universität wien

DIPLOMARBEIT

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„Untersuchung der zytotoxischen Wirkung des Schimmelpilzmetaboliten KR6-SynchromycinX“

verfasst von

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DEDICATION

I would like to express the deepest appreciation to my professor Mrs. Univ.-Prof. Mag. Dr. **Lemmens-Gruber**.

I am also grateful to Mrs. **Pakiza Rawnduzi** who showed me the road and helped to get me started on the path to this degree and she was always available for my questions.

I can't also forget my parents and my wife who have supported me all the way since the beginning of my study,their enco uragement, and faith in me.

Finally, this thesis is dedicated to all those who believe in the richness of learning.

Content

A- General overview and aim of the study

In December 2007, and as a part of a mycological research, a rare fungal species of the genus ***Chrysosporium*** was discovered. Extracts of this fungus were screened for antimicrobial activity by Romer Labs Division Holding GmbH; 3430 Tulln, Austria. In further consequence, about 300 extracts were tested for their cytotoxic effect at the Department of Pharmacology and Toxicology, University of Vienna. Nearly 20 percent of them were cytotoxic. **KR6-SX** is an isolated substance from one of these cytotoxic extracts.

In this study, the cytotoxic effect of **KR6-SX** on various cancer cell lines and non-cancer cell lines was investigated as well as reversibility of the effect. All investigations should serve to get first clues of whether **KR6-SX** is a potential lead compound with anti tumor activity.

B - Introduction

1- What is apoptosis?

The term apoptosis had been coined in order to describe the morphological processes leading to controlled cellular self-destruction. Apoptosis was firstly introduced to publications by (Kerr *et al*, 1972). The apoptotic mode of cell death is an active and defined process which plays an important role in the development of multicellular organisms and in the regulation and maintenance of the cell populations in tissues upon physiological and pathological conditions. It should be stressed that apoptosis is a well-defined and possibly the most frequent form of programmed cell death, but that other, non-apoptotic types of cell death also might be of biological significance (Leist and Jäättelä 2001).

2- Physiological significance of apoptosis.

Apoptosis or programmed cell death is a normal physiological process, which plays an important role in the development and morphogenesis, cellular homeostasis and the deletion of damaged and autoreactive cells (Fig. 1). e.g. the formation of the digits, the development of the brain and reproductive organs, the negative selection of T cells in the thymus and the deletion of infected and cancer cells. In the gut, apoptosis is important in the homeostasis of the epithelium by regulating cell numbers of epithelial cells and in the elimination of lamina propria T lymphocytes (LPLs) to prevent an excessive immune response because of the constitutive examination of luminal antigens (Verstege 2010).

Programmed cell death is a wide-spread phenomenon occuring in kinds of metazoans, such as mammals, insects, helminthes and amphibians (Reed and Tomaselli 2000).

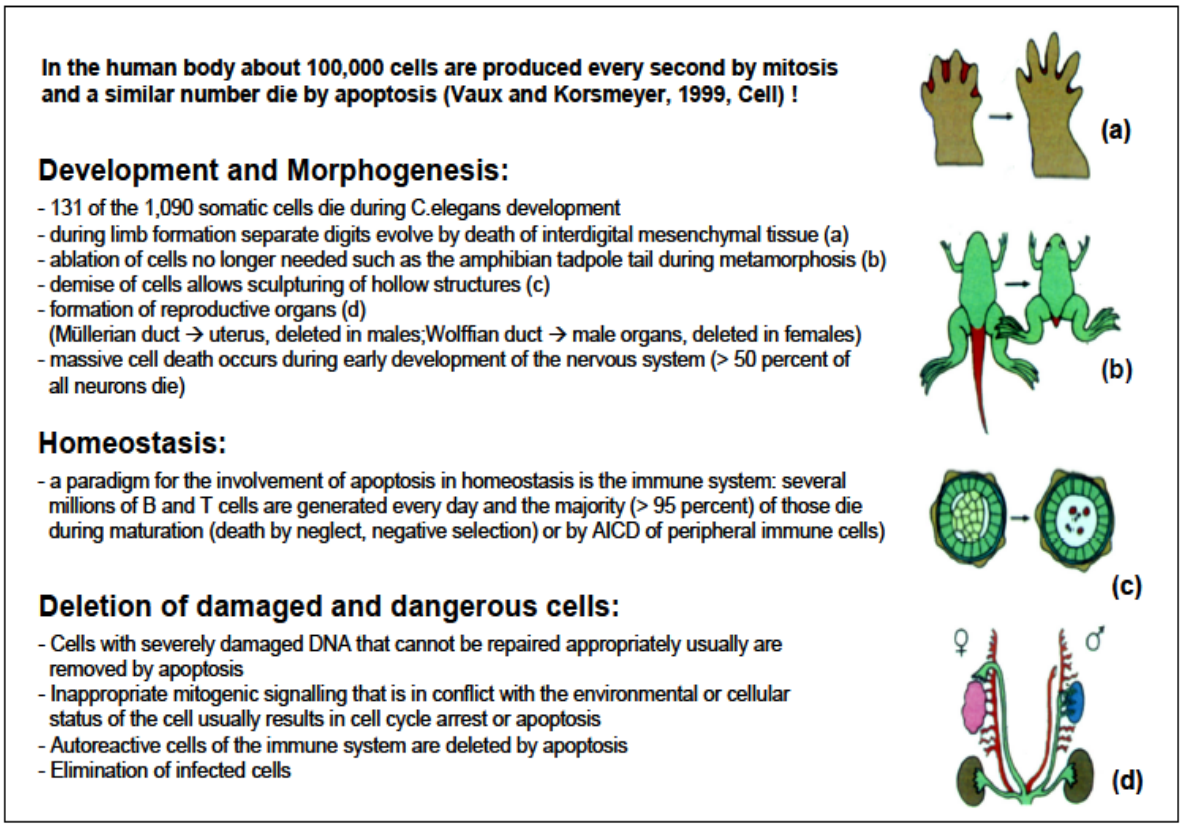

Figure 1: Examples of physiological apoptosis (Vaux and Korsmeyer 1999)**.**

3- Characterization of apoptosis.

Programmed cell death (PCD) may occur in multicellular organisms. Biochemical events lead to characteristic cell changes (morphology) and death. Apoptotic cells can be recognized by some morphological changes: the cell shrinks, shows deformation and loses contact to its neighboring cells. Its chromatin condenses and marginates at the nuclear membrane, the plasma membrane is blebbing or budding, and finally the cell is fragmented into compact membrane-enclosed structures, called 'apoptotic bodies' which contain cytosol, the condensed chromatin, and organelles (Fig. 2) (Hiquchi et al 2003).

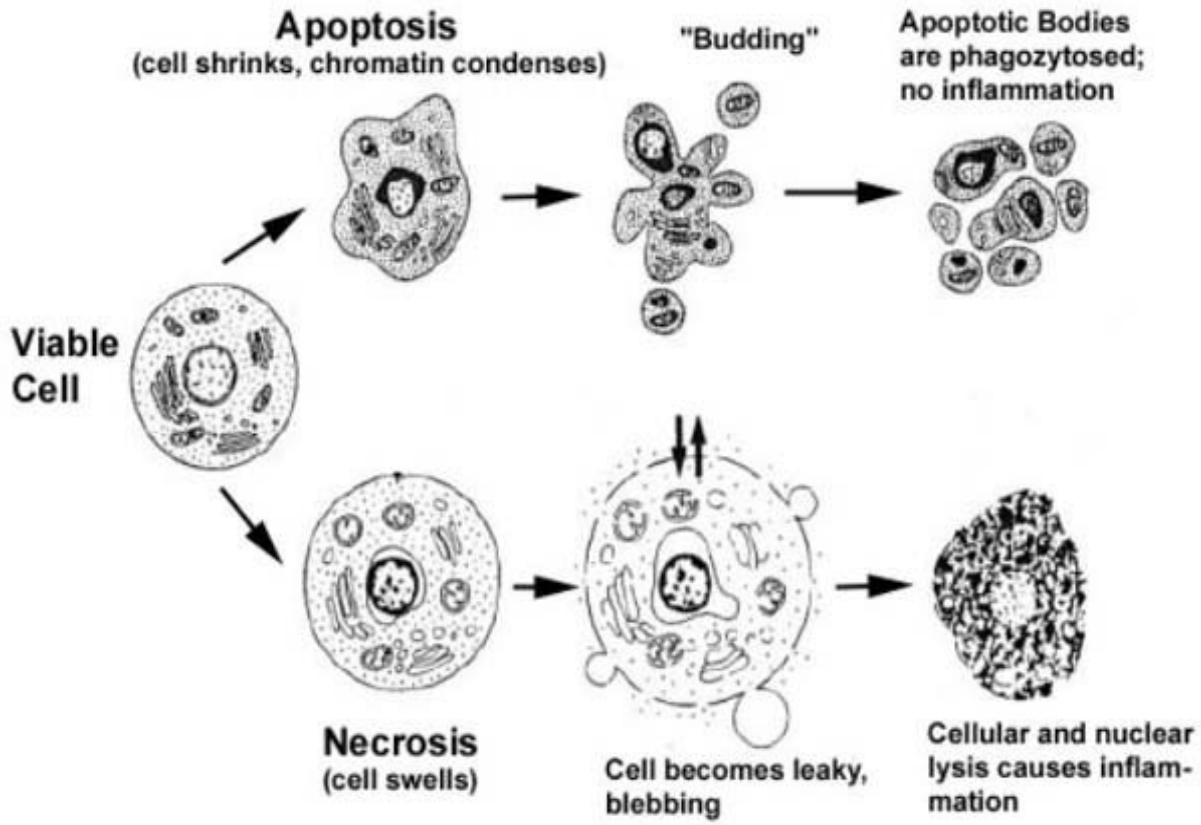

Figure 2: Hallmarks of the apoptotic and necrotic cell death process.

4- Necrosis.

Necrosis (from the Greek *νεκρός*, "dead", *νέκρωσις*, "death, the stage of dying, the act of killing") is the premature death of cells and living tissue. Necrosis is caused by factors external to the cell or tissue, such as infection, toxins, or trauma. This is in contrast to apoptosis, which is a naturally occurring cause of cellular death. While apoptosis often provides beneficial effects to the organism, necrosis is almost always detrimental and can be fatal. Cells that die due to necrosis do not usually send the same chemical signals to the immune system that cells undergoing apoptosis do. This prevents nearby phagocytes from locating and engulfing the dead cells, leading to a build-up of dead tissue and cell debris at or near the site of the cell death. For this reason, it is often necessary to remove necrotic tissue surgically, a process known as debridement (Proskuryakov et al 2003).

The apoptotic cells are phagocytosed without triggering inflammatory processes. The necrotic cell swells, becomes leaky and finally is disrupted and releases its contents into the surrounding tissue resulting in inflammation (Fig. 3, modified from (Van Cruchten, 2002). The most common differences between apoptosis and necrosis are summarized in Table 1.

