PALACKÝ UNIVERSITY OLOMOUC
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Lysosomal sequestration of anticancer drugs and multidrug resistance in cancer cells

DIPLOMA THESIS

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Abstract	The diploma thesis deals with multidrug resistance (MDR) and its mechanisms in cancer cells. In the theoretical part, the most important knowledge about the hallmarks of tumor cells, their treatment, and MDR, with the focus on lysosomal sequestration, are summarized. In the experimental part, the cells resistant to sunitinib (SUN) were established from the cells of chronic myeloid leukemia (K562 cells) and glioblastoma multiforme (T98G cells). The resistant cells were further characterized and the mechanisms of resistance, including lysosomal sequestration, senescence phenotype, and involvement of heat shock proteins (HSPs) were studied. In resistant T98G cells decreased proliferation, and changes in the morphology of lysosomes were observed. Nevertheless, the results did not unequivocally show the involvement of any of the studied mechanisms in the acquired SUN resistance of T98G and K562 cells.
Keywords	Multidrug resistance, lysosomal sequestration, tyrosine kinase inhibitors, lysosomal biogenesis, glioblastoma multiforme, sunitinib
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Abstrakt	Předložená diplomová práce se zabývá mnohočetnou lékovou rezistencí a jejími mechanismy (MDR) u nádorových buněk. V teoretické části jsou shrnuty nejdůležitější poznatky o charakteristických znacích rakoviny, její léčbě a MDR se zaměřením na lysosomální sekvestraci. V experimentální části byly získány rezistentní buňky k sunitinibu (SUN), odvozené z chronické myeloidní leukémie (buňky K562) a glioblastoma multiforme (buňky T98G). Rezistentní buňky byly dále charakterizovány a byly studovány mechanismy získané rezistence, zahrnující lysosomální sekvestraci, senescenční fenotyp a zapojení proteinů teplotního šoku (HSPs). V rezistentních T98G buňkách byla pozorována snížená proliferace a změny v morfologii lysozomů. Nicméně, výsledky jednoznačně neprokázaly zapojení žádného ze studovaných mechanismů u získané SUN rezistence buněk T98G a K562.
Klíčová slova	Mnohočetná rezistence, lysozomální sekvestrace, inhibitory tyrozinkináz, lysosomální biogeneze, multiformní glioblastom, sunitinib
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I hereby declare that I wrote this thesis independently and all used sources are included in the list of literature.

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TABLE OF CONTENTS

1 INTRODUCTION

The main cause of cancer development is the DNA mutations leading to the development of all necessary cancer hallmarks. These characteristics include replicative immortality, sustaining proliferative signaling, resisting cell death, and much more. The established tumors differ from each other in their genotype and phenotype. Based on their histochemistry, tumors may be divided into different groups according to the tissues they affect. Generally, they are divided into sarcomas (connective tissues), carcinomas (epithelial tissues), lymphomas with leukemias (lymphatic and hematopoietic tissues), and blastomas (neural tissue). One of many examples of brain tumors is the so-called glioblastoma multiforme (GBM). It is one of the most serious types of cancer as it is lethal within three months for patients without any treatment (Krex *et.al.,* 2007; Mačák *et.al.,* 2012).

Multidrug resistance (MDR) represents a crucial mechanism for cancer treatment failure. The whole process of MDR is based on cross-resistance between two or more drugs that are not necessarily related to one another. Sensitive cancer cells, therefore become resistant to a whole class of drugs that are neither structurally nor functionally different. The mechanisms of the acquired resistance involve mainly the mutation in the targeted sites, defects in the apoptotic pathway, or altered DNA pathway (Gottesman *et al*., 2002). Apart from these "classical" mechanisms, there can be other mechanisms such as lysosomal sequestration or acquired senescence phenotype involved as well. The mechanism that was studied in this diploma thesis is called lysosomal sequestration. The lysosomal inner environment is highly acidic so the hydrophobic weak bases, such as sunitinib, may freely pass through the lysosomal membrane. After the entry into the lysosomes, they become protonated and can start accumulating within thy lysosome (MacIntyre and Cutler, 1988; Zhitomirsky and Assaraf, 2016). During lysosomal sequestration, two phenomena including lysosomal biogenesis or exocytosis may occur.

In this diploma thesis, we observed and evaluated mechanisms of resistance in our established sunitinib resistant cells derived from chronic myeloid leukemia (K562) and glioblastoma multiforme (T98G). Sunitinib is indolinone-derivative and a multi-kinase inhibitor, that was proved to be sequestrated within the lysosomes of cancer cells (Gotink *et.al.,* 2015). Therefore, we observed the mechanisms of lysosomal sequestration, together with other mechanisms that could be involved in the sunitinib resistance such as senescent phenotype or heat-shock proteins overexpression.

2 GOALS OF THE THESIS

- Summarize the knowledge about the hallmarks of tumor cells, current treatment methods, causes of failure of anticancer treatment, and mechanisms of resistance.
- To establish SUN resistant K562 and T98G cells.
- To find out whether the lysosomal biogenesis is involved in the acquired resistance to SUN.
- To find out whether the senescence phenotype may be involved in the acquired resistance to SUN.
- To find out whether the heat shock proteins are involved in the acquired resistance to SUN.

