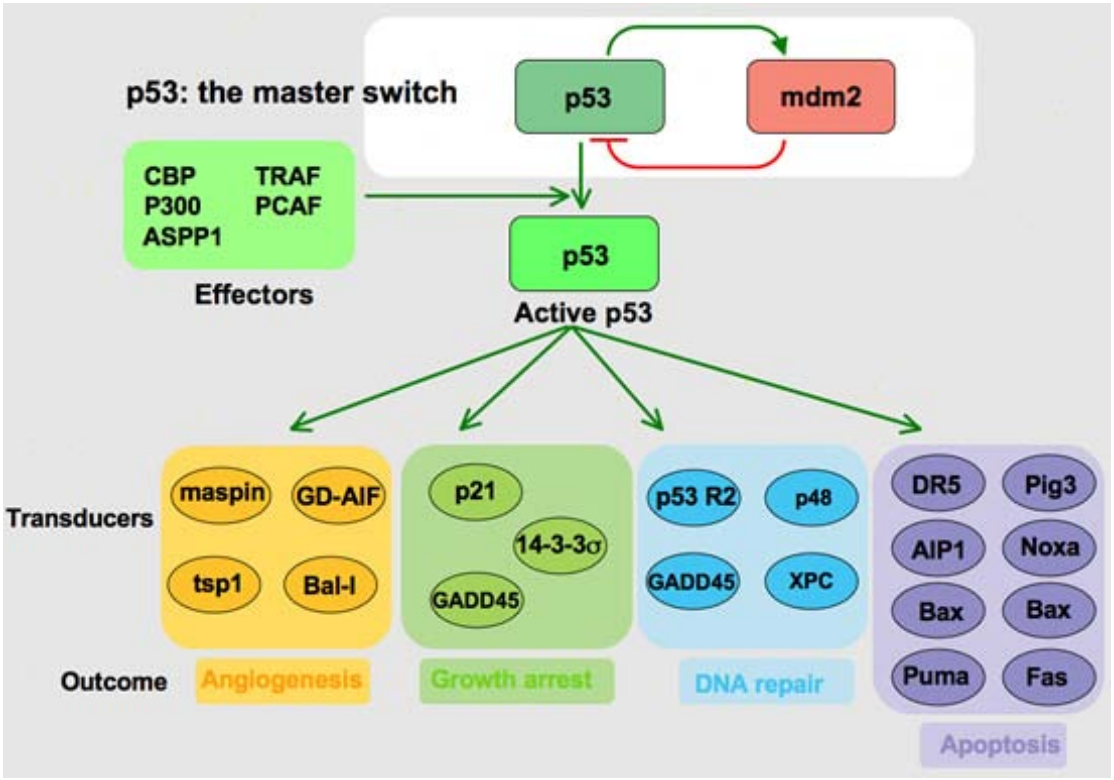

Figure 5: p53 pathways (Levine et al, 2006).

MATERIAL AND METHODS:

a) The test substance:

The test substance KR6-SX was provided by Dr. Roman Labuda (Romer Labs Division Holding GmbH; 3430 Tulln, Austria). KR6-SX was dissolved in DMSO at the desired concentrations for the attempt to obtain appropriately diluted with the nutrient medium. All the tests were conducted with a suspension. Therefore, the liquid dilution had been done before each dilution, and in addition always had to be well mixed to insure an equal distribution of KR6-SX. There were 8 different concentrations used (Table 2).

concentrations used of KR6sx									
µM	0,0001	0,0003	0,001	0,003	0,01	0,03	0,1	0,3	1

Table 2: overview of used concentrations of KR6sx

b) Cell lines and associated culture media:

The toxicity of the substance was tested on four cancer cell lines and on one non-cancer cell line.

c) Cancer cell lines:

- HCT116: Colon carcinoma cells (provided by Dr. Vogelstein, John Hopkins Baltimore University)

and the HCT116 cell line with homozygous deleted p53, p21, or bax gene:

- HCT116 p53/Ko
- HCT116 p21/Ko
- HCT116 bax/Ko

d) Non-cancer cell line:

- HEK-293: Human embryonic kidney cells (from ATCC)

e) Culture media:

The HCT116 cell lines were cultured using McCoy medium (Gibco®), to which 10% fetal bovine serum and 1% penicillin-streptomycin was added.

The HEK cells were cultured using DMEM/F12 (1x) (Gibco®), to which 10% fetal bovine serum and 1% penicillin-streptomycin was added.

f) Cultivating the cells:

General Information:

The cells were cultured in the incubator at 37°C (5% CO_2) in 25 cm^2 and 75 cm^2 culture shallow neck angle flasks. The small bottle was filled with culture medium up to 5 ml, the larger up to 10 ml. The culture medium was previously heated to 37°C in the heating bath.

All cell lines grew adherent, which means that the floating cells in the culture medium are deposited with time and stick onto the bottom of the culture vessel, where they grow and multiply. Dead cells become detached from the ground and float freely in the medium.

g) Thaw the cells:

The various cell lines were frozen in nitrogen liquid and thawed when needed. Each cell line was located in a separate Eppendorf tube.

The respective tube was removed from the nitrogen container and has been warmed briefly on the water bath until the contents liquefied. The cell suspension was removed by a disposable pipette and transferred into a previously labelled culture flask with a suitable nutrient medium. Then the flask was incubated at 37°C. The next day, DMSO was removed which was added to the cells before freezing, and exchanged by culture medium,.

h) Change of the culture medium:

The ingredients of the culture medium in the course of time, are metabolized by the cells, and therefore had to be changed 1-2 times per week.

For this purpose the old culture medium was aspirated and replaced with fresh medium. The dead cells which are floating freely in the culture medium were also already removed. However, the living cells remained in the culture flask, as they adhered to the ground.

i) The Passage:

As the cells continuously proliferate in the culture flask, after a few days the whole bottom of the flask is confluent. Under these conditions, the cells cannot proliferate anymore and die. Therefore, the cells were controlled every day under the microscope. As soon as the ground was covered with approximately 90%, a portion of the cells was removed and transferred to a new culture flask. This process is called passenger operation. The number of passages was listed on the culture vessels.

In order to remove the cells, they must first detach from the bottom of the culture flask. This was achieved by the addition of trypsin (trypsin/0.53 µM EDTA, vitalcell®). The culture medium was removed from the culture flask and the remaining medium was washed out by some trypsin / EDTA mixture, which was sucked off again after a short swing immediately. Then again, trypsin was added (700 µl for 25 cm^2 culture flasks, 2 ml for culture flasks of 75 cm^2), and depending on the cell line cells were incubated for 2 to 10 minutes until all the cells were detached from ground. For this purpose, the culture vessel was taken every 1 to 2 minutes from the incubator and checked under the microscope. Then the action of trypsin was stopped, by 2 to 4 ml of culture medium which was added by pipette. It is important that the cells are not incubated with trypsin too long, because it is cytotoxic and cells would die.

The cell suspension thus obtained was transferred into a part of a fresh culture vessel, and the vessel was filled with 5 or 10 ml fresh medium. This process is called splitting. The new passage thus obtained was further cultured in the incubator. The unneeded cell suspension was aspirated and discarded to the old culture flask.

j) Determination of cell number:

Insertion of the cells into the microliter plates requires knowledge of the cell number per ml of cell suspension.

The cell suspension obtained by trypsinization was transferred into a centrifuge tube and centrifuged for 5 min at 1000 U/min to remove the trypsin by subsequent aspiration of the supernatant. To the obtained cell pellet, about 5 ml of medium were added with a pipette and suspended by vortexing the cells evenly.

To determine the cell count, 10 µl of suspension were then removed, mixed with 10 µl trypan blue (Trypan Blue solution 0.4; SIGMA®) and then 10 µl were transferred under the cover of a Bauer counting chamber (Fig. 6). The addition of trypan blue was used to stain dead cells. The count was done under the microscope:

The chamber provides a large network of four squares, in order to facilitate counting. One square again is divided into 16 smaller squares (Fig. 7). Living cells are seen as bright spots on a dark background. Blue dead cells were not counted.