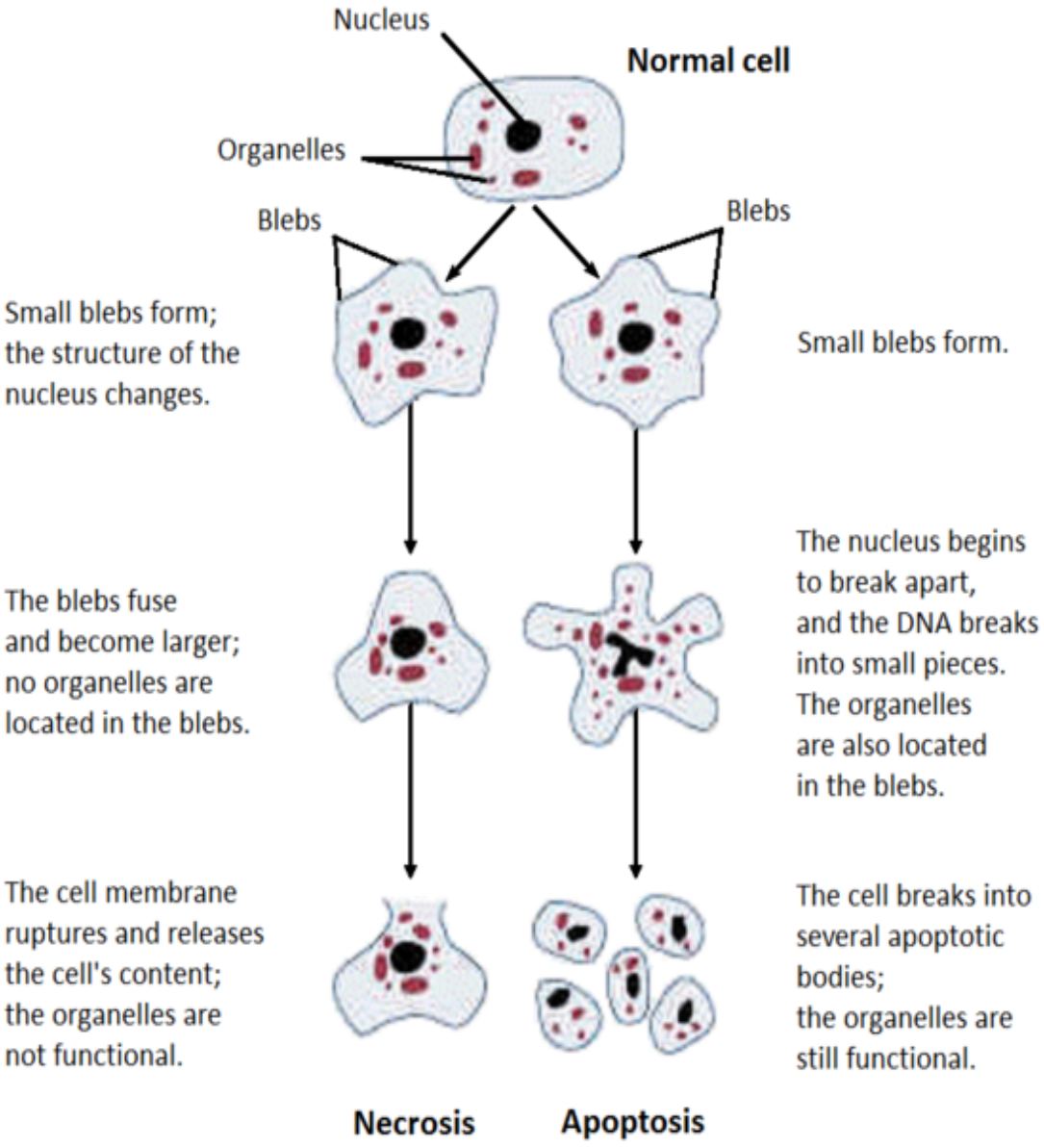

Figure 3: Structural changes of cells undergoing necrosis or apoptosis (Goodlett and Horn 2001)**.**

5- Apoptosis versus Necrosis.

	Apoptosis	Necrosis
Natural	Yes	caused by factors external to the cell or tissue, such as infection, toxins, or trauma
Effect	Beneficial	Detrimental
Introduction	Apoptosis programmed cell death (PCD) in humans & multicellular organisms. PCD involves a series of biochemical events leading to a cell destruction and death.	Necrosis is the premature death of cells and living tissue. Necrosis is caused by external factors, such as infection, toxins or trauma. This is in contrast to apoptosis, which is a naturally occurring cause of cellular death.
Result	Can prevent tumor formation (homeostasis between cell death rate and mitosis rate)	Necrosis results in inflammation, which could become chronic.
Definition	Programmed cell death	The cell or tissue damage due to external factors.
Process	Membrane blebbing, shrinkage of cell nuclear collapse (nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation), appoptopic bodyformation. Then, engulf by white blood cells	Membrane disruption, respiratory poisons and hypoxia which cause ATP depletion, metabolic collapse, cell swelling and rupture leading toinflammation.

Table 1: Apoptosis vs. Necrosis

6- Methods for distinguishing apoptotic from necrotic (necroptotic) cells.

In order to perform analysis of apoptotic versus necrotic (necroptotic) cells one can do analysis of morphology by time-lapse microscopy, flow fluorocytometry, and transmission electron microscopy. There are also various biochemical techniques for analysis of cell surface markers (phosphatidylserine exposure versus cell permeability by flow fluorocytometry), cellular markers such as DNA fragmentation (flow fluorocytometry), caspase activation, Bid cleavage, and cytochrome c release (Western blotting). It is important to know how primary and secondary necrotic cells can be distinguished by analysis of supernatant for caspases, HMGB1, and release of cytokeratin 18. However, no distinct surface or biochemical markers of necrotic (necroptotic) cell death have been identified yet, and only negative markers are available. These include absence of apoptotic parameters (caspase activation, cytochrome c release, and oligonucleosomal DNA fragmentation) and differential kinetics of cell death markers (phosphatidylserine exposure and cell membrane permeabilization) (Krysko et al 2008).

7- Apoptosis pathways.

There are at least two broad pathways that lead to apoptosis, an "Extrinsic" and an "Intrinsic" Pathway. In both pathways, signaling results in the activation of a family of cysteine peptidases, named caspases that act in a proteolytic cascade to dismantle and remove the dying cell (Reyes-Zurita et al 2013).

a- Extrinsic pathways.

Extrinsic apoptosis signalling is mediated by the activation of so called “death receptors” which are cell surface receptors that transmit apoptotic signals after ligation with specific ligands. Death receptors belong to the tumor necrosis factor receptor (TNFr) gene superfamily, including TNFr-1, Fas/CD95, and the TRAIL receptors DR-4 and DR-5 (Ashkenazi 2002).

All members of the TNFr family consist of cysteine rich extracellular subdomains which allow them to recognize their ligands with specificity, resulting in the trimerization and activation of the respective death receptor (Naismith 1998).

Subsequent signalling is mediated by the cytoplasmic part of the death receptor which contains a conserved sequence termed the death domain (DD). Adapter molecules like FADD or TRADD themselves possess their own DDs by which they are recruited to the DDs of the activated death receptor, thereby forming the so-called death inducing signalling complex (DISC).

In addition to its DD, the adaptor FADD also contains a death effector domain (DED) which through homotypic DED-DED interaction sequesters procaspase-8 to the DISC (Figure: 5).

As described above, the local concentration of several procaspase-8 molecules at the DISC leads to their autocatalytic activation and release of active caspase-8. Active caspase-8 then processes downstream effector caspases which subsequently cleave specific substrates resulting in cell death. Cells harboring the capacity to induce such direct and mainly caspase-dependent apoptosis pathways were classified to belong to the so called type I cells (Scaffidi 1998). In type II cells, the signal coming from the activated receptor does not generate a caspase signalling cascade strong enough for execution of cell death on its own. In this case, the signal needs to be amplified via mitochondria-dependent apoptotic pathways. The link between the caspase signalling cascade and the mitochondria is provided by the Bcl-2 family member Bid. Bid is cleaved by caspase-8 and in its truncated form (tBID) translocates to the mitochondria where it acts in concert with the proapoptotic Bcl-2 family members Bax and Bak to induce the release of cytochrome c and other mitochondrial proapoptotic factors into the cytosol (Figure 4, Luo 1998).

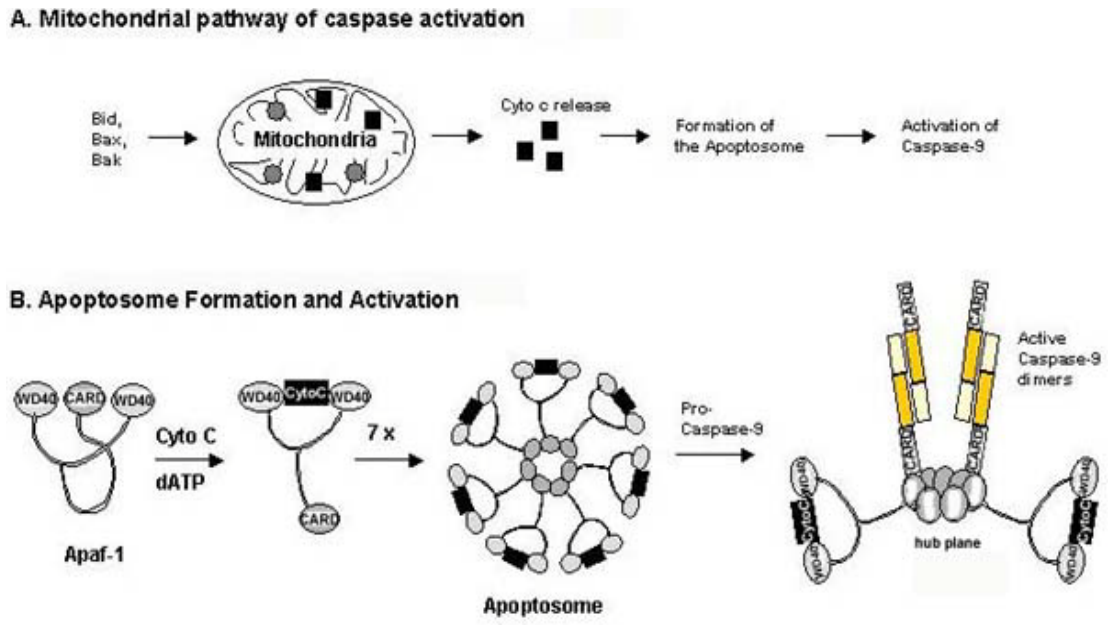

Figure 4: Mitochondria-mediated caspase activation at the apoptosome (Luo 1998)**.**

Apoptotic stimuli trigger the release of apoptogenic factors from the mitochondrial intermembrane space to the cytosol, such as cytochrome c which induces the formation of the apoptosome and the activation of procaspase-9. B. By the action of cytochrome c (Cyto C) and dATP the Apaf-1 protein adopts a conformation that allows the formation of a heptameric, wheel-like structure, the apoptosome. Procaspase-9 molecules can bind to the inner “hub” region of the apoptosome and are activated by dimer formation. Active caspase-9 dimers further mediate activation of effector caspases (Acehan 2002).

Cytosolic cytochrome c is binding to monomeric Apaf-1 which then, in a dATP-dependent conformational change, oligomerizes to assemble the apoptosome, a complex of wheel-like structure with 7-fold symmetry, that triggers the activation of the initiator procaspase-9 (Figure 5). Activated caspase-9 subsequently initiates a caspase cascade involving downstream effector caspases such as caspase-3, caspase-7, and caspase-6, ultimately resulting in cell death (Slee 1999).

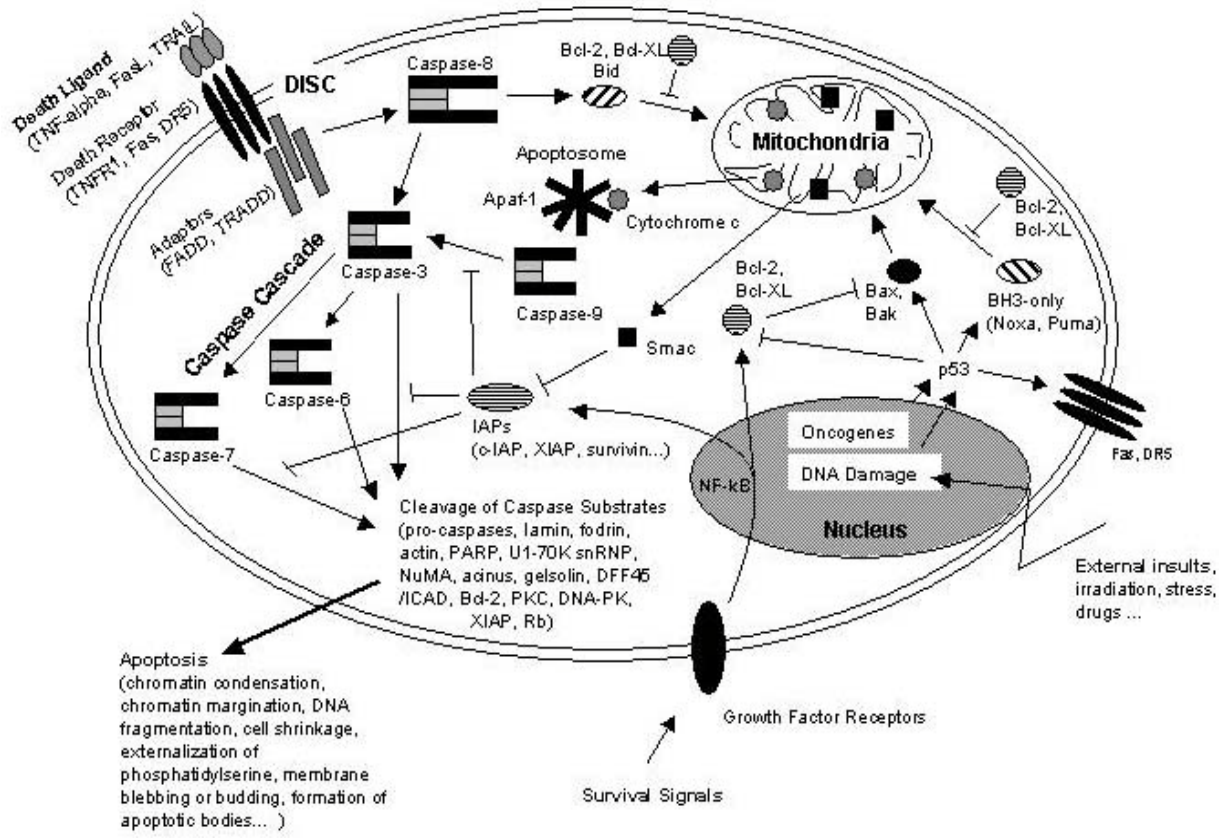

Figure 5: Schematic representation of some major apoptotic signalling pathways (Acehan 2002)**.**

Apoptosis can be induced in response to various signals from inside and outside the cell, e.g. by ligation of so called death receptors or by cellular stress triggered by oncogenes, irradiation or drugs. Signals emanating from death receptors initially activate the Death Inducing Signalling Complex (DISC) which mediates activation of the initiator caspase-8. Activated caspase-8 initiates a caspase cascade by processing the effector caspases-3, -6, and -7 which in turn cleave a number of protein substrates. Cleavage of caspase substrates eventually leads to the characteristic morphological and biochemical features of apoptosis.