3 THEORETICAL PART

3.1 Cancer and carcinogenesis

Cancer is one of the most common diseases causing the death of millions of people every year. Cancer cells are characterized by DNA mutations that lead to an increased rate of proliferation. These mutations are accumulated during life, for example in a form of point mutations, which may subsequently lead to activation of oncogenes and inactivation of tumor-suppressors genes, both leading to the cell transformation. Commonly known mechanisms of point mutations include deletion or insertions of a single base pair. Alterations in genes may also occur by rearrangement of whole chromosomes by mechanisms such as chromosomal translocation. Emerged cancer cells are then different in their genotype, as well as a phenotype in comparison to the normal body cells. (Renan, 1993; Vogelstein and Kinzler, 2004; Sarkar *et al.,* 2013).

Proto-oncogenes are inactive forms of oncogenes that take part in the differentiation and proliferation of cells. They can be aberrantly activated by mutation varying from point mutation to the mutation of whole chromosomes. In most cases, the mutation of proto-oncogenes happens in somatic cells, and alteration in only one single allele of the gene is sufficient for its manifestation as they have a dominant phenotypic effect. First oncogenes were discovered in retroviruses and up till now there have been more than 70 human oncogenes identified, each of them being linked with a certain type of human cancer (Weinberg, 1994; Vogelstein and Kinzler, 2004; Slabý *et. al*., 2015).

On the other hand, tumor suppressor genes, so-called antioncogenes, produce proteins responsible for the suppression of proliferation and for keeping terminally differentiated somatic cells in G_0-phase of the cell cycle. The genetic alteration must inactivate the tumor suppressor genes to contribute to the tumor formation. However, for the tumor phenotype to be shown, there must be both alleles of certain gene mutated or lost since they have recessive effect in phenotypes. The progress of gene inactivation is different in diseases inherited to diseases that occur sporadically. This phenomenon is described by Knudson's two-hit hypothesis (Fig. 1). The sporadic form works with the theory, that spontaneous mutation of one of the functional alleles occurs. Then the process of, so-called loss of heterozygosity (LOH) occurs, causing the inactivation or loss of second functional allele resulting in the homozygous state. In the case of hereditary diseases, the formation of a tumor, therefore, happens earlier, as there is already one of the alleles inactivated. This hypothesis was formulated already in 1971 by Alfred G. Knudson, who analyzed retinal tumor formation and the mutations in the RB1 gene. This gene together with other well know tumor suppressors and oncogenes are listed in the tables below (Tabs. 1, 2) (Vogelstein and Kinzler, 2004, Giacinti and Giordano, 2006; Morris and Chan, 2015; Slabý *et. al*., 2015).

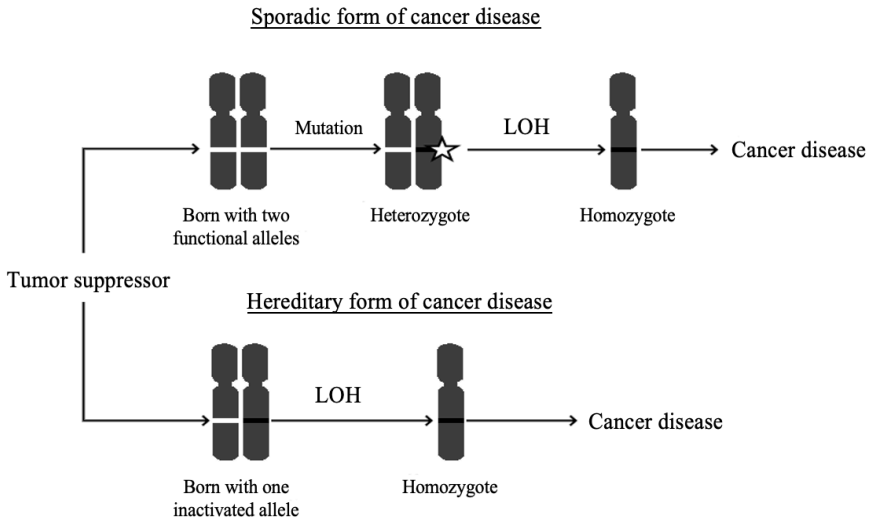


Fig. 1: Knudson's two-hit hypothesis. Healthy individual is born with two functional alleles of tumor suppressor gene. During the time, spontaneous mutation of one of the alleles takes place resulting in its inactivation. After some time, another mutation takes place resulting in LOH or so called, loss of heterozygosity. Only in this case the tumor phenotype develops. In comparison to individuals with already inherited inactivated allele, only LOH needs to take place to develop the tumor phenotype (After Slabý *et. al*., 2015)

Tab. 1: Examples of viral oncogenes and their characterization (After Slabý *et.al*, 2015).