The cells which are accurately located on an edge line of a square were not double counted, only the dots were observed per field in the left and top border line. Cells in each of the four large squares were counted separately, the numbers of cells was recorded, and then the average of the four values were calculated (Table 3).

As the cell suspension was pre-diluted 1:1 with trypan blue, the average has been multiplied by the factor 2. The product obtained was again multiplied by 10^4. The result is the number of cells per ml of cell suspension.

Figure 6: Counting and determining the viability of cultured cells

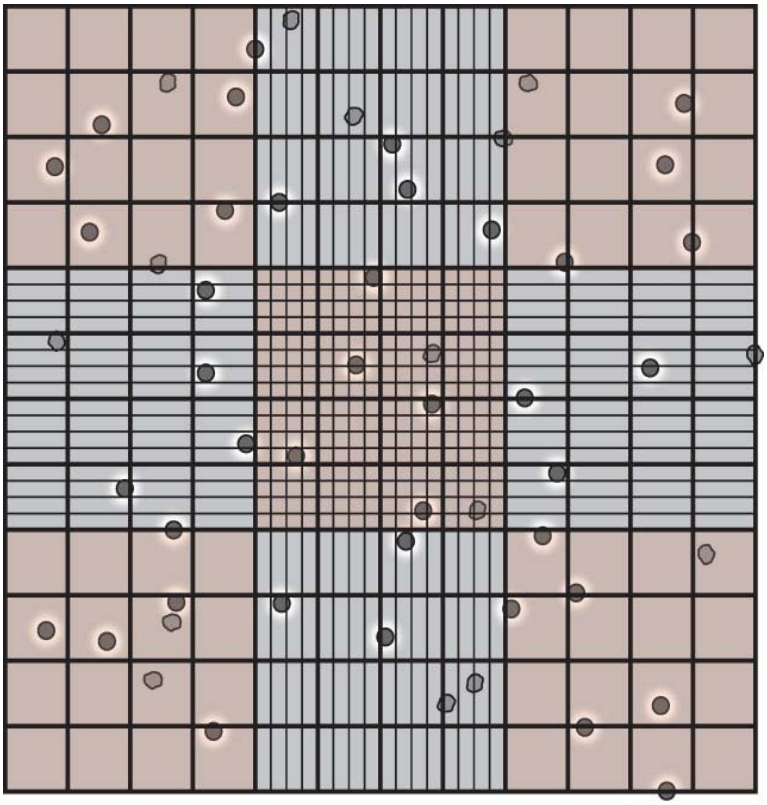

Figure 7: The hemocytometer (counting chamber)

Example:

	Cell number:	**Average:**	**Dilution factor:**	**Result:**
1. square	**80**	**85**	**× 2**	**170×10^4**
2. square	**88**			
3. square	**90**			
4. square	**82**			

Table 3: Calculation of cell number per ml of cell suspension

k) EZ4U-cytotoxicity test:

Test principle:

Living cells have the ability to reduce tetrazolium salts to red-coloured formazan derivatives (Fig. 8). Depending on the intensity of the color, the proportion of living and dead cells therefore can be determined [Text EZ4U "Easy for you" nonradioactive cell proliferation and cytotoxicity assay; Biomedica Medical Products GmbH & co KG].

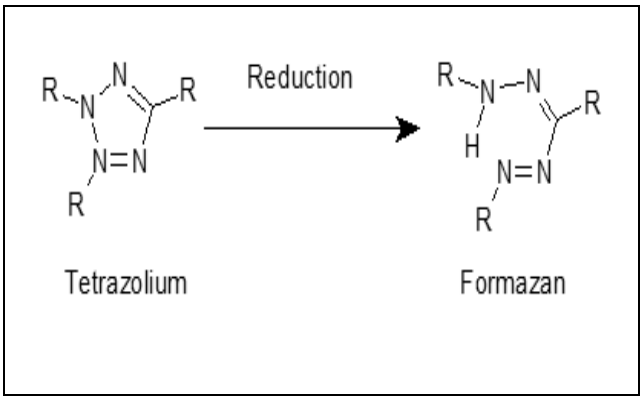

Figure 8: Tetrazolium→Formazan

Implementation:

Attaching the cells to 96-well plates:

After determination of the number of cells, the cell suspension was diluted to the desired concentration (Table 3) and 100µl were transferred into each well of the set. The edge of the plate was filled with pure medium in order to ensure, that all the cells are cultured under the same condition. The plates were then incubated for one day, until the cells have settled to the bottom.

		cell lines	
		HCT 116	HEK
Incubation period (h)	4, 8, 16	5×10^4 cells/ ml	1×10^5 cells / ml
	24, 48, 72	2×10^4 cells /ml	5×10^4 cells / ml

Table 4: Cell number/ml as a function of incubation time and the respective cell line

Addition of the test substance:

The toxin was diluted with medium to the desired concentrations.

There were three wells filled with the same concentration of toxin. Per well 100 µl were added. In the first well row only pure medium was pipetted to obtain a control value. The plates were incubated and then evaluated after the desired incubation period.

l) Analysis using EZ4U-Test:

The EZ4U-substrate was first dissolved in 2.5 ml of the activator and then diluted 1:10 with nutrient medium. The medium in the plates was aspirated and 100 µl of this mixture per well were added. There were 3 wells where there was no cell performed. The plates were then incubated for 1.5 to 3 hours. Once the control was stained brick red, the plates were analyzed using a photometer.

The absorbance of the individual wells was measured at 450 nm (with 620 nm as reference wavelength).

m) Calculation:

From the three sets corresponding wells, the means were calculated. From these sets the blank value was then subtracted. The resulting values were converted to percent, the control was considered as a base line (=100%). The result describes how much percent of the cells survive.

The calculations and creating the graphics were performed using Excel and Sigma plot.

RESULTS AND DISCUSSION:

The cells were exposed to various concentrations of KR6-SX and certain percentage of survivors was detected by incubation. It was tested to determine the cytotoxic effect on the cell.

a) Cytotoxicity on HEK cells:

In the human embryonic kidney cell line HEK, with the smallest concentration of 0.0003 µM strongly proliferating cells can be seen, with an increase in the number of living cells after 8 hours till 120% and after 48 hours till 123% (Fig. 9). As the concentration increased, the number of the dead cells exposed to KR6-SX increased. At a concentration of 0.03 µM the number of living cells reached 24% after 48 and 72 hours of incubation. Approximately all the cells were killed by KR6-SX at a concentration of 0.3 µM with an incubation period of 24 hours. At a concentration of 1 µM and an incubation period of 8 hours all the cells were dead.