In some cell systems, this direct caspase cascade is sufficient to elicit apoptosis on its own (type 1 signalling), whereas in other cases the signal coming from the DISC must be amplified by the proteolytic activation of the BH3-only protein Bid by caspase-8 with subsequent induction of apoptotic events at the mitochondria (type 2 signalling).

Mitochondrial apoptotic signalling includes the release of cytochrome c from the mitochondrial intermembrane space to the cytosol where it contributes to the formation of the apoptosome which consists of cytochrome c, Apaf-1 and dATP. The apoptosome activates caspase-9 which is another initiator caspase and thus is able to mediate the caspase cascade by activating caspase-3. Another mitochondrial proapoptotic factor is Smac which acts by inhibiting the IAPs from blocking caspase activity.

IAPs are a family of proteins with antiapoptotic activity by directly inhibiting caspases. IAP expression can be upregulated in response to survival signals such as those coming from growth factor receptors, e.g. by activation of the transcription factor NF-kB, therefore providing a means to suppress apoptosis signalling. Of central importance are the antiapoptotic Bcl-2 family members such as Bcl-2 and Bcl-XL which counteract the action of BH3-only proteins such as Bid but also of proapoptotic Bax and Bak and thus can inhibit mitochondrial proapoptotic events.

Apoptotic signals coming from the inside of the cell frequently have their origin within the nucleus, being a consequence of DNA damage induced by irradiation, drugs or other sort of stress. DNA damage in most cases eventually results in the activation of the p53 transcription factor which promotes expression of proapoptotic Bcl-2 members and suppresses antiapoptotic Bcl-2 and Bcl-XL.

Other organelles besides mitochondria and the nucleus, such as the ER and lysosomes also have been implicated in apoptotic signalling pathways, and it should be kept in mind that

presumably hundreds of proteins are part of an extremely fine-tuned regulatory network consisting of pro- and antiapoptotic factors.

b- Intrinsic pathway

The intrinsic signaling pathway for programmed cell death involves non-receptor–mediated intracellular signals, inducing activities in the mitochondria that initiate apoptosis (Figure 6).

Stimuli for the intrinsic pathway include viral infections or damage to the cell by toxins, free radicals, or radiation. Damage to the cellular DNA can also induce the activation of the intrinsic pathway for programmed cell death. These stimuli induce changes in the inner mitochondrial membrane that result in the loss of transmembrane potential, causing the release of pro-apoptotic proteins into the cytosol. Pro-apoptotic proteins activate caspases that mediate the destruction of the cell through many pathways. These proteins also translocate into the cellular nucleus, inducing DNA fragmentation, a hallmark of apoptosis.

The regulation of pro-apoptotic events in the mitochondria occurs through activity of members of the Bcl-2 family of proteins and the tumor suppressor protein p53 (Hanahan and Weinberg 2000).

Members of the Bcl-2 family of proteins may be pro- or anti-apoptotic. The anti-apoptotic proteins are Bcl-2, Bcl-x, Bcl-x_L, Bcl-X_S, Bcl-w, and BAG. Some of these proteins are currently under investigation as potential targets for anticancer therapy. Pro-apoptotic proteins include Bcl-10, Bax, Bak, Bid, Bad, Bim, Bik, and Blk. It has been suggested that upregulation of these proteins or their increased activation may offer an approach for cancer therapy (Rastogi et al 2009).

Cellular pathways that modulate the activities of the p53 protein are also currently being evaluated as targets for potential anticancer therapies (Fulda et al 2004).

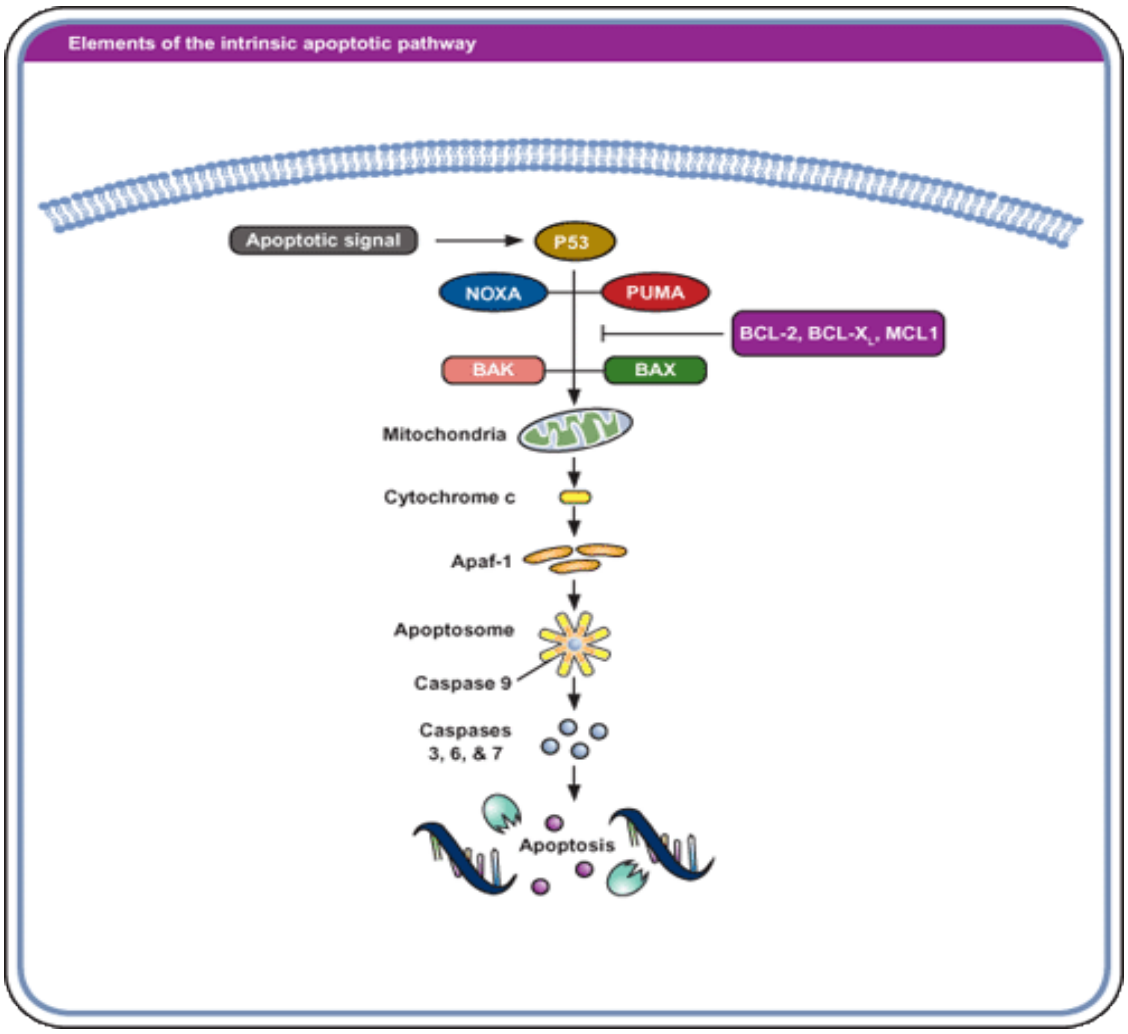

Figure 6: Elements of the intrinsic apoptotic pathway (Roy and Nicholson 2000).

8- Apoptosis in cancer

Tumors often form due to cancer cells developing the ability to suppress or inhibit apoptosis, making them immortal and dangerous. Excessive or reduced apoptotic responses can have potentially fatal consequences. Cancer is a disease that is often characterized by a lack of apoptosis. Cancer cells are able to live on and continuously replicate themselves by evading the cell-suicide process, leading to the formation of tumors, typically because these cells possess a number of mutations. The mutations not only allow them to become more proliferative than normal (by ignoring cellular signals regulating their growth), but also prevent them from undergoing apoptosis, the normal response to DNA mutations. Research suggests that mutations in certain genes, such as the p53 gene, are pivotal to the development

of tumors. Interest in the p53 gene in particular has grown recently as it has been found to be an especially important tumor suppressor gene. It is often damaged in cancer cells, preventing apoptosis from occurring and causing the development of potentially deadly tumors. This underlines the significance of apoptosis triggering genes: a greater knowledge of how apoptosis is regulated in tumor development would undoubtedly help in the development of treatments for cancer (Wong 2011).

C - Materials and Methods

1- 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 and then diluted with the nutrient medium - according to the cell line under investigation - to obtain the desired concentrations for the experiments.

During the addition of the DMSO, the test substance KR6-SX was deposited and therefore all the tests were conducted with the suspension form. The test substance had to be mixed well before dilution and addition of nutrient medium to ensure a uniform distribution of KR6-SX.

The molecular weight was given as 682 g / mol. Based on this value, the serial dilutions were made.

Nine different concentrations were used to identify the toxic effect of the test substance on all cell lines. In the following table (Table 2), the concentrations used in ng/ml and µM are listed:

Concentrations of the test substance KR6-SX									
µM	**0.0001**	**0.0003**	**0.001**	**0.003**	**0.01**	**0.03**	**0.1**	**0.3**	**1**
ng/ml	**0.068**	**0.025**	**0.682**	**2.046**	**6.82**	**20.46**	**68.21**	**204.6**	**682.1**

Table 2: **Overview of the used concentrations of the test substance KR6-SX**

2- Cell lines and nutrient media

In the frame of this work, the cytotoxicity of the test substance KR6 SX will be examined on five cell lines, three of them are cancer cell lines and the other two are non-cancer cell lines.

a- Cancer cell lines:

- **KB-3-1:** Cervical adenocarcinoma cells (from ATCC).
- **A549:** Adenocarcinoma human alveolar basal epithelial cells (Lung carcinoma cells) (from ATCC).
- **CACO2:** Human epithelial colorectal adenocarcinoma cells (Colon carcinoma cells) (from ATCC).

b- Non- cancer cell lines:

- **FHs74Int:** Human intestinal epithelial cells (from ATCC).
- **MH-S:** Murine alveolar macrophage cells (from ATCC).

3- Nutrient media

Selection of a suitable nutrient medium is dependent on cell type, culture conditions and degree of chemical definition required for the cell culture application.

- For the cancer cell lines (**KB-3-1**/ **A549**/ **CACO2**) we used the **RPMI-1640** medium (Sigma®), which is prepared by adding fetal bovine serum (Sigma®) in percentage of 10 % and Penicillin-Streptomycin mixture (Sigma®) in percentage of 1% to the original medium RPMI-1640 (Sigma®) to be suitable for culturing the cancer cell lines and to promote their growth.

- For the non-cancer cell lines **(FHs74Int**/ **MH-S)** we used the **RPMI-1640** medium (Sigma®), which is prepared by adding calf serum (Sigma®) in percentage of 10 % and Penicillin-Streptomycin mixture (Sigma®) in percentage of 1% to the original medium RPMI-1640 (Sigma®) to be suitable for culturing then non-cancer cell lines and to promote their growth.