Oncogenes				
Viral Oncogene	Cellular Protooncogene	Normal Function	Typical Somatic Mutation	Examples of Tumors
v-erb-b	EGFR/ERBB1	Receptor Tyrosine Kinase	Point mutation	Gliomas, Colorectal carcinoma
v-erb-b2	HER2/ERBB2	Receptor Tyrosine Kinase	Point mutation	Breast and Ovarian Carcinoma
v-ki-ras2	KRAS	Cell Signaling	Point mutation	Colorectal and Lung Carcinoma
v-abl	ABL1	Protein Kinase	Translocation	Leukemia
v-myc	MYC	Transcription Factor	Translocation, Gene Amplification	Neuroblastoma, Burkitt Lymphoma

Tab. 2: Examples of tumor suppressor genes and their characterization (After Vogelstein and Kinzler, 2004; Slabý *et al.,* 2015).

Tumor suppressor genes			
Gene	Normal Function	Localization within the cell	Examples of Tumors
APC	Regulation/degradation of free β-catenin	Cytoplasm	Familial adenomatous polyposis
RB1	Bonding of E2F transcription factor	Nucleus	Retinoblastoma, Osteosarcoma
TP53	Transcription factor	Nucleus	Carcinomas of intestines, breasts, lungs, Osteosarcoma
BRCA1	DNA repair	Nucleus	Breast and Ovarian Carcinoma
BRCA2	DNA repair	Nucleus	Breast Carcinoma

3.1.1 Characteristics of cancer cells

Cells of all cancer diseases can be distinguished by their unique properties. With continuous research in the past decades, there were six main hallmarks of cancer proposed. They comprise of evading growth suppressors, resisting cell death, sustaining of proliferative signaling, activation of invasion and metastasis, induction of angiogenesis, and lastly replicative immortality (Fig. 2). With our expanding understanding of the cancer mechanism, there are other hallmarks emerging, such as reprogramming of cell metabolism and evasion of destruction by the immune system (Hanahan and Weinberg, 2011; Pavlova and Thompson, 2016).

These hallmarks ensure that cancer cells can proliferate and survive in general. However, they are acquired in different time segments and their acquirement is enabled by genomic instability of cancer cells and by tumor-promoting inflammation. So-called “tumor microenvironment”, which is created by normal cells, contributes to the acquisition of all the cancer cells’ hallmarks as well (Hanahan and Weinberg, 2011).

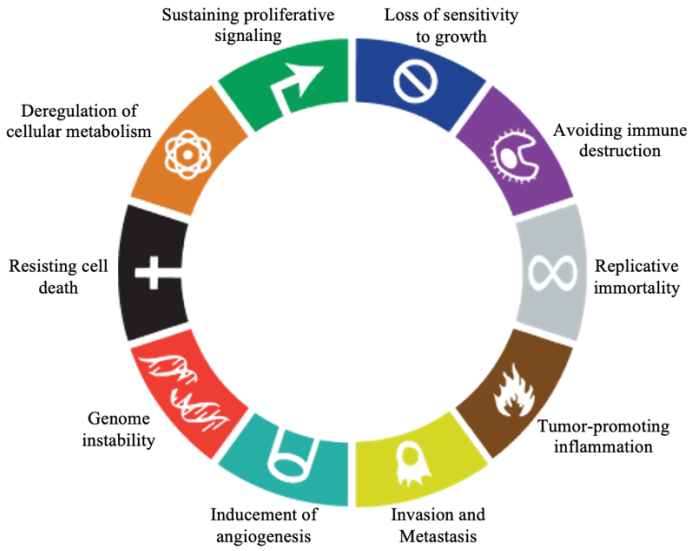


Fig. 2: The eight hallmarks of cancer with two enabling characteristics consisting of genome instability and tumor-promoting inflammation that facilitate the acquisition of the hallmarks (After Hannah and Weinberg, 2011).

3.1.1.1 Sustaining proliferative signaling

The increasing number of cells, which is closely accompanied by cell division, is called cell proliferation. In normal cells, it is a strictly controlled process, and it is dependent on signal molecules called growth factors or mitogens. These factors are usually produced by one type of cell and stimulate the cells nearby by so-called paracrine signaling. They are also able to bind to the receptors located on the cell surface and trigger intracellular signaling pathways, which then stimulate the cell cycle. Examples of growth factors are platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), or vascular endothelial growth factor (VEGF) (Hanahan and Weinberg, 2011; Yang *et. al.;* 2014; Alberts *et. al.,* 2015).

Nevertheless, antiproliferative signalization plays an important role as well. A typical representative of this signalization is induced by the transforming growth factor-beta family (TGF-β). This TGF-β family is responsible for tissue-specific control of proliferation and differentiation across embryonic and adult cells. Signalization by TGF-β starts with its bond to TGF-β receptor (TGF-βR) located in the cell membrane. This bond results in the phosphorylation of transcription factors belonging to the SMAD family. Specifically, factors SMAD-2 and SMAD-3 are activated and together form a common mediator SMAD complex (Co-SMAD). The whole complex is then translocated into the nucleus resulting in activation of transcription of specific genes, one of them being the *p15* gene. The p15 protein is an important inhibitor of cyclin-dependent kinase 4 (CDK4) and cyclin-dependent kinase 6 (CDK6). When p15 specifically binds to CDK4/6 it disables its bond with cyclin D. It is also able to displace an important tumor suppressor protein, p27, from its complexes with D-CDK4/6, allowing p27 to

inhibit other complexes such as A/CDK2 or E/CDK2 (Fig. 3). All these inhibitions result in TGF-β induced G_1-phase arrest. Nevertheless, many types of cancer exhibit inactivation in the TGF-β signaling pathway (Slabý *et.al*, 2015; Tzavlaki and Moustakas, 2020).