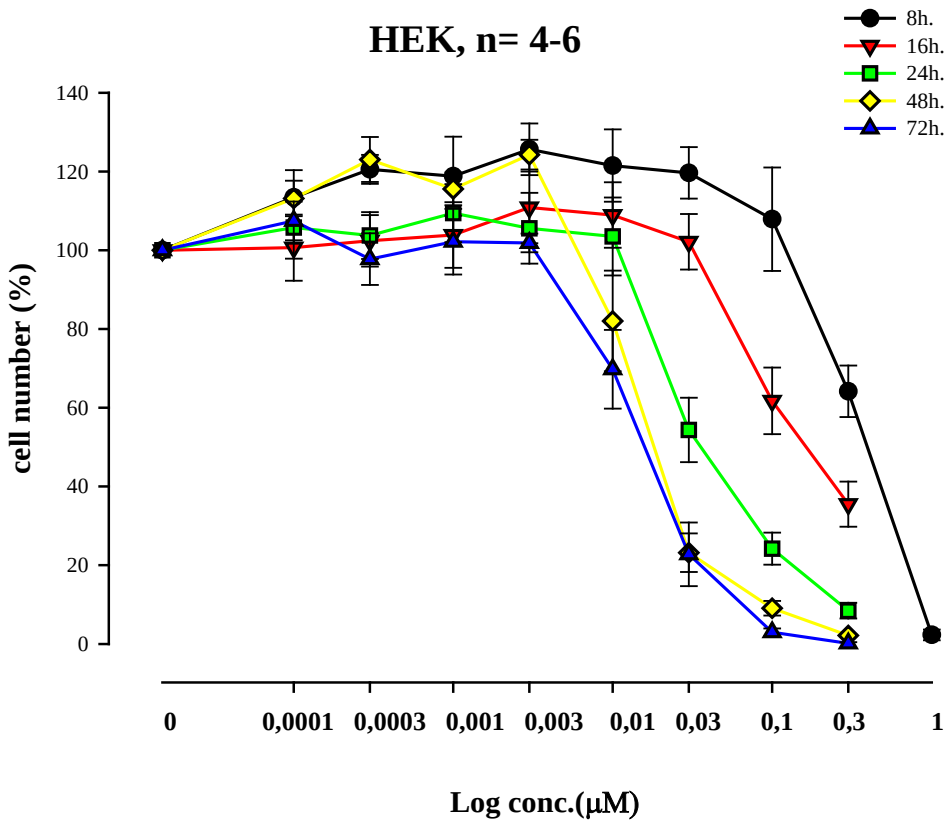

Figure 9: Cytotoxicity on HEK cells in response to the concentration of KR6-SX and duration of exposure.

b) **Cytotoxicity on HCT 116 cells:**

In cancer colon cells the cytotoxicity was highly dependent on the duration of exposure to KR6-SX. For example, after 8 hours of incubation at the lowest concentration of 0.0001 µM the cell number increased to more than 100% (Fig. 10). When increasing the concentration the cell number decreased. After 16 hours of incubation, at the concentration 0.01 µM the cell number was 80% but by increasing the concentration till 0,3 µM the percentage of the cell number decreased to 10%, and this occurred also after 24 hours of incubation. Increasing the duration of exposure of KR6-SX decreased the survival rate of the cells to zero %. At a concentration of 0.3 µM all cells were killed by KR6-SX after 48 and 72 hours of incubation.

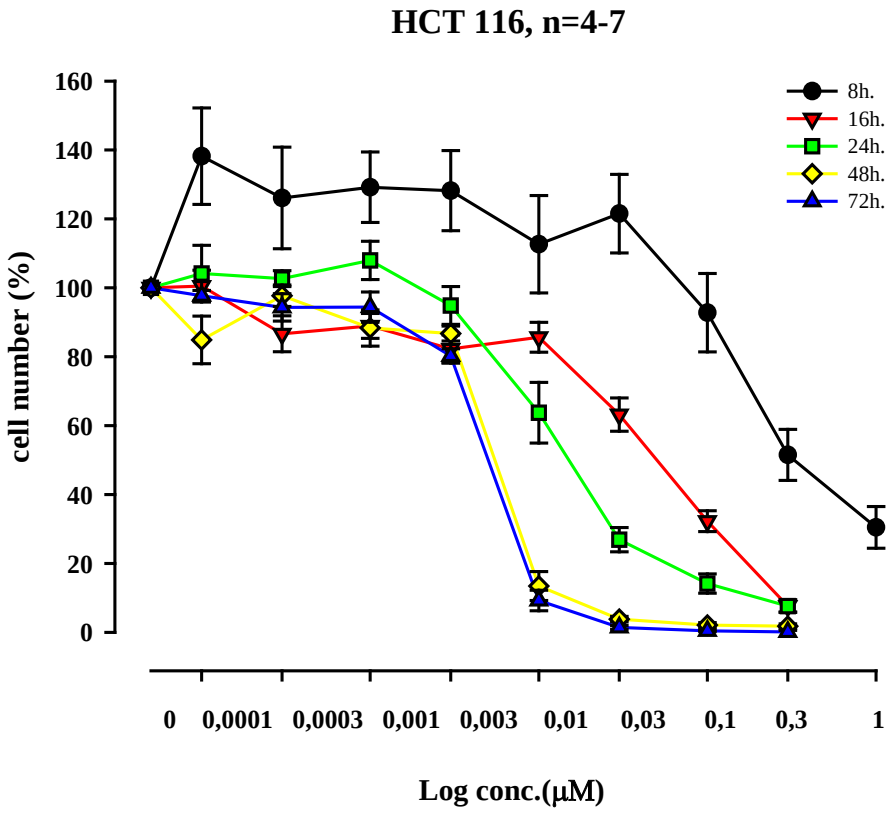

Figure 10: Cytotoxicity on HCT116 cells in response to the concentration of KR6-SX and duration of exposure.

c) **Cytotoxicity on HCT 116 p21/ko, HCT 116 p53/ko and HCT 116 bax/ko cells:**

Figures 11, 12 and 13 demonstrate the concentration- and incubation time-dependent effect of KR6-SX in HCT 116 p21, p53 and bax knock-out cells.

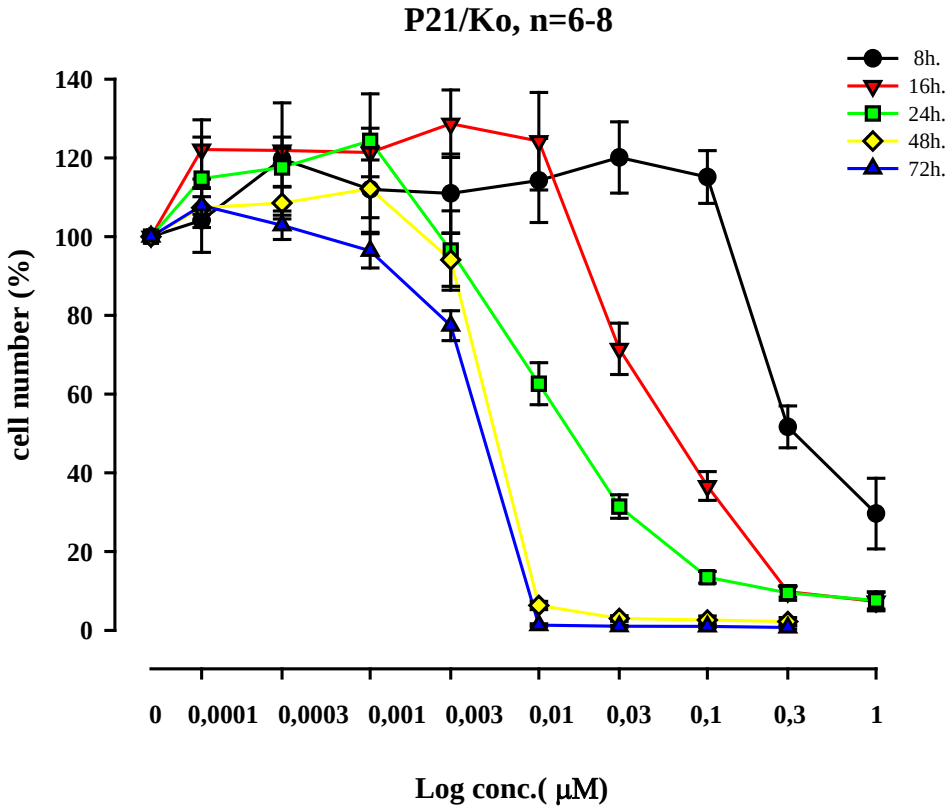

Figure 11: Cytotoxicity on HCT116 p21/ko cells in response to KR6-SX and duration of exposure