4- Cultivation of the cells

a- General overview

Most of our cell lines grow as a single thickness cell layer or sheet attached to a plastic or glass substrate. Once the available substrate surface is covered by cells, growth slows and then ceases. Thus, in order to keep the cells healthy and actively growing, it is necessary to subculture them at regular intervals. Usually, this subcultivation process involves breaking the bonds or cellular “glue” that attaches the cells to the substrate and to each other by using proteolytic enzymes such as trypsin. The loosened cells are then removed from the culture vessel, counted, diluted and subdivided into new vessels. Cells then reattach, begin to grow and divide, and, after a suitable incubation period, again reach saturation or confluency. At this point, the subcultivation cycle can be repeated.

b- Listed below some tools and materials which should be available to be able to work in the cell culture laboratory:

- Water bath
- Centrifuge
- Refrigerator and freezer (-20°C)
- Cell counter (e.g., haemocytometer)
- Inverted microscope
- Liquid nitrogen freezer
- Aspiration pump
- Cell culture vessels (e.g., flasks)
- Pipettes
- Waste containers
- Media and reagents
- Cells
- Laminar flow (hood)
- Alcohol 70 %
- Gloves

c- General guidelines to be followed before, during, and after work in cell culture:

1- Disinfect the hood using 70% ethanol before starting.
2- Disinfect gloves by washing them in 70% ethanol and allowing to air dry.
3- Put all materials and equipment into the hood prior to starting work after disinfecting the exterior surfaces with 70% ethanol.
4- While working, do not contaminate hands or gloves by touching anything outside the hood (especially face and hair). If gloves become contaminated re-sanitize with 70% ethanol as above before proceeding.
5- Discard gloves after handling contaminated cultures and at the end of all cell culture procedures.
6- Equipment in the hood or that which will be taken into the hood during cell culture procedures (media bottles, pipette tip boxes, pipette aids) should be wiped with tissue soaked with 70% ethanol prior to use.
7- After completing work disinfect all equipment and material before removing from the hood. Spray the work surfaces inside the hood with 70% ethanol and wipe dry with tissue.
8- Disinfect the hood with 10 – 30 min UV light.
9- Water bath temperature is (37°C).
10- Incubation temperature is (37°C) and CO_2 saturation is (5%).

All of the above guidelines are required to ensure that all cell culture procedures are performed to a standard that will prevent contamination from bacteria, fungi and mycoplasma, and cross contamination with other cell lines.

d- Resuscitation of Frozen Cell Lines

It is vital to thaw cells correctly in order to maintain the viability of the culture and enable the culture to recover more quickly. Some cryoprotectants, such as DMSO, are toxic above 4ºC. Therefore, it is essential that cultures are thawed quickly and diluted in culture medium to minimize the toxic effects.

Procedure:

1- Remove ampoule from liquid nitrogen and place in a water bath at an appropriate temperature for the cell line, e.g. 37ºC. Submerge only the lower half of the ampoule. Allow to thaw with constant agitation until a small amount of ice remains in the vial - usually 1-2 minutes. Transfer to the hood immediately.
2- Wipe the outside of the ampoule with a tissue moistened (not excessively) with 70% alcohol; hold tissue over ampoule to loosen lid.
3- Slowly, drop wise, pipette cells into pre-warmed growth medium to dilute DMSO.
4- Incubate cells overnight. Change medium the next morning. Removal of DMSO is critical.
5- Examine cells microscopically after 24 hours and sub-culture as necessary.

e- Changing the medium

One of the most important factors required for culturing cells, is the culturing medium, which can vary in pH, glucose concentration, growth factors, and the presence of other nutrients, and because the ingredients of the culture medium are metabolized in the course of time, the medium should be changed once or twice weekly at least.

In the case of adherent cultures, the media can be removed directly by aspiration and replaced. For culturing cells in small flasks ($25cm^2$) we used 5 ml medium and 10 ml for culturing in large flasks ($75cm^2$).

f- Subculturing or passaging cells

Adherent cell lines will grow in the flask until they have covered the surface area available or the medium is depleted of nutrients. The degree of adhesion varies from cell line to cell line, but in the majority of cases proteases, e.g. Trypsin, is used to release the cells from the flask. However, this may not be appropriate for some lines where exposure to proteases is harmful or can lead to damage of the surface receptors of the cells .Viewing the cells should be done on daily basis using an inverted microscope to assess the degree of the growth and confirm the absence of bacterial and fungal contaminants.

The growth of a culture can be estimated by comparing the amount of space covered by cells with the unoccupied spaces. If the growth is more than 80%, subculturing is required. To subculture the cells they need to be brought into suspension.

Procedure:

1- Remove spent medium by aspirator.

2- Wash the cell monolayer with (Trypsin/EDTA, Vitacell®) mixture. In our laboratory we use 0.800µl for small flask (25cm^2) and 2ml for large flask (75 cm^2).

3- Remove the Trypsin/EDTA mixture immediately by aspirator.

4- Pipette Trypsin/EDTA mixture again onto the washed cell monolayer using the same amount mentioned in the step (2), and rotate the flask to cover the monolayer with the mixture.

5- Incubate in the hood for 2-10 minutes, according to the cell line under investigation.

6- Once the cells start to sheet and the media becomes cloudy, move on to the next step. You may examine the cells using an inverted microscope to ensure that the cells are detached and floating. It may help to "slap or tap" the flasks gently to release any remaining attached cells.

7- Resuspend the cells in a small volume of the medium (around 4 ml for small flasks and 8 ml for large flasks) to inactivate the trypsin.

8- Triterate the cells (this is a process to disaggregate clumps or sheets of cells) by running the suspended cells in the medium three to four times with the tip of the pipette on the bottom corner of the flask.

9- Transfer the required number of cells to a new labeled flask containing the required amount of the pre-warmed medium (5 ml for small flasks and 10 ml for large flasks), and this called splitting.

10- The remaining amount of the cell suspension is used in the next step to be counted and then plated in the microplate (96 well) to examine the cell viability after exposure to different concentrations of the test substance.

g- Cell Counting

To determine growth rates or set up cultures at known concentrations it is necessary to count the cell suspension. Heamocytometers can be used in the presence of a vital dye like trypan Blue. The reactivity of trypan blue is based on the fact that the chromophore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable, i.e. dead cells are stained blue with the dye.

Procedure:

Before plating the cells for a new experiment:

1- Spin down remaining cell suspension for 5minutes at 1000 rpm at room temperature.
2- Aspirate the supernatant.
3- Resuspend cells pellet in 5ml of the medium.
4- Vortex the cell suspension to ensure the equal distribution of the cells in the suspension.
5- Before the cells have a chance to settle put about 10 µl of the cell suspension into a small tube, and add to it 10 µl of (Trypan blue stain 0.4 % Sigma ®) and mix gently for about 2 minutes using a small pipette.
6- Ensure that the haemocytometer is clean using 70% ethanol.
7- Using the pipette, draw up 10 µl from cell suspension containing trypan blue. Carefully fill the haemocytometer by gently resting the end of the pipette tip at the edge of the chambers. Allow the sample to be drawn out of the pipette by capillary action; the fluid should run to the edges of the grooves only. Re-load the pipette and fill the second chamber if required.
8- Count the number of non-blue cells in the (4, 4x4 squares).
9- Focus on the grid lines of the haemocytometer using the microscope. Focus on one set of 16 corners square.
10- Using a hand tally counter, count the number of cells in this area of 16 squares. When counting, always count only live cells that look healthy (unstained by trypan blue). Count cells that are within the square and any positioned on the right hand or bottom boundary line as indicated in Figure 7.
11- Move the haemocytometer to another set of 16 corner squares and carry on counting until all 4 sets of 16 corner squares are counted.

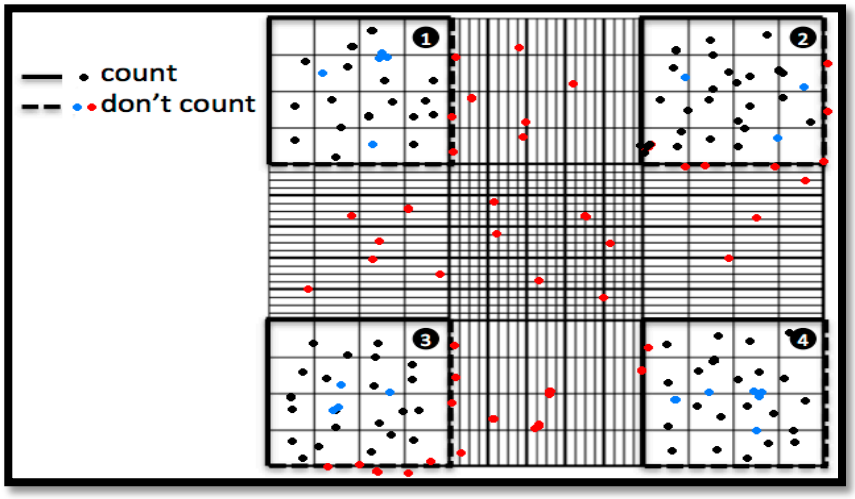

Figure 7: Schematic illustration of a counting chamber

The haemocytometer is designed so that the number of cells in one set of 16 corner squares is equivalent to the number of cells x 10^4 / ml. Therefore, to obtain the count we should calculate the total count from 4 sets of 16 corner = (square 1 + square 2 + square 3 + square 4) x104, then divide the count by 4 to calculate the mean, and then multiply by 2 to adjust for the 1:2 dilution in trypan blue. The result is equivalent to the number of cells x 10^4 / ml. As an example see Table 3

	Cell count	Total	Divided / 4	Multiplied / 2	Result
First square	100	348	87	174	174 x 10^4 cells/ml
Second square	90				
Third square	85				
Forth square	73				

Table 3: Calculation of the cell count per ml cell suspension

h- Plating cells for cytotoxicity test using EZ4U proliferation assay

By knowing the amount of the cells in the cell suspension, we can make the appropriate dilution, to seed appropriate number of cells as shown in Table 4, in order to get quantitative

information about cell survival after exposure to different concentrations of the test substance at different time intervals (4, 8, 16, 24, 48, and 72 hours).

Cell line	Incubation time (h)		
	4-8-16	24-48-72	
KB-3-1	5x 10^4 cells/ml	2x 10^4 cells/ml	The required number of the cells
A549	5x 10^4 cells/ml	2x 10^4 cells/ml	
CACO2	5x 10^4 cells/ml	2x 10^4 cells/ml	
FHs74Int	5x 10^4 cells/ml	5x 10^4 cells/ml	
MH-S	1x 10^4 cells/ml	5x 10^4 cells/ml	

Table 4: Number of cells /ml required in relation to the incubation time for every cell line under investigation

Once the cells are resuspended in the correct amount of culture media, make sure the cells are well mixed by using a pipette. Pipette 100 µl from the cell suspension tube into each well of the required 96 well culture plates. These plates should be labeled with the type of cells, number of cells, incubation period, assay number, plate number and date. Place cell culture plates into a 37°C CO_2 incubator overnight before using for an assay.

5 Addition of the test substance KR6-SX

By knowing the molecular weight of the test substance and the amount provided from the Laboratory we can calculate the molarity of the test substance and then we can prepare multiple stock solutions to examine the effect of different concentrations of the test substance

on the cell line under investigation. Therefore, we have prepared different stock solutions as shown in Table 5.

Stock solution I	0,1 µmol		112 0 µg /200ml
Stock solution II	100 µmol	12,18 µl (from I)/1ml	68,21µg/1ml
Stock solution III	10 µmol	100µl (from II) /1ml	6,821 µg/ml
Stock solution IV	0,1 µmol	10 µl (from III) /1ml	0,0682 µg/ml

Table 5: Used stock solutions of the test substance KR6-SX

Procedure:

After incubating the cells all over the night, the test substance should be diluted with medium to the desired concentrations. The most important thing is to put into account the 1:1 dilution in the plate (100µl of the cell suspension: 100 µl of the test substance), and therefore, the toxin concentration should always be multiplied by 2. Three wells should be loaded with the same toxin concentration.

Every well should be filled with 100 µl of the cell suspension and 100 µl of the test substance, and so every well will contain 200 µl. The wells in the first row filled only with pure medium to obtain a control value. The plates then should be incubated and analyzed after the desired incubation period.

Table 6 illustrates both of the calculations for different concentrations of the test substance KR6-SX, and their stock solutions.

Stock solution	Concentration of KR6-SX µmol/ml	100µl	200µl	ng/ml
From IV	**0,0001**	1	2	0,068
	0.0003	3	6	0.025
	0,001	10	20	0,682
	0,003	30	60	2,046
From III	**0,01**	1	2	6,82
	0,03	3	6	20,46
	0,1	10	20	68,21
	0.3	30	60	204.6
	1	100	200	682.1

Table 6: Toxin concentration in µmol/ml and related stock solutions

6- Analysis using EZ4U proliferation assay

Test principle:

The test is based on the finding that an intracellular reduction system in mitochondria of living cells reduces slightly yellow colored Tetrazolium salts to intense red colored Formazan derivatives. These derivatives are excreted into the culture medium and the absorbance is measured with a microplate reader. The amount of colored Formozan derivatives correlates with the amount of living cells.