Cancer cells can sustain indefinite proliferation since they are not dependent on mitogenic, or antiproliferative signalization. They can achieve this attribute in many alternative ways. One of them is the production of their own growth factors resulting in not paracrine, but autocrine signaling. Another possibility is to target the receptors on their surface. Cancer cells can either increase receptor expression, or change their structural conformation (Hanahan and Weinberg, 2011). Proliferation in cancer cells can be also sustained by mutations and defects in negative feedback pathways. Typically, somatic mutations appear in RAS and RAF gene families causing the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) mitogen-activated protein kinase pathway (MAPK) (Lake *et.al.,* 2016). Mutations of RAS and RAF family genes occur in more than 30% of all human cancers. Mutations of *RAS* and *RAF* genes were reported in 90% of pancreatic adenocarcinomas and 45% of papillary thyroid carcinomas, respectively (Santarpia *et. al.,* 2012).

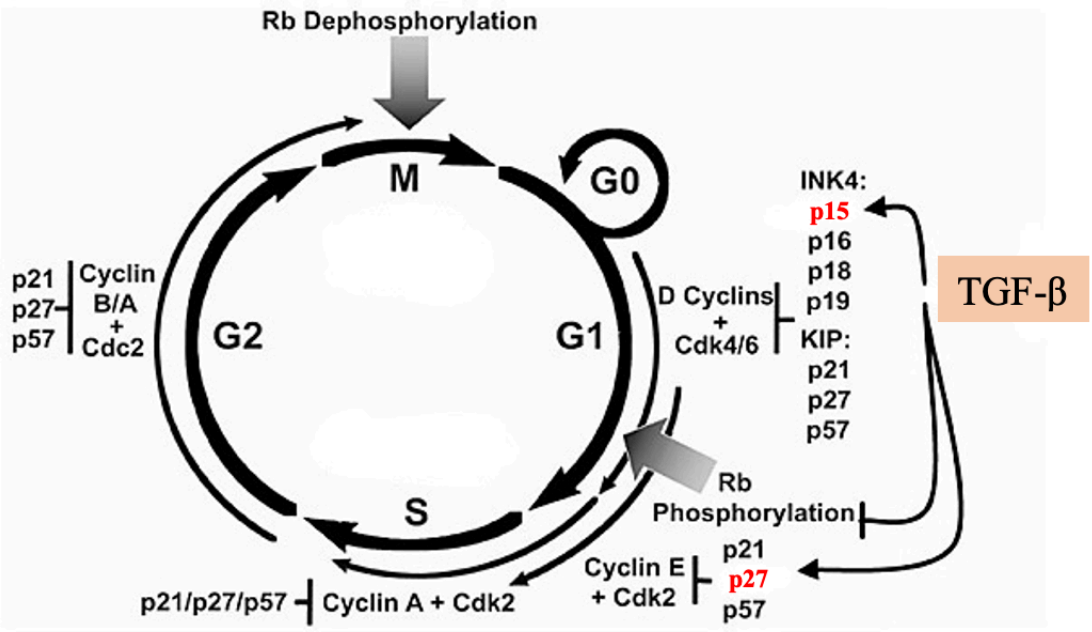


Fig. 3: The cell cycle. Cell cycle progression is governed by cyclin-dependent kinases (cdks), the activities of which are regulated by binding of cyclins, by phosphorylation and by the cdk inhibitors [the inhibitor of cdk4 (INK4) family: p15, p16, p18 and p19; and the kinase inhibitor protein (KIP) family: p21, p27 and p57] (After Donovan and Slingerland, 2000).

3.1.1.2 Loss of sensitivity to growth suppressors

One of the most typical characteristics of cancer cells is uncontrollable proliferation. Since the process of cell division is highly controlled and regulated by a lot of systems, cancer cells do have to avoid them to survive. One way is to lose the ability to respond to the growth suppressors that not only negatively regulate cell proliferation, but also control and repair damages in DNA (Hanahan and Weinberg, 2011; Yang and Karin, 2014).