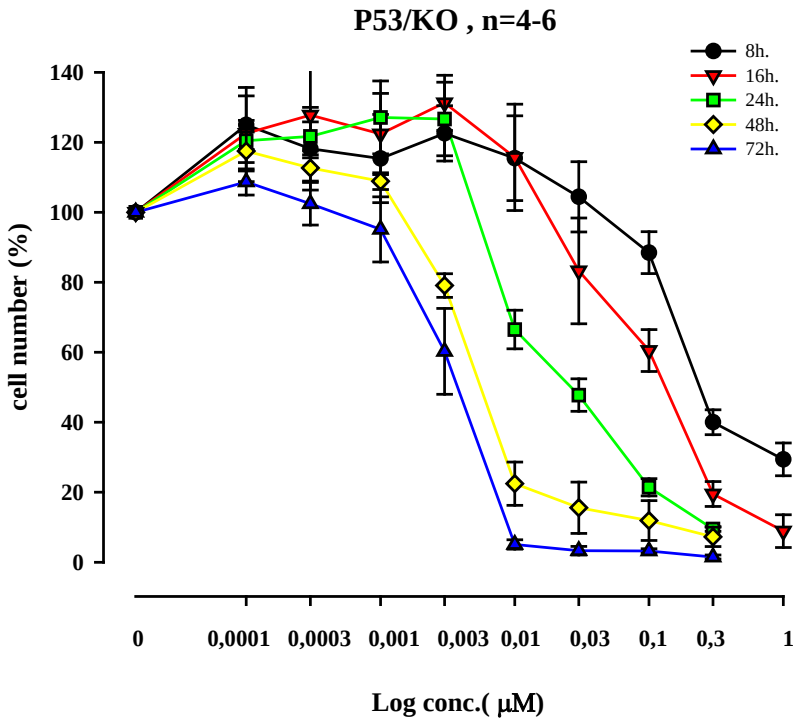

Figure 12: Cytotoxicity on HCT116 p53/ko cells in response to the concentration of KR6-SX and duration of exposure

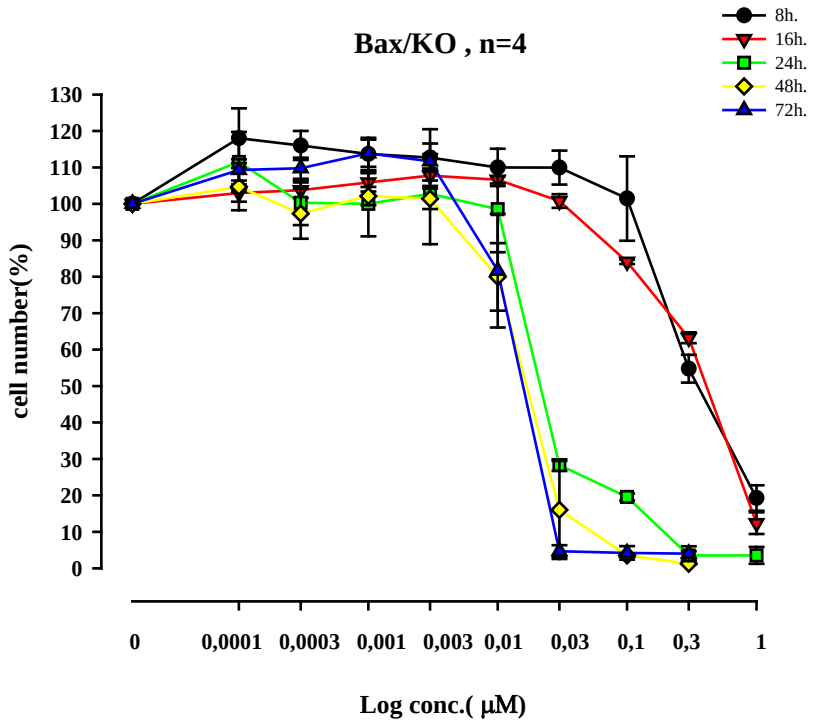

Figure 13: Cytotoxicity on HCT116 bax/ko cells in response to the concentration of KR6-SX and duration of exposure.

d) <u>Comparison of cytotoxicity between the HCT 116 mother cell line and sub cell lines at different incubation times:</u>

For better comparability, the dose-dependent effect curves of each HCT116 cell line were illustrated for incubation periods of 16 (Fig. 14), 24 (Fig. 15), 48 (Fig. 16) and 72 hours (Fig. 17). It was shown that bax and p53 play a role in apoptosis caused by KR6-SX at short incubation times. However, at prolonged exposure to KR6-SX only bax had an influence on cytotoxicty.

Incubation time 16h

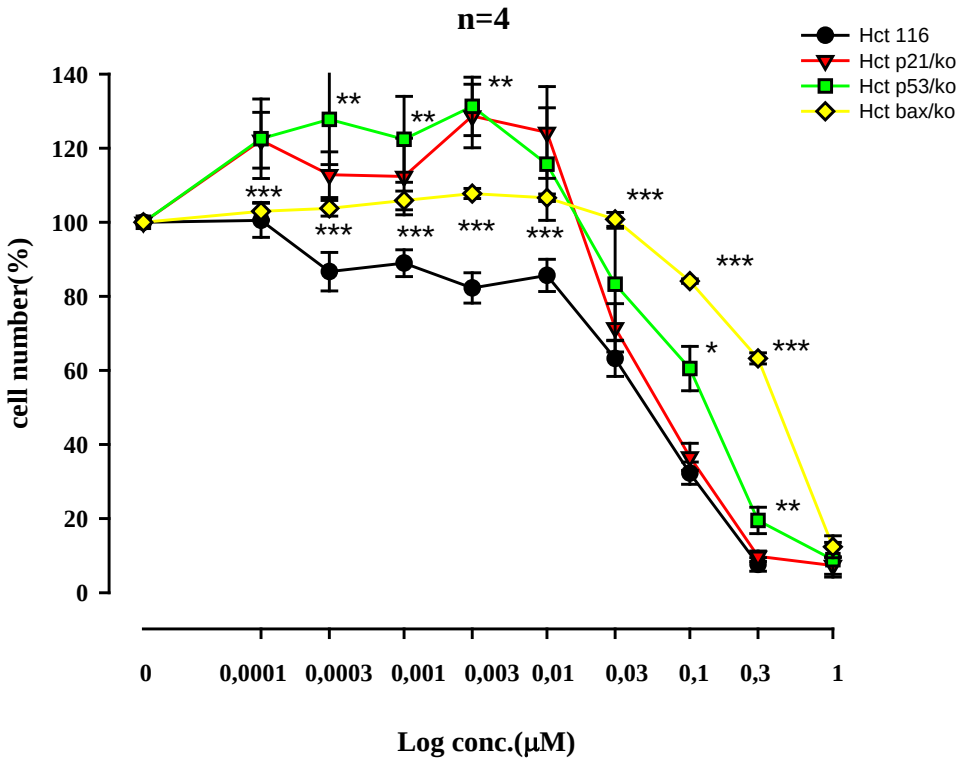

Figure 14: Comparison between the different HCT116 cell lines after 16 hours of exposure and increasing concentrations of KR6-SX.

Symbols: *****..... statistically significant ($p<0.05$)
******… statistically significant ($p<0.01$)
*******... highly statistically significant ($p<0.001$)

Comparison between the mother cell line and the sub cell lines showed statistically significant differences in sensitivity to KR6-SX, which was always taken in relation to the mother cell line.

After 16 hours of exposure, a significant difference in the sensitivity of different cell lines HCT116 was observed. The growth of the mother cell line HCT116 was most strongly inhibited. The cell line without bax gene showed significantly less sensitivity to the cytotoxic effect KR6-SX. This can be explained in stressful situations that bax promotes apoptosis by it.