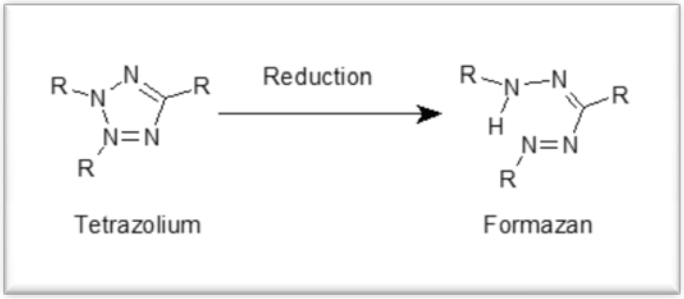

Figure 8: Reduction of Tetrazolium to Formazan

Procedure:

Firstly, dissolve the EZ4U substrate in 2.5 ml of activator, and then by a factor of 1:10 dilute it with the culture medium. The medium in the plates should be aspirated at first and then each well should be loaded with 100µl of this mixture (substrate + activator + medium). In order to obtain a blank value, the mixture should be added to around three border-wells, in which there are no cells. The plates after that should be incubated for 2-5 hours, and as soon the control wells are stained brick red, the plates should be analyzed using a photometer. The absorption of each well should be measured at 450 nm (with 620 nm as a reference wavelength).

7- Computation

The mean of the blank values should be calculated, and then each reading should be subtracted from the mean of the blank value (control and test). The mean value can be calculated through using the readings of three wells which is filled with the same concentration of the test substance (after subtraction of the blank value). The resulting values then should be converted to percent, and the control value should be considered as 100%. The results describe the survival percentage of the cells after exposure to the different toxin concentrations. The calculations and the graphics can be performed using excel or Sigma Plot.

8- Wash-out experiments

Test principle

These experiments try to show whether the cells, which were in contact for a short period with the test substance, and were then further cultured in fresh medium, can recover from the toxic effect of the test substance.

Procedure:

After seeding the cells and addition of the test substance, and after a specific incubation period, the test substance was sucked off using aspirator and the wells were rinsed with culture medium. Then the culture medium was sucked off again and the cells were re-incubated with fresh nutrient medium. The cytotoxicity can be determined after 24 h of the

first addition of the test substance (i.e. after 16 hours of washing the cells) using EZ4U proliferation assay as mentioned before.

D- Results and Discussion

The cells were exposed to different concentrations of the test substance KR6-SX, and the survival percentage was detected after specific incubation time using EZ4U assay and microplate reader. A cytotoxic effect was observed on all cell lines under investigation.

1- Cytotoxicity on KB-3-1 cell line

In the cervical carcinoma cell line **KB-3-1**, it is apparent that the cytotoxicity is highly dependent on the duration of exposure to KR6-SX. As an example: a concentration of 0.03 µM KR6-SX stimulated cell proliferation after 4, 8 and 16 h of incubation. But after longer exposure times, the cells started to die; after 24 hours 90% of the cells were still living, after 48 hours 70 %, and after 72 hours only 35% of the cells were still living after exposure to KR6-SX. By the highest tested concentration (1 µM) after 4 hours 60 % of the cells were killed, and after 8 and16 hours the survival rate approached 20%. By a concentration of 0.1 µM and incubation for 48 and 72 hours the survival rate approached zero % and nearly all the cells were killed. The survival rate reached zero% also after 24 hours of incubation with a concentration of 0.3 µM of KR6-SX. The dose-response curves for 48 and 72 hours differ only slightly from each other (Figure 9, Table 7).

Table7: EC $_{25,50,75}$ values of KR6-SX on KB-3-1 (µM)

	EC_{75}	EC_{50}	EC_{25}
4	**-**	**0.724**	**0.277**
8	**0.844**	**0.3**	**0.21**
16	**0.8**	**0.25**	**0.172**
24	**0.1**	**0.073**	**0.047**
48	**0.0748**	**0.046**	**0.0234**
72	**0.052**	**0.0228**	**0.00992**

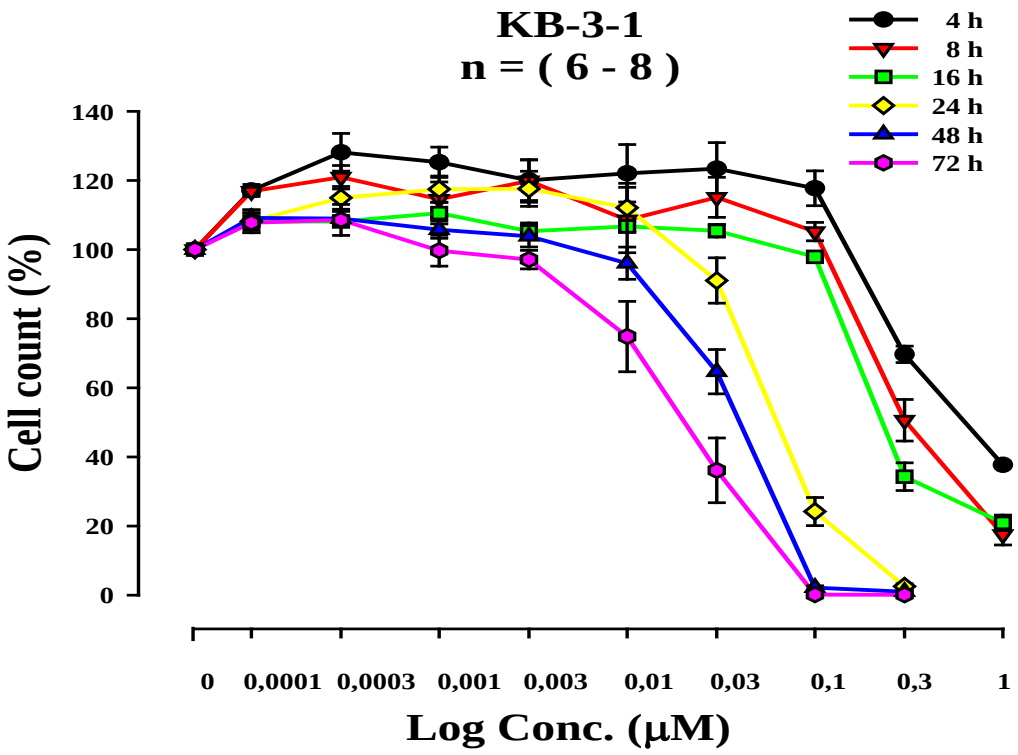

Figure 9: Cytotoxicity on KB-3-1 cell line in relation to KR6-SX concentrations at different exposure times.

2- Cytotoxicity on A549 cell line

In the lung carcinoma cell line **A549**, it is obvious that cytotoxicity is highly dependent on the exposure period and concentration of the test substance. From Figure 10 it can be seen that at a concentration of 0.003 µM KR6-SX the cells were first stimulated after 4-h incubation; the proliferation rate was near 130%. A similar process was observed at a higher concentration up to 0.03 µM. At a concentration of 0.03 µM KR6-SX it can be seen that the cells were more sensitive to KR6-SX. After 24 hours of incubation 25% of the cells were killed, and after 48 and 72 hours the survival rate approached zero %. Half of the cells were already killed after 8 h incubation at a concentration of 0.3 µM; and after 24 hours incubation it approached the zero line at a concentration of 0.3 µM KR6-SX. The dose-response curves of 48 and 72 hours differ only slightly from each other, i.e., the largest effect was expected after 48 hours incubation (Figure 10, Table 8).

Table 8: EC $_{25,50,75}$ values of KR6-SX on A549 (µM)			
	EC_{75}	EC_{50}	EC_{25}
4	-	-	0.278
8	-	0.50	0.138
16	0.296	0.212	0.122
24	0.0915	0.06	0.0281
48	0.027	0.0192	0.0112
72	0.0241	0.018	0.0115

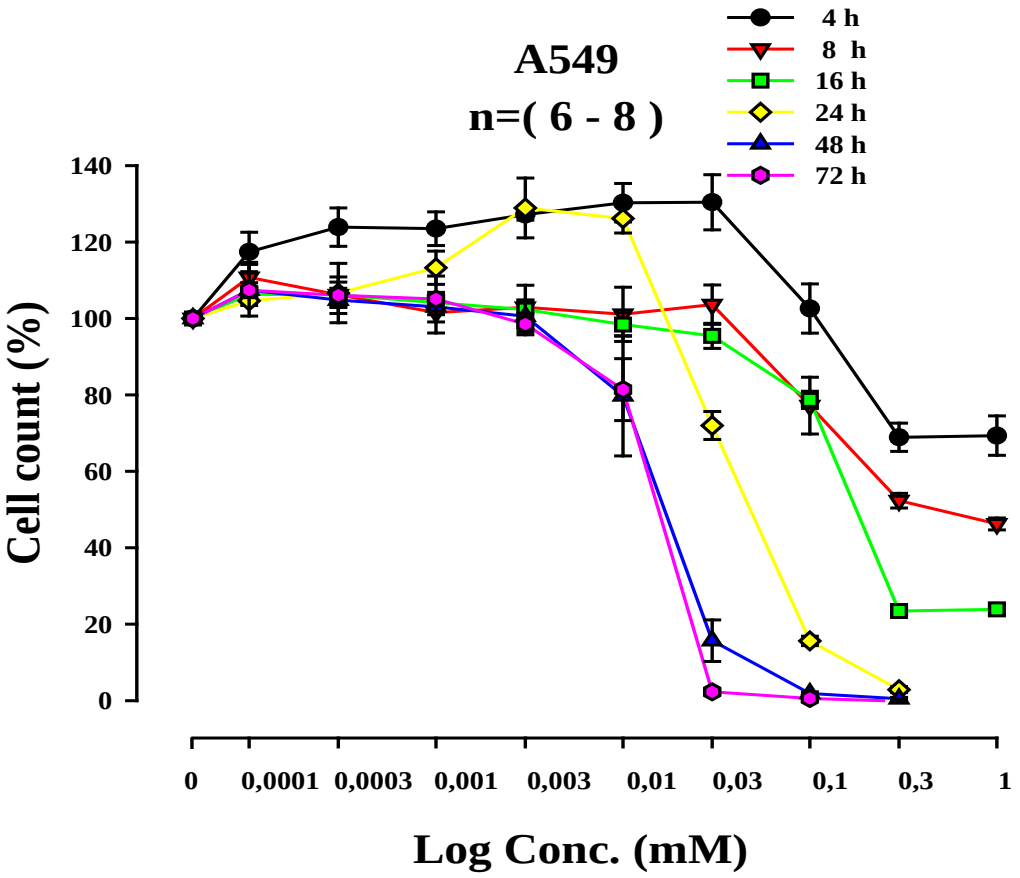

Figure 10: Cytotoxicity on A549 cell line in relation to KR6-SX concentrations at different exposure times.

3- Cytotoxicity on CACO2 cell line

In the colon carcinoma cell line **CACO2** it is obvious that the test substance stimulated the cells proliferation at low concentrations like 0.003 µM KR6-SX, and the cells continued to grow and the survival rate reached around 140% after 24 hours of incubation, but with a concentration of 0.01 µM KR6-SX we noticed a slight change in the cell proliferation as the cells began to die after 24 hours of incubation. At longer contact with KR6-SX, the number of the viable cells decreased rapidly and reached around 45 % after 48 hours and only 25 % after 72 hours of incubation. It is also apparent that the survival rate approached zero % at incubation times of 48 and 72 hours using a concentration of 0.03 µM KR6-SX. At the highest concentration of KR6-SX (0.3 µM) the dose-response relationship is well defined, i.e. by increasing the concentration of KR6-SX, the number of the viable cells dropped rapidly after short incubation periods, as it reached near 20 % after 8 and 16 hours incubation, and approached zero after 24 hours of incubation (Figure 11, Table 9).