Two proteins, retinoblastoma 1 protein (pRb) and tumor protein 53 (p53), encoded by tumor suppressor genes *RB1* and *TP53* may play a crucial role in cell-cycle regulation. They are the control points that decide whether the cell will carry on with proliferation or if the programs for cell death will be activated. pRb protein was firstly identified in a retina tumor called retinoblastoma. However, with time it was shown that inactivation of pRb, may be involved also in, formation of cervical cancer or Burkitt's lymphoma. Normal cells during the cell cycle go through "restriction point" (R point), where they decide if they proceed with their proliferation. This decision is mainly dependent on pRb protein which serves as the R point switch. pRb in the G1 phase is hypophosphorylated since it binds regulatory and transcription factors such as E2F. Complex between pRb and E2F blocks proliferation. When the expression of cyclin D increases, the subsequent activation of cyclin-dependent kinases causes the phosphorylation of pRb protein. With this phosphorylation, the E2F transcription factor that is necessary for gene expression and G1/S transition, is released from its bond with pRb, and products necessary for S-phase progression can form. Therefore, mutation or loss of the pRb protein function can cause deregulation of the cell cycle and cause malignant phenotypes (Weinberg, 1995; Giacinti and Giordano, 2006; Hanahan and Weinberg, 2011; Yang and Karin, 2014).

On the other hand, p53 tumor suppressor has been called "the guardian of the genome" based on the fact, that it plays an important role in cellular stress response and DNA damage. Under normal conditions, the concentration of p53 is very low since it interacts with Mdm2 protein. This protein acts as a ubiquitin ligase and therefore p53 is rapidly degraded. If the p53 protein is phosphorylated, Mdm2 cannot bind to this protein and the expression of targeted genes is increased. During small genome damage, p53 increases the transcription of p21 and Bax proteins. The p21 protein is a negative regulator that is involved in the control of the G1 and G2 checkpoint of the cell cycle. The concentration level of p53 arises in conditions where genome damage is excessive or when levels of necessary nutrients are suboptimal. In these situations, p53 can either cause cell-cycle arrest, senescence, or initiate apoptosis. Mutations or complete loss of p53 were discovered in more than 50% of human cancer cells. Clinical research also shows that inactivation or deletion of p53 causes inflammation which can induce cancer development (Mayo *et.al.,* 2002; Hanahan and Weinberg, 2011; Yang and Karin, 2014).

3.1.1.3 Resisting cell death

In mature organisms, not only mechanisms for maintenance and development of the cells are important. Destroying not only unwanted cells but also normal cells within the multicellular organisms plays just as an important role. Highly regulated and programmed cell death is called apoptosis, and its deregulation is one of the key hallmarks of cancer. During the process itself, the cells show typical morphological changes such as chromatin condensation with subsequent DNA fragmentation, cell shrinkage, the collapse of the cytoskeleton, and budding of the plasma membrane. The two most important types of the cell death are apoptosis and autophagy. Nevertheless, there exist different types such as ferroptosis or pyroptosis as well (Sarkar *et. al.,* 2013; Alberts *et. al.,* 2015; Pistritto *et. al.,* 2016).

The process of apoptosis can take place by either extrinsic or intrinsic pathways. The extrinsic pathway is initiated by extracellular signal proteins that bind to the cell-surface receptors of death. Therefore, this pathway is called death receptor dependent. The receptors on the plasma membrane belong to the tumor necrosis factor receptor superfamily (TNRF) and involve, for example, *Fas* receptor (FasR) or TNR-related apoptosis-inducing ligand receptor-R2 (TRAIL-R2). These receptors have a signature death domain (DD) which is structurally defined as an intracellular protein-protein interaction site. It has approximately 80 amino acids and is crucial for signal transmission to induce apoptosis. In case that the corresponding ligand, for example, Fas ligand (FasL), binds to the death domain of Fas receptor, formation of death-inducing signaling complex (DISC) takes place resulting in the crucial caspase cascade activation. In the extrinsic pathway, the first pro-caspase to be activated is procaspase 8, which is activates apical caspase 8. This caspase then activates effector caspases 3, 6, and 7, which contribute to the apoptotic mechanism (Sarkar *et. al.,* 2013; Pistritto *et. al.,* 2016; Yanumula and Cusick; 2020).

On the other hand, the intrinsic pathway is triggered from inside of the cell as a direct response to internal signals, such as stress or DNA damage. This pathway is also called mitochondrial as it is dependent on the release of cytochrome c from mitochondria. Regulation is based on keeping constant levels of anti-apoptotic (Bcl-2, Bcl-X_L, Bcl-W, Mcl-1, A1) and proapoptotic (Bax, Bak, Bim/Bid activators) proteins of the Bcl-2 family. The Bid activator can interact with other proteins such as Bax. When Bax and Bak are released from their inhibition by anti-apoptotic proteins, they disrupt the integrity of the outer mitochondrial membrane, causing the release of cytochrome c. It acts as a proapoptotic signaling protein and binds to apoptotic protease activating factor-1 (Apaf1). This bond then causes oligomerization of Apaf1 and the formation of the apoptosome, which is required for caspase-cascade activation. Apoptosome is a complex made up of Apaf1, ATP, and procaspase 9. The whole complex then allows activation of procaspase 9 to the apical caspase 9 resulting in activation of effector caspases (Lowe *et.al.,* 2004; Kile, 2009; Hanahan and Weinberg, 2011; Sarkar *et. al.,* 2013).