Incubation time 24h

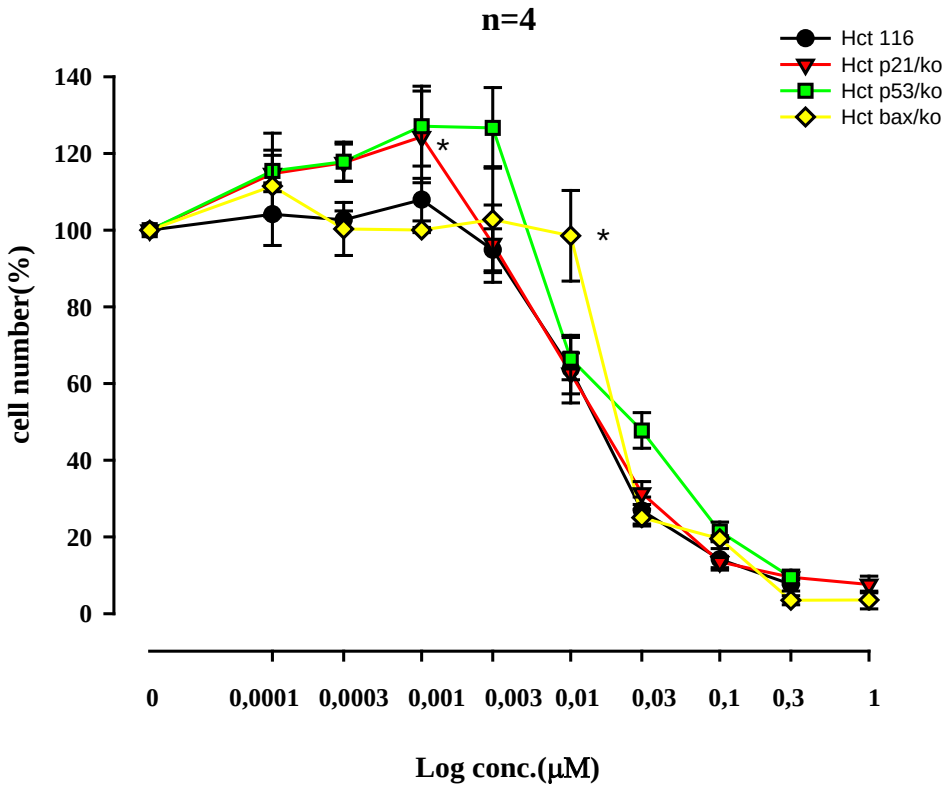

Figure 15: Comparison between the different HCT116 cell lines after 24 hours of exposure and increasing concentrations of KR6-SX.

*..... statistically significant ($p<0.05$)
**…. statistically significant ($p<0.01$)
***... highly statistically significant ($p<0.001$)

After 24 hours incubation only at 0.1 µM KR6-SX a significant difference between the mother cell line and the corresponding bax knock out cell line was observed.

Incubation time 48h

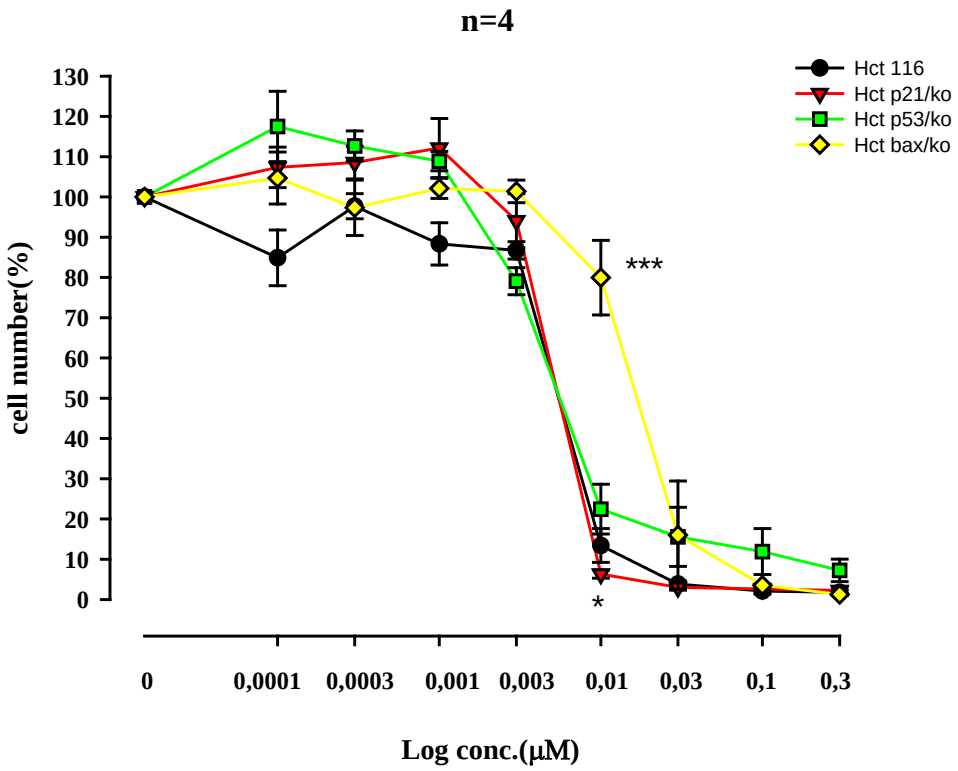

Figure 16: Comparison between the different HCT116 cell lines after 48 hours of exposure at increasing concentrations of KR6-SX.

*..... statistically significant ($p<0.05$)
**…. statistically significant ($p<0.01$)
***... highly statistically significant ($p<0.001$)

After the 48 hours incubation there was a difference in sensitivity of cell lines to KR6-SX, especially in the cell line without bax gene at a concentration of 0.01µM.

Incubation time 72h

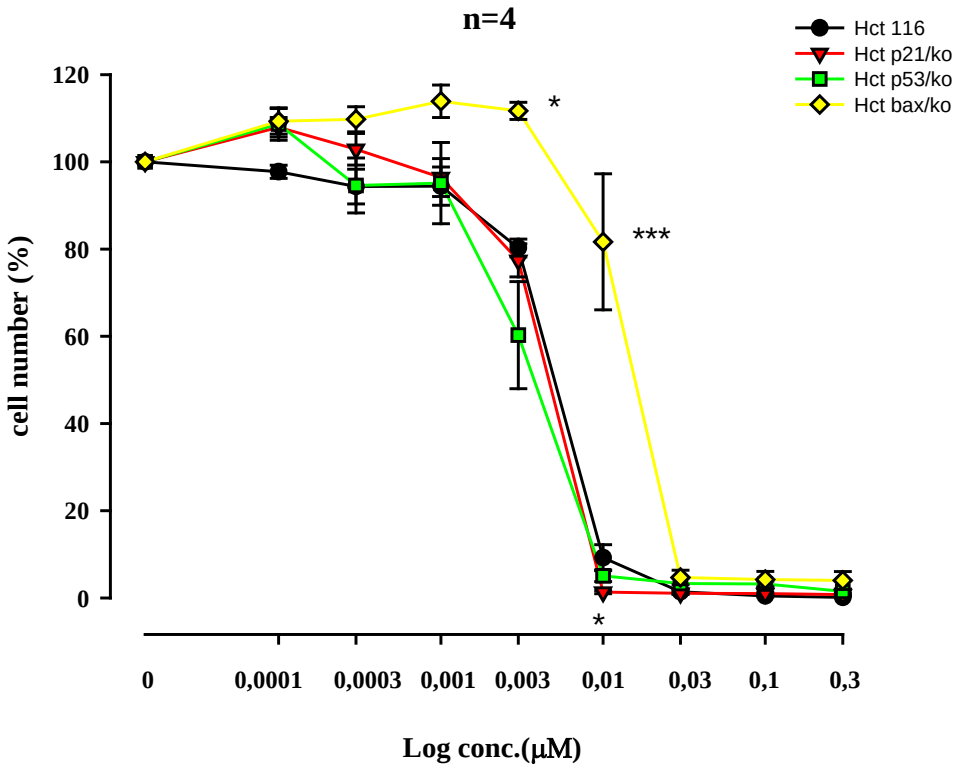

Figure 17: Comparison between the different HCT116 cell lines after 72 hours of exposure at increasing concentrations of KR6-SX.

*****..... statistically significant ($p<0.05$)
******…. statistically significant ($p<0.01$)
*******... highly statistically significant ($p<0.001$)

After 72 hours incubation the dose-dependent effect curves are almost identical, except the cell lines without bax gene. This was the most sensitive cell line, showing statistically significant differences to the mother cell line at 0.03 and 0.01 µM.

e) <u>Comparison of the cytotoxicity on the cancer cell lines HCT116, HCT116 p21/ko, HCT116 p53/ko, and HCT116 bax/ko based on EC_{25}:</u>

As shown in Table 5 and Fig. 18 there were no significant differences in EC_{25} in all tested cell lines except HCT116 bax/ko.