Table 9: $EC_{25,50,75}$ values of KR6-SX on CACO2 (µM)

	EC_{75}	EC_{50}	EC_{25}
8	**0.3**	**0.2284**	**0.158**
16	**0.283**	**0.180**	**0.0884**
24	**0.074**	**0.0272**	**0.0176**
48	**0.02**	**0.0094**	**0.007**
72	**0.0098**	**0.0075**	**0.00515**

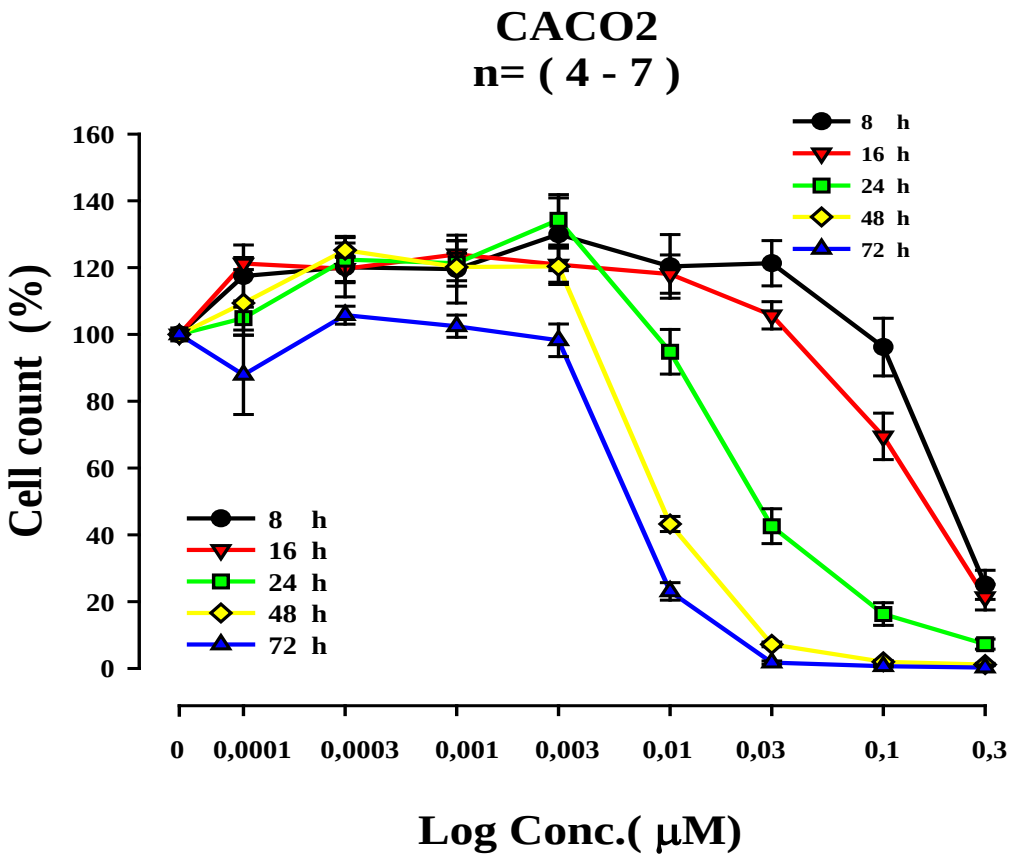

Figure 11: Cytotoxicity on CACO2 cell line in relation to KR6-SX concentrations at different exposure times.

4- Cytotoxicity on Macrophages (MH-S) cell line

In this type of non-cancer cells, it is apparent that the use of low concentrations of KR6-SX, for example 0.0001-0.001µM of the test substance, were not considered as immunotoxic, but there was a proliferating effect observed at all incubation times. However, after using a concentration of 0.003 µM KR6-SX, it is apparent that the cells firstly have a proliferating action at incubation times of 8, 16 and 24 hours. Then, by increasing the concentration of KR6-SX to 0.01 µM, the percentage of the viable cells reached around 70 %. But after 24 hours of incubation around 50 % of the cells were killed after longer exposure periods to KR6-SX (48 and 72 hours). All cells were killed after 48 and 72 hours of incubation using a concentration of 0.03 µM KR6-SX, or by using 0.1 µM KR6-SX for 24 hours only. More than 85 % of the cells were killed after 8 hours of incubation using the highest concentration of KR6-SX (0.3 µM), which may prove that KR6-SX is a highly immunotoxic substance. The dose-response curves for 48 and 72 hours incubation were almost the same, and so the largest effect was expected after 48 hours (Figure 12, Table 10).

Table 10: EC $_{25,50,75}$ values of KR6-SX on MH-S (µM)			
	EC_{75}	EC_{50}	EC_{25}
8	0.256	0.159	0.0864
16	0.168	0.0818	0.05
24	0.0296	0.018	0.00875
48	0.0209	0.00948	0.005
72	0.02	0.00895	0.0044

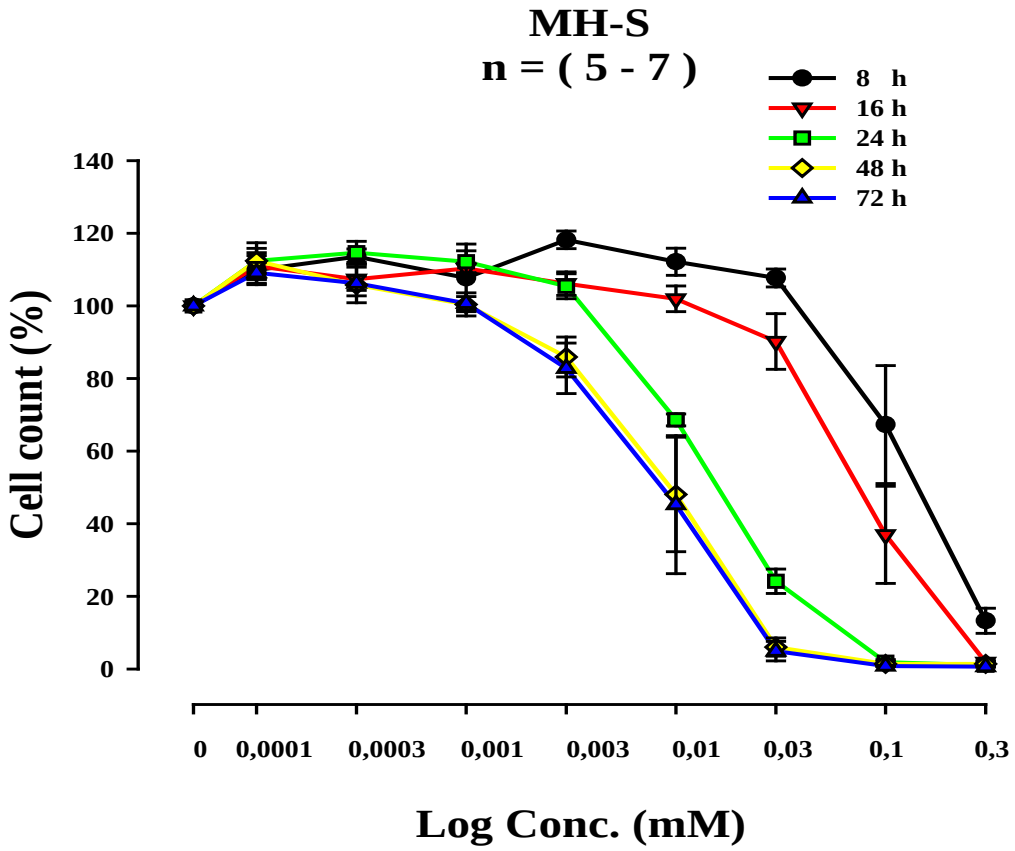

Figure 12: Cytotoxicity on macrophages (MH-S cell line) in relation to KR6-SX concentrations at different exposure times.

5- Cytotoxicity on FHs74Int cell line

The non-cancer intestinal cell line FHs74Int was very difficult to handle due to their slow growth rate and their yield was always very small. In addition, they have a strange behavior toward our test substance KR6-SX, as all cells were killed just after 24 hours of incubation using the highest tested concentration of KR6-SX (1 µM). Therefore, there weren't any viable cells to complete the experiments after 48 and 72 hours of incubation. From the graph we can see that by using a concentration of 0.01µM KR6-SX the cells showed proliferating pattern and the test substance stimulated the cells to grow rapidly reaching a survival rate of around 120 %, but by increasing the concentration of KR6-SX to 0.03µM, the survival rate decreased dramatically after 16 and 24 hours of incubation approaching 10 %. But after 8 hours incubation only 20 % of the cells were killed using the same concentration. By increasing the dose to 0.1µM 50 % of the cell population was killed after 16 hours of incubation, while all the cells were killed after longer exposure periods (16 and 24 hours). The curve for the short incubation period (4 hours) shows that there was a gradual decrease in the survival rate by low concentrations of KR6-SX. But the survival rate started to drop rapidly after using a concentration of 0.1µM, and at 0.3µM nearly 80 % of the cells were killed. This means that this type of cells is very sensitive to KR6-SX.

Table 11: $EC_{25,50,75}$ values of KR6-SX on FHs74Int (µM)

	EC_{75}	EC_{50}	EC_{25}
4	**0.278**	**0.189**	**0.102**
8	**0.234**	**0.0945**	**0.036**
16	**0.0248**	**0.0185**	**0.012**
24	**0.0275**	**0.0224**	**0.0167**

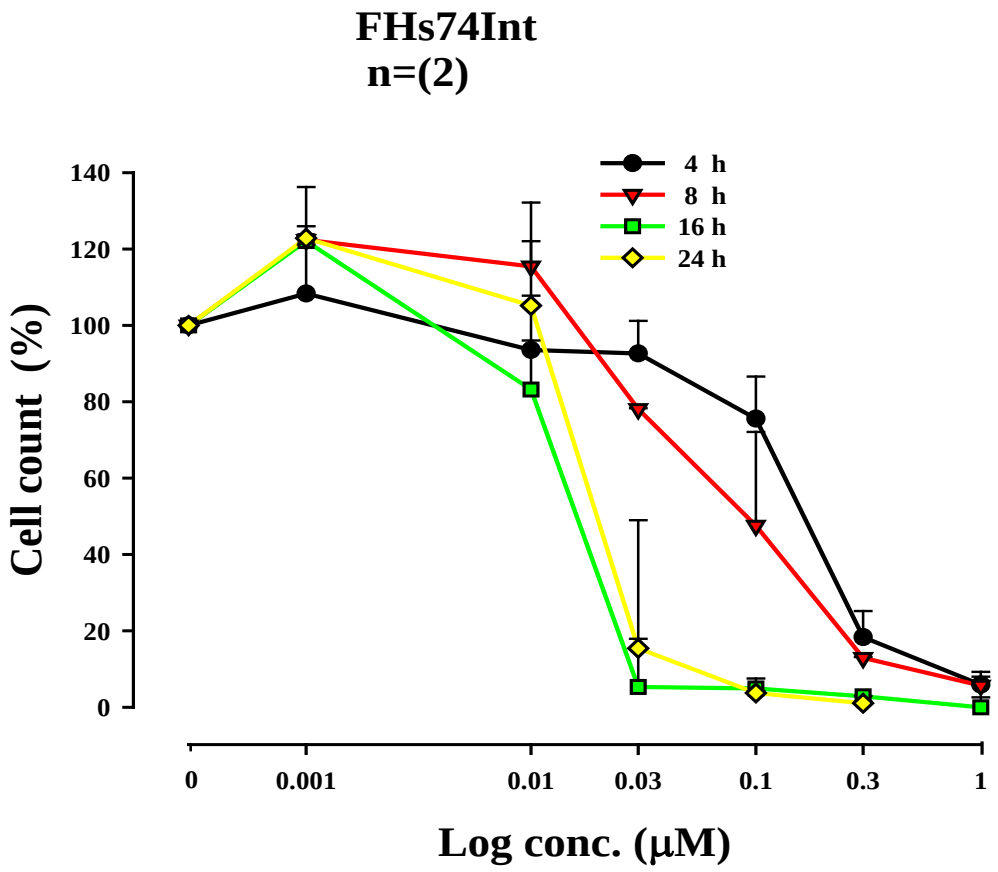

Figure 13: Cytotoxicity on FHs74Int cell line in relation to KR6-SX concentrations at different exposure times.

6- Reversibility of KR6-SX effects on CACO2 cell line

According to the dose-response curves, the cells continued to die even after washing out the test substance KR6-SX from the culture plate. The cytotoxic effect was apparent after washing the cells from the highest concentration of KR6-SX (0.3 µM). At this concentration all cells were killed 16-h after wash-out, i.e. the same behavior to the cells which were in contact with the test substance KR6-SX for 24 hours. In wash-out experiments with the low concentration of 0.03 µM KR6-SX, only 20 % of the cells were killed 16 hours after incubation in absence of the test compound, compared to more than 50 % of the cells which were in contact with KR6-SX for the same period. The survival rate reached around 25 % after incubation for 8 hours at the highest used concentration of KR6-SX (0.3 µM). Although

the short period of contact of the cells with the test substance KR6-SX, the cells get damaged and killed and can't recover. As a consequence, it may be assumed that the cytotoxic effect of KRSX is irreversible (Figure 14).