However, apoptosis is not the only representative of cell death. During normal physiological conditions, autophagy operates on low levels the same way as apoptosis does. Cells usually use autophagy for the elimination and degradation of damaged or old organelles. Nevertheless, cells can highly induce the level of autophagy as a response to intracellular or extracellular stress such as nutrient deprivation, hypoxia, or pathogenic infection. During insufficient nutrition, cells can increase the breakdown of organelles, such as mitochondria and ribosomes, and use the catabolites as a new source for energy metabolism. Autophagy can be divided into microautophagy, macroautophagy, and chaperone-mediated autophagy (Galluzzi *et.al*.2017). Microautophagy is characterized as the direct enclosing of degraded cell components with vacuolar or lysosomal membrane. On the other hand, macroautophagy is linked with the formation of intracellular vesicles that fuse with lysosomes and form so-called autophagosomes (Feng *et.al.*2014). Inhibition of autophagy by a pathway involving PI3-kinase, mTOR kinases, and AKT is like the inhibition during apoptosis. Autophagy can also be induced by BH3 only protein family. These proteins inhibit the anti-apoptotic proteins and activate the proapoptotic ones. During cell death, cells undergoing autophagy are considerably morphologically different from those undergoing necrosis or apoptosis. In recent studies, autophagy-dependent cell death was specified as regulated cell death depending only on the autophagy machinery, without involving other death pathways. (Morselli *et. al.,* 2009; Hanahan and Weinberg, 2011; Bialik *et.al.,* 2018; Yun and Lee, 2018).

3.1.1.4 Replicative immortality

Normal human cells have a finite capacity for replication, and after a certain amount of cell cycles, they stop replicating. This maximum number of cell cycles, that one somatic cell can undergo, is described as the Hayflick limit (Shay and Wright, 2000). Cells usually divide up to 50 – 70 times, then they either initiate process of a cell death or enter the state of replicative senescence. In this state, they decrease their metabolic activity and consumption of nutrients. Generally, the aging of the cells is caused by the shortening of telomeres which are structures at the end of both ends of chromosomes protecting the genome for example from nucleolytic degradation. Nevertheless, 90% of cancer cells can express high levels of telomerase, which is a specialized reverse transcriptase, that can elongate telomeres in cancer cells. In embryonic and adult stem cells the telomerase expression is very high. However, in differentiated somatic cells telomerase cannot be detected as it is transcriptionally silenced by the telomerase reverse transcriptase (*TERT*) gene. Therefore, in cancer cells, the reactivation of telomerase expression occurs by transcriptional derepression of *TERT* (Kim *et.al.,* 1994; Hanahan and Weinberg, 2011; Li and Tergaonkar, 2016).

When the level of telomere shortening in somatic cells exceeds a critical value, cells trigger p53 and pRb pathways. The cell cycle is then arrested in the G1/S phase and cells enter the state of senescence, or the process of apoptosis is initiated. Since cancer cells can elongate their telomeres by their highly active telomerase, or by alternative telomere lengthening (ALT), they gain infinite replicative and proliferative potential (Fig. 4) (Blasco, 2005; Sobinoff and Pickett, 2017).

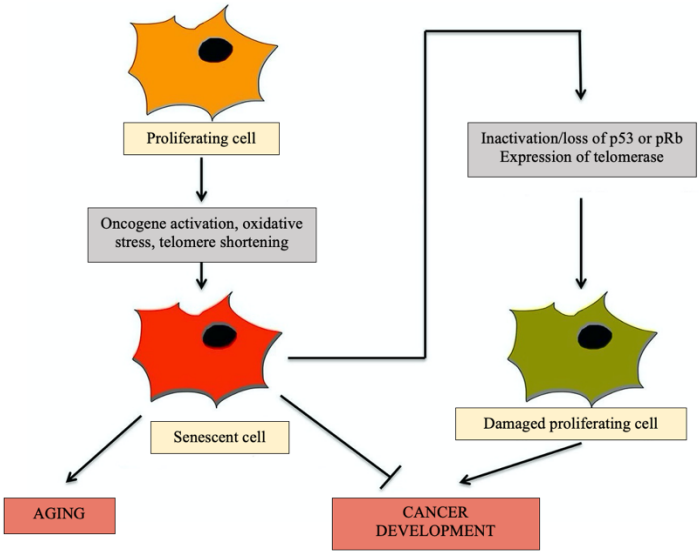


Fig. 4.: Senescence in tumor suppression mechanism, by blocking proliferation of cancer cells, and promotes aging via loss of macroscopic structure and inflammation (After Bhatia-Dey *et.al.;* 2016).

3.1.1.5 Induction of angiogenesis

During embryonic development, new blood vessels can be formed by either vascularization or angiogenesis. The process of angiogenesis is very complex and results in the formation of new capillaries from existing ones by sprouting. In adults, the process is arrested and occurs mainly during female reproductive cycles (Hanahan and Folkman, 1996; Hanahan and Weinberg, 2011).