Cell line	**EC_{25} (µM)**				
	8h	**16h**	**24h**	**48h**	**72h**
HCT116	0.20	0.03	0.02	0.004	0.003
HCT116 bax/ko	0.21	0.20***	0.02	0.010**	0.010**
HCT116 p21/ko	0.23	0.03	0.01	0.005	0.003
HCT116 p53/ko	0.12	0.06	0.01	0.004	0.002

Table 5: Comparison of EC_{25} values for the cytotoxicity of KR6-SX on the cancer cell lines HCT116, HCT116 p21/ko, HCT116 p53/ko, and HCT116 bax/ko.

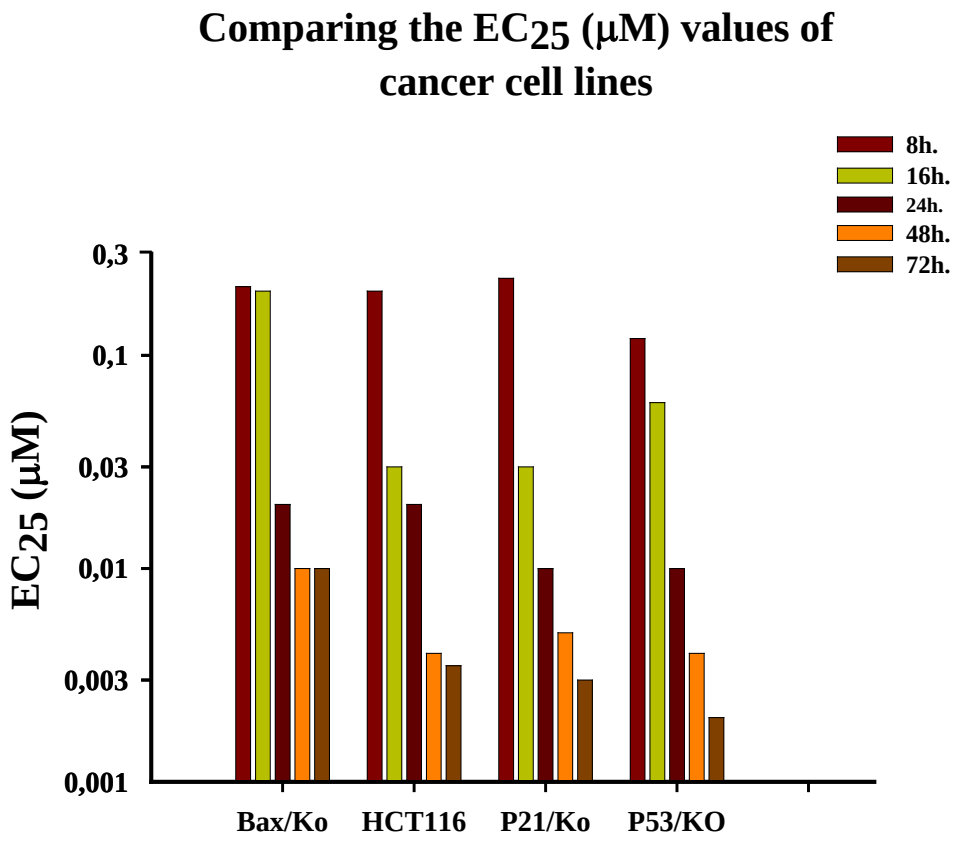

Figure 18: Comparison of EC_{25} values of KR6-SX on various cancer cell lines

f) Comparison of the cytotoxicity on the cancer cell lines HCT116, HCT116 p21/ko, HCT116 p53/ko, and HCT116 bax/ko based on EC_{50}:

As shown in Table 6 and Fig. 19 there were no significant differences in EC_{50} in all tested cell lines except HCT116 bax/ko.

Cell line	EC_{50} (µM)				
	8h	**16h**	**24h**	**48h**	**72h**
HCT116	0.4	0.06	0.030	0.006	0.006
HCT116 bax/ko	0.4	0.48**	0.024	0.019***	0.018***
HCT116 p21/ko	0.3	0.06	0.020	0.006	0.005
HCT116 p53/ko	0.3	0.20*	0.030	0.007	0.004

Table 6: Comparison of EC_{50} values for cytotoxicity on the cancer cell lines HCT116, HCT p21/ko, HCT116 p53/ko, and HCT116 bax/ko

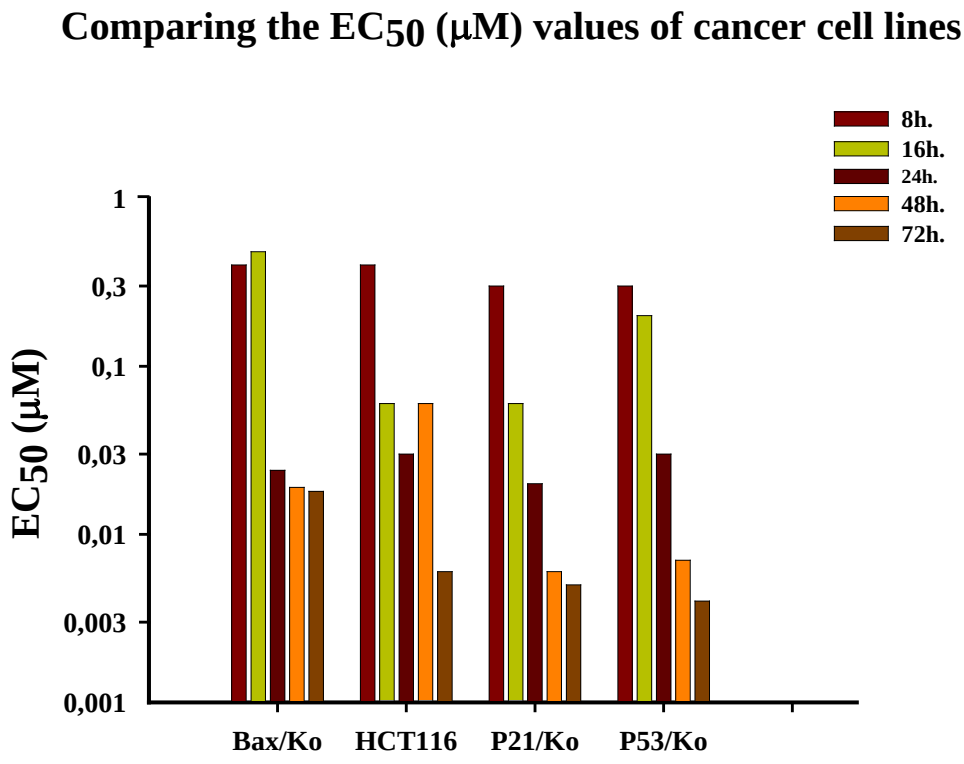

Figure 19: Comparison of EC_{50} values of KR6-SX on various cancer cell lines

g) Comparison of the cytotoxicity on the cancer cell lines HCT116, HCT116 p21/ko, HCT116 p53/ko, and HCT116 bax/ko based on EC_{75}:

Comparing EC_{75} for cytotoxicity of KR6-SX on the cancer cell lines revealed that HCT116 bax/ko is the least sensitive HCT116 cell line. After 8 hours incubation EC_{75} was less than 1 µM KR6-SX, but even after prolongation of the duration of exposure till 72 hours the EC_{75} was less than 0.03 µM KR6-SX (Table 7, Fig. 20).