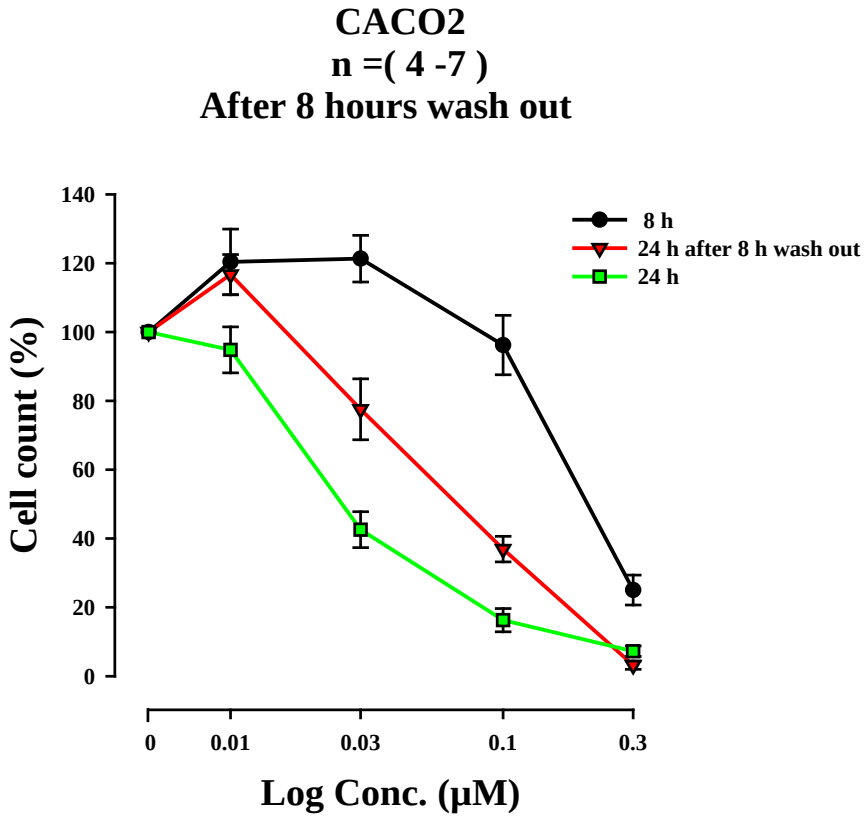

Figure 14: Reversibility of the cytotoxic effect of KR6-SX in CACO2 cells.

7- Comparison of EC_{75} values on different cell lines

Based on EC_{75} values, it is apparent that the non-cancer intestinal cells FHs74Int was the most sensitive cell towards KR6-SX, as to reach 75 % cell death of the cells population after 16 hours of exposure, it only required 0.0248 µM KR6-SX, whereas it took for the other cell lines more than 0.168 µM KR6-SX to reach the same result. By comparing the cancer cell lines, the least sensitive cell line appeared to be KB-3-1 cervical carcinoma cells, since it required 16 hours of exposure, and a concentration of 0.8 µM KR6-SX (near to the highest tested concentration) to reach 25 % of viable cell population. There was a slight difference in the EC_{75} values between all the cancer cell lines after 16 hours of incubation, but it is apparent that with longer incubation time, the colon carcinoma cell line CACO2 was the most sensitive to KR6-SX, as it required just 0.0098 µM KR6-SX to reach the 25 % of the cell count after 72

hours of incubation, whereas it took for the other cancer cell lines more than 0.0241 µM KR6-SX to be killed to 75% (Figure 15, Table 12).

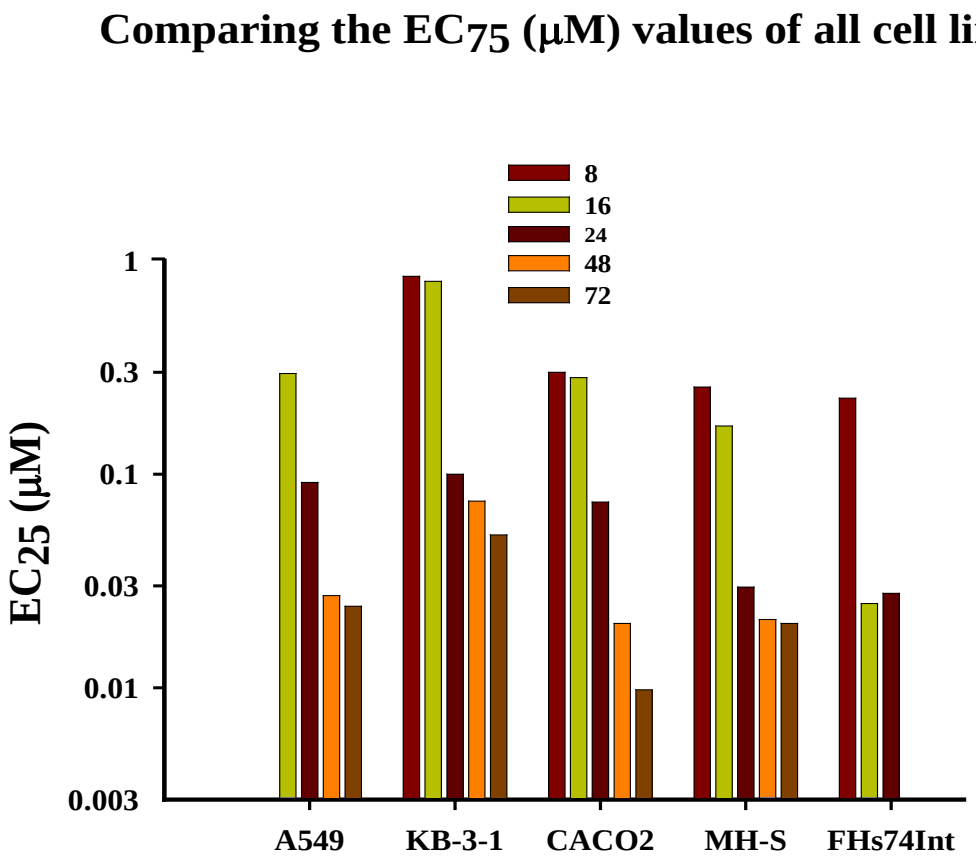

Figure 15: Comparing the EC_{75} (µM) values of all cell lines

Table 12: values of EC_{75} for all cell lines (µM)					
	A549	KB-3-1	CACO2	MH-S	FHs74Int
8	-	0.844	0.3	0.256	0.227
16	0.296	0.8	0.283	0.168	0.0248
24	0.0915	0.1	0.074	0.0296	0.0277
48	0.027	0.0748	0.02	0.0209	-
72	0.0241	0.052	0.0098	0.02	-

8- Comparison of EC_{50} values on different cell lines

In direct comparison between all cell lines under investigation, it is possible to recognize that the most sensitive cell towards our test substance KR6-SX was the non-cancer intestinal cells FHs74Int, as to kill 50 % of the cells after 8-hours of exposure. It only required 0.093 µM KR6-SX, whereas it took for the other cell lines more than 0.159 µM KR6-SX to reach the same result. This was also the same after 16 hours of incubation, as it required only 0.0184 µM KR6-SX to kill 50 % of FHs74Int cells, whereas it took for the other cell lines more than 0.0818 µM KR6-SX to be killed to 50 %. By comparing the cancer cell lines, there was a slight difference in the EC_{50} values between them in the short exposure time (8,16, and 24 hours), but it is apparent that with longer incubation time, the colon carcinoma cell line CACO2 was the most sensitive to KR6-SX, as it required just 0.00895µM KR6-SX to reach the 50 % of the cell count after 72 hours of incubation, whereas it took for the other cancer cell lines more than 0.018 µM KR6-SX to be killed to 50 %. The least sensitive cell line appears to be A549 lung carcinoma cells, since it required after 8 hours of exposure, a concentration of 0.5µM KR6-SX (near to the highest tested concentration) to be killed to 50% (Figure 16, Table 13).

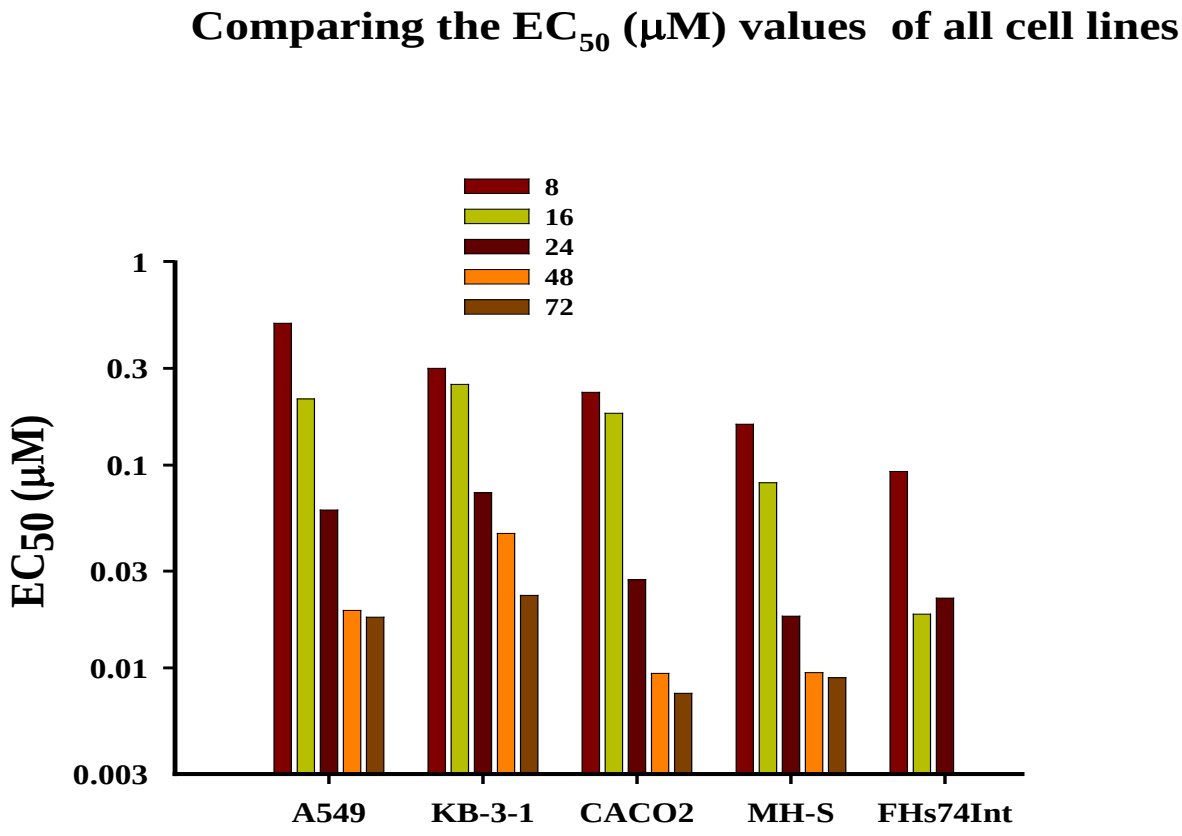

Figure 16: Comparing the EC_{50} (µM) values of all cell lines

Table 13: values of EC_{50} for all cell lines					
	A549	**KB-3-1**	**CACO2**	**MH-S**	**FHs74Int**
8	**0.5**	**0.3**	**0.2284**	**0.159**	**0.093**
16	**0.212**	**0.25**	**0.18**	**0.0818**	**0.0184**
24	**0.06**	**0.073**	**0.0272**	**0.018**	**0.0221**
48	**0.0192**	**0.046**	**0.0094**	**0.00948**	**-**
72	**0.018**	**0.0228**	**0.0075**	**0.00895**	**-**

9- Comparison of EC_{25} values on different cell lines

As expected like all the previous graphs, it is possible to say that the most sensitive cell towards our test substance KR6-SX is the non-cancer intestinal cells FHs74Int, as to reach cell death rate of 25 % after 8 hours of exposure, it only required 0.036 µM KR6-SX, whereas it took for the other cell lines more than 0.0864 µM KR6-SX to reach the same result. This was also the same after 16 hours of incubation, as it required only 0.012 µM KR6-SX to kill 25 % of FHs74Int cells, whereas it took for the other cell lines more than 0.05µM KRSX to be killed by 25%. But after 24 hours of incubation, there was a different result, as the most sensitive cell was the Macrophages MH-S cell line, because it required only 0.00875 µM KR6-SX to reach the cell count of 75 %, whereas it took for the other cell lines more than 0.0167 µM KR6-SX to be killed by 25 %. And by comparing the cancer cell lines, there is a slight difference in the EC_{25} values between them in the short exposure time (8 and 16 hours), but it is apparent that with longer incubation time, the colon carcinoma cell line CACO2 was the most sensitive to KR6-SX, as it required just 0.00515 µM KR6-SX to reach the 75 % of the cell count after 72 hours of incubation, whereas it takes for the other cancer cell lines more than 0.00992 µM KR6-SX to be killed by 75 %. The least sensitive cell line appears to be KB-3-1 cervical carcinoma cells, since it required after 8 hours of exposure, a concentration of 0.21 µM (Figure 17, Table 14).