Tumors in the human body can grow dependently on nutrients and oxygen from its surroundings only up to approximately 1-2 mm^3. When reaching this critical size, tumors can initiate a process of angiogenesis, which not only helps them with necessary supplies but also helps with dissemination and metastasis. This change from non-angiogenic to angiogenic phenotype does not develop gradually but happens by the so-called angiogenic switch (Hanahan and Folkman, 1996; Baeriswyl and Christofori, 2009). Angiogenesis is controlled by angiogenic and antiangiogenic factors. The most typical angiogenic and antiangiogenic factors are vascular endothelial growth factor (VEGF) and thrombospondin-1 (TSP-1), respectively. Expression of angiogenic factors is usually initiated by hypoxia. Low oxygen supply then stabilizes the hypoxia-

induced factor 1α (HIF1α), which activates the transcription of the *VEGF* gene (Tosetti et.al., 2002). During normoxia, HIF1α is hydroxylated by prolyl-4-hydroxylase. This allows bond formation between HIF1α and von Hippel-Lindau (VHL) tumor suppressor, which can mark HIF1α with ubiquitin and therefore cause its subsequent degradation. While this process can take place only in a presence of oxygen. When hypoxia occurs, prolyl-4-hydroxylase is inactive and HIF1α cannot be degraded. On the contrary, HIF1α is stabilized and translocates from cytoplasm to the nucleus where it induces the gene expression of angiogenic factors such as VEGF (Tosetti et.al., 2002; Ke and Costa, 2006).

One of the most significant angiogenesis inhibitors is thrombospondin-1 (TSP-1). It is a large glycoprotein in the extracellular matrix that binds to the endothelial CD36 receptor. TSP-1 transcription was shown to be regulated by p53 protein in fibroblast, as well as in mammary epithelial cells. In the case of p53 loss, the concentration of TSP-1 decreases and enables the initiation of angiogenesis (Dameron *et.al.,* 1994; Hanahan and Folkman, 1996). Other angiogenesis inhibitors can be derived from either plasminogen or from collagen 6 and 18 (Hanahan and Weinberg, 2011; Slabý *et.al*, 2015).

Already in Hanahan and Weinberg's article from 2011, it was mentioned, that pericytes are one of tumor neovasculature components. Pericytes are cells located along the walls of capillaries. In the central nervous system, they play an important role, for example, in blood vessel formation or brain blood flow (Attwell *et.al.,* 2016). It is now generally known, that pericytes play an important role within the tumor microenvironment, which ensures the growth and dissemination of the tumor. During physiological angiogenesis, pericytes are recruited into the blood vessel sites by platelet-derived growth factor subunit B (PDGF-B). When PDGF-B is overexpressed, the number of pericytes closely appose to the vessels is higher, and they help with the stabilization of new vasculatures (Ambrasson *et.al.,* 2003; Armulik *et.al.,* 2011; Hanahan and Weinberg, 2011).

With their role in angiogenesis, pericytes are together with fibroblasts and endothelial cells good targets for drug therapy. During angiogenesis, the therapy can be aimed either on inhibition of neovasculature, destruction of existing vessels, or stabilization of tumor blood vessels for their better uptake of cytostatic agents (Slabý *et.al*, 2015). Pharmacological treatments involve the usage of tyrosine kinase inhibitors (TKIs), such as sunitinib or imatinib, which can inhibit receptors of PDGF-F or VEGF (Kuhnert *et.al.,* 2008; Meng *et. al.,* 2015).

3.1.1.6 Invasion and metastasis

Generally, tumors can be divided into two groups based on their ability to spread within the organism. Benign tumors are usually well defined from their surroundings and their treatment is manageable by surgical removal. On the other hand, malign tumors represent a much bigger threat

to the organisms as they grow rapidly. They are also able to invade their surrounding (invasion) and spread to a distant part of the body through the blood and lymphatic vessels (metastasis). This ability is usually connected with decreased expression and loss of E-cadherin molecules, which are responsible for cell-to-cell adhesion. Normal cells can perceive contact with not only other cells but also with the extracellular matrix (ECM). When they are detached from ECM, a special type of apoptosis, called anoikis, is triggered. Contact inhibition between the cells can inhibit proliferation (CIP) and locomotion (CIL). CIP is based on the inhibition by cell density and its mechanism is damaged in several types of cancer. Transformed cells can overcome the contact inhibition and therefore continue to proliferate even after reaching the confluence. (Talmadge and Fidler, 2010; Hanahan and Weinberg, 2011; Mendonsa *et.al.,* 2018).

The migration of cancer cells in the organism is a highly coordinated process and is separated into specific steps. All together are these steps called invasion-metastasis cascade. Cells that are released from the primary tumor must overcome the ECM and basal membrane to get into the bloodstream. The cell then may leave its primary site, either as a single cell, or in clusters called micrometastasis. The blood circulation and lymphatic vessels mediate cell translocation to their new site, where they can exit into a new tissue or organ. At the new site, translocated cells once again pass through the basal membrane and ECM. This can result in the formation of a new tumor, like the primary one, just in different location (Hanahan and Weinberg, 2011; Micalizzi *et. al.,* 2010; Lamouille *et.al.,* 2014; Zeeshan and Mutahir; 2014).