Cell line	**EC_{75} (µM)**				
	8h.	**16h.**	**24h.**	**48h.**	**72h.**
HCT116	-	0.16	0.05	0.009	0.008
HCT116 bax/ko	0.89	0.82*	0.05	0.027**	0.020**
HCT116 p21/ko	-	0.20	0.06	0.008	0.008
HCT116 p53/ko	-	0.27	0.07	0.009	0.008

Table 7: Comparison of EC_{75} values for cytotoxicity on the cancer cell lines HCT116, HCT p21/ko, HCT116 p53/ko, and HCT116 bax/ko

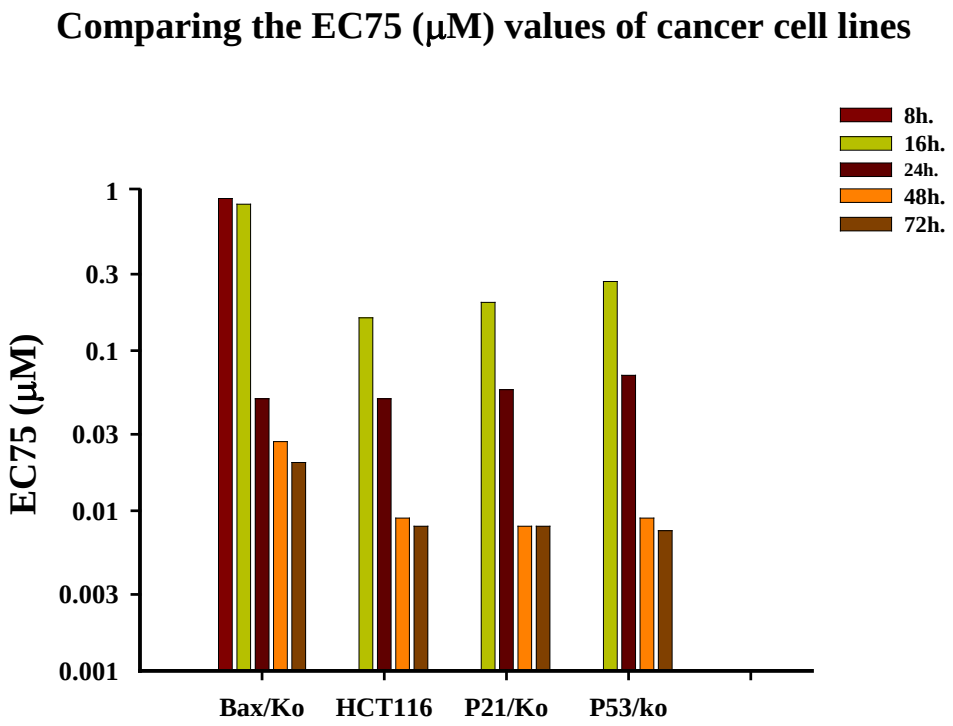

Figure 20: Comparison of EC_{75} values of KR6-SX on various cancer cell lines using

h) Comparison of the cytotoxicity on the non-cancer cell line HEK, and on the cancer cell line HCT116 based on the EC_{25}:

Comparing the cytotoxic effect of the KR6-SX between the mother cancer cell line HCT116 and non-cancer cell line HEK showed that HCT116 is more sensitive to KR6-SX at longer incubation periods of 48 and 72 hours (Table 8, Fig. 21).

Cell line	**EC_{25} (µM)**				
	8h.	**16h.**	**24h.**	**48h.**	**72h.**
HEK	0.25	0.07	0.02	0.022	0.0900
HCT116	0.19	0.03	0.02	0.004**	0.0035***

Table 8: Comparison of EC_{25} for the cytotoxicity of KR6-SX on the cancer cell line HCT116 and the non-cancer cell line HEK

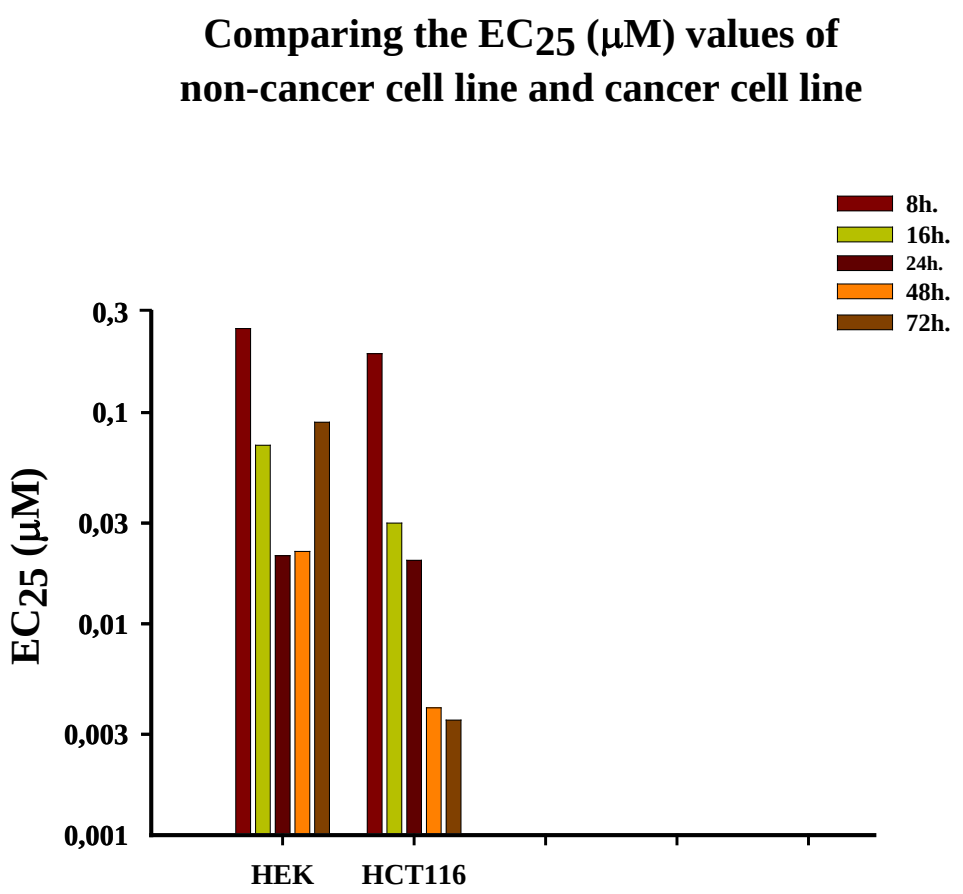

Figure 21: Comparison of EC_{25} values for the effect of KR6-SX on non-cancer cell line and cancer cell line

i) **Comparison of the cytotoxicity on the non-cancer cell line HEK, and on the cancer cell line HCT116 based on EC_{50}:**

Comparing EC_{50} for the cancer cell line HCT116 and HEK cell shows that HCT116 is more sensitive to KR6-SX at 72 hours incubation time (Table 9, Fig. 22).

Cell line	**EC_{50} (µM)**				
	8h.	**16h.**	**24h.**	**48h.**	**72h.**
HEK	0.47	0.19	0.039	0.04	0.019
HCT116	0.35	0.06	0.030	0.06	0.006*

Table 9: Comparison of EC_{50} for the cytotoxicity of KR6-SX on the cancer cell line HCT116 and the non-cancer cell line HEK