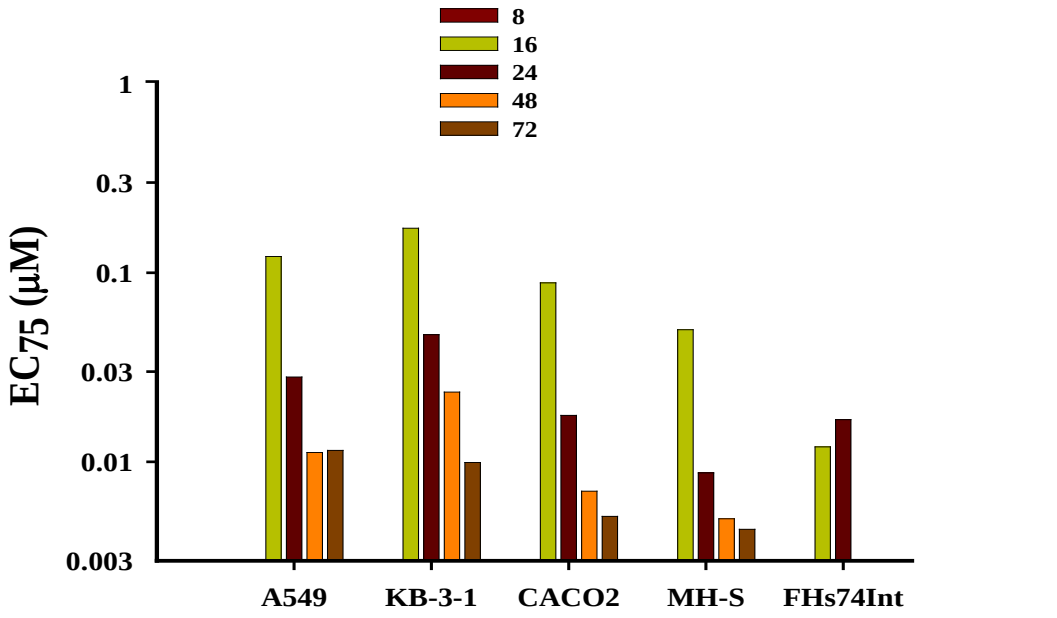

Figure 17: Comparing the EC_{25} (µM) values of all cell lines

Table 14: values of EC_{25} for all cell lines					
	A549	KB-3-1	CACO2	MH-S	FHs74Int
8	0.138	0.21	0.158	0.0864	0.036
16	0.122	0.172	0.0884	0.05	0.012
24	0.0281	0.047	0.0176	0.00875	0.0167
48	0.0112	0.0234	0.007	0.005	-
72	0.0115	0.00992	0.00515	0.0044	-

10- Cytotoxic effect of KR6-SX on the intestinal cell lines CACO2 and FHs74Int

Comparing the cytotoxic effect of KR6-SX on intestinal cell lines, unfortunately it appears that the non-cancer cell line FHs74Int is more sensitive toward KR6-SX, for example, to kill half of the cells, it required only 0.0184 µM KR6-SX after 16 hours of incubation, whereas it required around 0.180 µM KR6-SX to kill 50% of the cancer cell line CACO2 in the same period of time (Figure 18, Table 15).

Comparing the cytotoxic effect of KR6-SX on intestinal cell line based on the values of EC_{50} (µM)

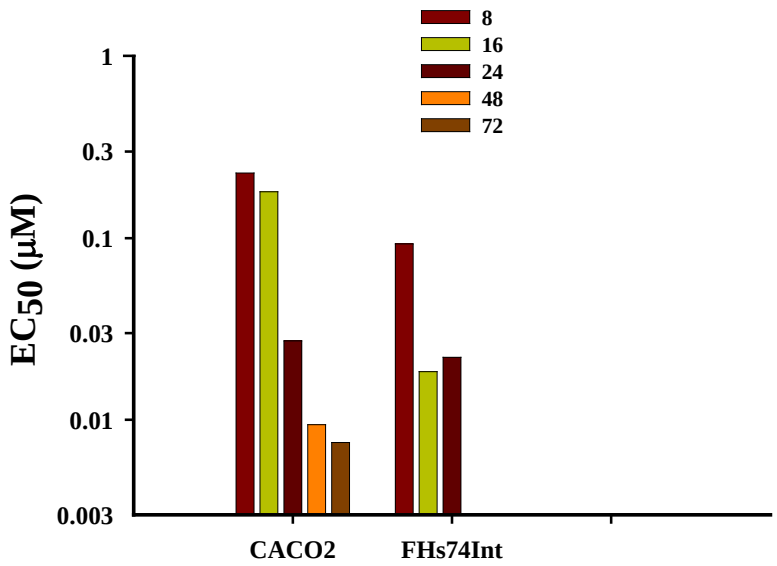

Figure 18: Cytotoxic effect of KR6-SX on intestinal cell lines

Table 15: values of EC_{50} for intestinal cell line		
	CACO2	FHs74Int
8	0.2284	0.093
16	0.18	0.0184
24	0.0272	0.0221
48	0.0094	-
72	0.0075	-

11- Cytotoxic effect of KR6-SX on the non-cancer cell lines MH-S and FHs74Int

Comparing the cytotoxic effect of KR6-SX on non-cancer cell lines, the graph showed that the non-cancer cell line FHs74Int is more sensitive toward KR6-SX than MH-S, for example, to kill half of the cells, it required only 0.0184 µM KR6-SX after 16 hours of incubation, whereas it required around 0.0818µM KR6-SX to kill 50% of the non-cancer cell line MH-S in the same period of time (Figure 19, Table 16).

Comparing the cytotoxic effect of (KR6-SX) on non-cancer cell lines based on the values of EC_{50} (µM)

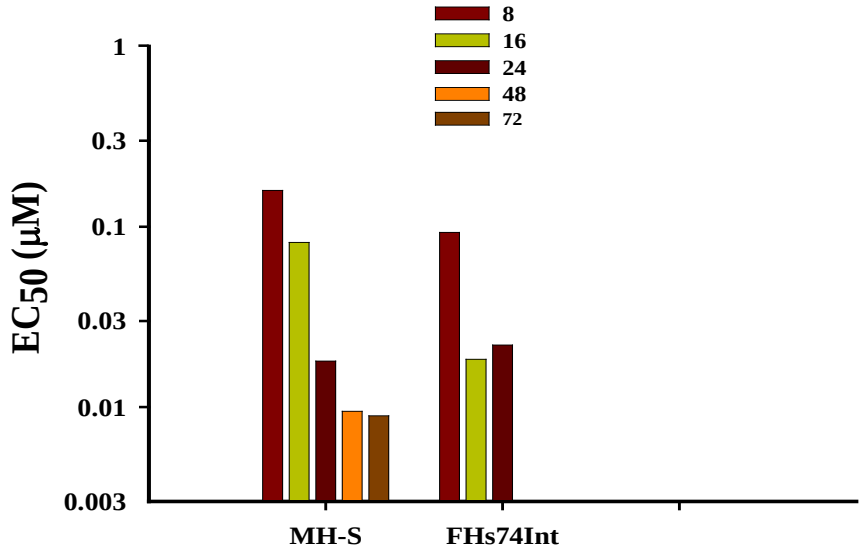

Figure 19: Cytotoxic effect of KR6-SX on the non-cancer cell lines

Table 16: values of EC_{50} for non-cancer cell lines

	MH-S	FHs74Int
8	0.159	0.093
16	0.0818	0.0184
24	0.018	0.0221
48	0.00948	-
72	0.00895	x

E- Conclusion

KR6-SX has a cytotoxic effect on all cell lines under investigation (cancer cell lines: A549, KB-3-1, CAC02; and non-cancer cell lines: MH-S, FHs74Int). The most sensitive cells towards our test substance were unfortunately the non-tumorigenic intestinal cells (FHs74Int) and macrophages (MH-S), which would lead to a high immunotoxic effect if it is applied on humans. The mechanism by which KR 6-SX leads to apoptosis, remains unclear. The cytotoxicity by KR6-SX is irreversible. As a result, and despite of the short contact with the cells, the cells were damaged and it couldn't to be repaired even after washing out the toxin. Furthermore, the cells continued to die after the process of washing.

The experiments show that there is a significant increase in the rate of the cells division before they die partially, this is most probably due to the fact that by adding the toxin KR6-SX, the cells were stimulated for proliferation in order to save their lives.

KR6-SX is therefore inappropriate as chemotherapeutic agents, because it is not toxic only on the cancer cell lines but also on the non-cancer cell lines, and with high extent. It shows also a high immunotoxic effect.

The metabolites that have a structure similar to this fungus, to KR6-SX, or one of its derivatives may be suitable to be screened as a cytostatic agent, and it would be interesting to study this.

F - Abstracts

Abstract - English

From a fungal species of the genus *Chryosporium* the metabolite KR6 SX Synchromycin was isolated. It was noted that KR6-SX has high cytotoxic activity. To prove this, and within the frame of my Diploma thesis, the cytotoxic effect of KR6-SX on three cancer cell lines (CACO2, KB-3-1, A549) as well as two non-cancer cell lines (FHs74Int, MH-S) was tested. It is proved that KR6 SX is cytotoxic to all these cell lines. The most sensitive cells to KRSX were the non-cancer cell lines (FHs74Int and MH-S). The cytotoxicity by KR6-SX is irreversible. As a result, and despite of the short contact with the cells, the cells were damaged and couldn't to be repaired even after washing out the toxin. Furthermore, the cells continued to die after the process of washing. KR6-SX is therefore inappropriate as chemotherapeutic agents, because it is not toxic only on the cancer cell lines but also on the non-cancer cell lines, and with high extent. It shows also a high immunotoxic effect. The metabolites that have a structure similar to this fungus or to KR6-SX or one of its derivatives may be suitable to be screened as a cytostatic agent, and it would be interesting to investigate this.

Abstract - Deutsch

Der Metabolit KR6-SynchromycinX wurde aus einer Pilzart der Gattung *Chrysosporium* isoliert. In Vorversuchen wurde festgestellt, dass KR6-SX eine hohe zytotoxische Aktivität aufweist. Um dies genauer zu untersuchen, wurde im Rahmen meiner Diplomarbeit die zytotoxische Wirkung von KR6 -SX auf drei Krebs-Zelllinien (CACO2, KB-3-1, A549) sowie zwei Nicht-Krebs-Zelllinien (FHs74Int, MH-S) getestet. Es wurde bewiesen, dass KR6-SX zytotoxisch auf alle dieser Zelllinien wirkte. Die empfindlichsten Zellen gegenüber KR6-SX waren die Nicht-Krebs-Zelllinien FHs74Int und MH-S. Durch Auswaschversuche konnte festgetellt werden, dass auch ein kurzer Kontakt der Zellen mit KR6-SX und anschließender Erholungsphase in frischem Medium reicht, um die Zellen irreparabel zu schädigen und in weiterer Folge abzutöten. Deshalb ist die Zytotoxizität von KR6-SX unumkehrbar. KR6 -SX ist deshalb ungeeignet als Chemotherapeutikum, weil es nicht nur auf die Krebszelllinien stark toxisch wirkt, sondern auch in hohem Maße auf Nicht-Krebs-Zelllinien. Es zeigt auch eine starke immunotoxische Wirkung. Metaboliten, die eine ähnliche Struktur wie KR66-SX oder eines seiner Derivate haben, wären möglicherweise als Zytostatikum geeignet, weshalb es interessant wäre diese zu untersuchen.

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H-Figures index

I-Table’s index

J – Curriculum vita (CV)

Name: Elmoataz Elmoner

Education:

1998-2003	Bachelor of Chemistry and Pharmaceutical Science, Tanta University
2009-2010	Master student in the field of Biopharmaceutics, Tanta University
2010-2011	University Preparation Program (German language preparation course), University of Vienna
2011-till now	Studies for the academic validation of the degree of Pharmacy, University of Vienna

Professional experience:

May 2003 - Nov 2003	Pharmacist at Suez Canal resort pharmacy in the northern coast of Egypt
Nov 2003 - Oct 2005	Pharmacist at a group of private pharmacies and ware houses
Jan 2004 - Till now	Governmental pharmacist at Almoatamadiah Hospital in Egypt
Nov 2005 – Sep 2010	Pharmacist at Almouwasat Medical Services Company in Saudi Arabia (joint commission international awarded health care facility)
2010 – Till now	Owner of a private pharmacy in Egypt