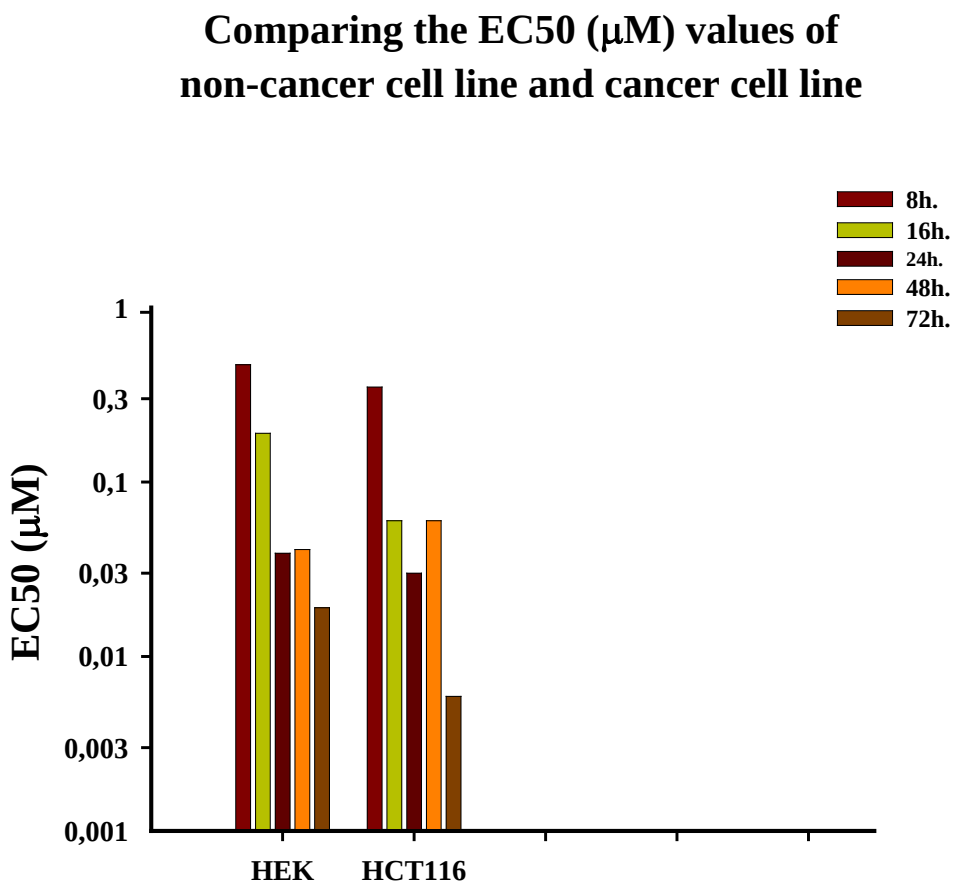

Figure 22: Comparison of EC_{50} values for the effect of KR6-SX on non-cancer cell line and cancer cell line

j) Comparison of the cytotoxicity on the non-cancer cell line HEK, and on the cancer cell line HCT116 based on EC_{75}:

Comparing the EC_{75} between the mother cancer cell line HCT116 and non-cancer cell line HEK showed that the HEK cell is more resistant to KR6-SX than HCT116 (Table 10, Fig. 23).

Cell line	**EC_{75} (µM)**				
	8h.	**16h.**	**24h.**	**48h.**	**72h.**
HEK	0.75	-	1.00	0.029	0.029
HCT116	-	0.16	0.05*	0.008*	0.008*

Table 10: Comparison of EC_{75} for the cytotoxicity of KR6-SX on the cancer cell line HCT116 and the non-cancer cell line HEK

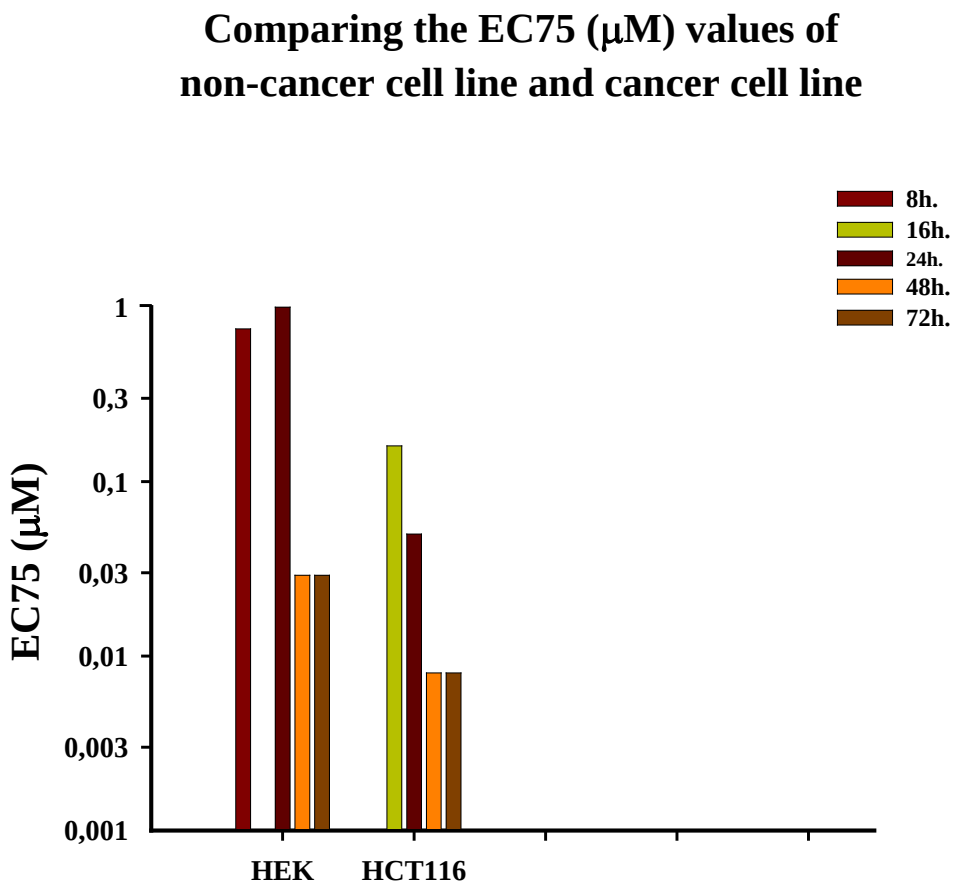

Figure 23: Comparison of EC_{75} values for the effect of KR6-SX on non-cancer cell line and cancer cell line

CONCLUSION

KR6-SX acts cytotoxic on all tested cell lines, the cancer cells HCT116, HCT p53/ko, HCT116 p21/ko, HCT116 bax/ko and on the non-cancer cell HEK.

The mechanism of action of KR6-SX remains unclear and the cytotoxicity of KR6-SX is irreversible. As a consequence in spite of the short toxin contact, the cells are irreparably damaged and killed. The cells show a significantly increased cell division rate before they die. This is probably due to the fact that high concentrations of KR6-SX suspend the cell a higher stress and thus the proliferation is stimulated in order to ensure the survival of the cell. Maybe the substance has a mutagenic potential. Furthermore, it was shown at the lowest tested concentration of KR6-SX that the number of the cell death is low, probably because the cells at the first exposure try to resist the substance. But with longer and higher exposure times the cells start dying.

Therefore, KR6-SX is an unsuitable chemotherapeutic drug, because it is also cytotoxic to the non-cancer cell at a similar concentration range.

ABSTRACT

The metabolite KR6-SX was isolated from a fungal species of the genus *Chrysosporium*.

The cytotoxicity of KR6-SX was examined on the cancer cells (HCT116, HCT116 p21/ko, HCT116 p53/ko, HCT116 bax/ko) and on non-cancer cells (HEK). The cytotoxic effect was shown on all these cell lines. The cell lines HCT116 with knockout p21 and p53 genes are most sensitive to the effect of KR6-SX at all test concentrations and incubation times, whereas bax knockout HCT116 cells were least sensitive. It was clear that the cytotoxic effect of KR6-SX is an irreversible effect which leads to cell the death.

Based on these results, it is suggested that KR6-SX is not suitable as a chemotherapeutic agent, because cytotoxicity affects both the cancer cells and the non-cancer cells.

ABSTRACT

Der Metabolit KR6-SX wurde vom Schimmelpilz *Chrysosporium* isoliert. Die Zytotoxizität von KR6-SX wurde an verschiedenen Krebszelllinien (HCT116, HCT116 p21/ko, HCT116 p53/ko, HCT116 bax/ko) und nicht-malignen Zellen (HEK) untersucht. In allen untersuchten Zelllinien wurde eine starke zytotoxische Wirkung festgestellt. Die Zelllinie HCT116 mit den knock out Genen p21 und p53 waren die empfindlichsten Zelllinien, bei allen getesteten Konzentrationen und Inkubationszeiten, wohingegen bax knockout HCT116 Zellen die geringste Empfindlichkeit aufwiesen. Der zytotoxische Effekt von KR6-SX war irreversibel und führte zum Zelltod. Aufgrund dieser Resultate scheint KR6-SX kein geeigneter Kandidat für ein Chemotherapeutikum zu sein, da es gleichermaßen stark zytotoxisch auf maligne und nicht-maligne Zellen wirkt.

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