3.1.1.7 Deregulation of cellular metabolism

Classic cellular metabolism is based on glucose being the main source energy. In normal cells, glucose is transported by glucose transporters 1-4 (GLUT1-GLUT4), metabolized by glycolysis into pyruvate, and then into carbon dioxide in the mitochondria (Hanahan and Weinberg, 2011).

Already in the 1920s, German physiologist Otto Warburg noticed, that cancer cells can reprogram their metabolism so that they shift their energy metabolism mainly to glycolysis even when the oxygen level is sufficient. This phenomenon was later called aerobic glycolysis or the Warburg effect (Warburg O., 1956; Vander Heiden *et al.,* 2009). During glycolysis in cytosol, only two molecules of ATP are produced per one molecule of glucose. On the other hand, total energy production, when including mitochondrial as well, comes to 36 molecules of ATP. However, the speed of aerobic glycolysis is approximately 100 times faster than mitochondrial oxidative phosphorylation. Therefore, when considering the same unit time, aerobic glycolysis is can generate more ATP than the classic oxidation of glucose. (Pfeiffer *et.al.,* 2001; Vaupel *et al.,* 2019). Increased rate of glucose consumption in cancer cells is used for visualization of many types of human cancer. The method of positron emission tomography (PET) works with

radioisotopes attached to a molecular probe that targets specific pathways in the metabolism of the cells. Considering the glucose consumption, radioactively labeled glucose analog, ^{18}F-fluorodeoxyglucose (^{18}F-FDG), is commonly used (Croteau *et.al.,* 2016).

Cancer cells usually upregulate the expression of glucose transporters (GLUT-1 and GLIT-3) for easier transport into the cytoplasm. Also, enzymes that catalyze the initial steps of glycolysis, such as hexokinase (HK) or phosphofructokinase (PFK), are overexpressed in more than 70% of cancer cells (Marín-Hernandez *et.al.,* 2009; Vaupel et al., 2019). A very important role in the upregulation of glucose transport holds activation of HIF1α and PI3K/Akt/mTOR-pathway. HIF1α may be activated either by hypoxic conditions or by reactive forms of oxygen (ROS). These forms of oxygen are produced in excessive amounts by cancer cells by the free electrons in mitochondria during low levels of oxygen. Whereas cancer cells are resistant to these high ROS levels, in normal cells the apoptosis would be triggered. Activation of these pathways may be triggered also by upregulation of heat-shock protein 90 (HSP90), by activation of oncogenes (Ras, Raf), or inactivation of tumor suppressors (p53, PTEN) (López-Lazaro; 2009; Vander Heiden *et al.,* 2009; Marín-Hernandez *et.al.,* 2009; Vaupel et al., 2019). The concentration of ROS, and therefore a higher probability of cancer cells surviving, may be lower by switching to aerobic glycolysis (Lu *et.al.,* 2015).

3.1.1.8 Avoiding immune destruction

The human immune system is very complex and is composed of cells, tissues, and organs, which can produce substances, that can fight and destroy foreign pathogens and infected or transformed cells. Generally, the immune system is divided into innate (macrophages, phagocytes, neutrophils, dendritic cells, natural killer cells) and acquired (T and B lymphocytes) systems, which differ in their specificity, memory, and response time (Alberts *et. al.,* 2015).

Process, where cancer cells can be recognized and destroyed by the immune system, is called immunosurveillance. However, it is now known, that cancer cells can avoid immunosurveillance and suppress the anticancer immune response to survive (Hanahan and Weinberg, 2011; Vinay *et.al.,* 2015). Cancer cell survival is carried out in three phases, all together being named cancer immunoediting. During the first phase, also called the elimination phase, active immunosurveillance identifies and destructs cancer cells. In the second, equilibrium phase, any cancer cells that survived the first phase, enter dynamic equilibrium with the host immune system. The immune system is capable to suppress tumor growth, yet it is not able to eradicate these cells. In this longest phase, many mutations, changes, and selection pressure occur with consequently shifting the equilibrium towards tumor growth. Subsequently, the third phase of escape or evasion occurs. In this stage, the immune system is not able to control the tumor

growth. Escape of the cancer cells is realized by either loss of antigen structures from the surface of cancer cells or by the tumor-induced immunosuppression. These cells then decrease or completely lose their expression of MHC class I. molecules (MHC I.) resulting in T-lymphocytes not being able to recognize them. By producing cytokines such as TGF-β or IL-10, cancer cells can inhibit the cytotoxic activity of macrophages or NK-cells (Dunn *et al.,* 2002; Vinay *et.al.,* 2015; Abbott and Ustoyev, 2019).

3.1.1.9 Tumor-promoting inflammation

Inflammation is a part of specific immunity which is involved not only in defense against pathogens but also in processes such as tissue regeneration and repair. Nonetheless, immune cells, that are engaged in inflammatory reactions, form together with fibroblasts and vascular cells so-called tumor microenvironment (TME). It is assumed, that nearly 20% of all cancer diseases are proceeded by chronic inflammation, which is presented in the tissue long before own tumor formation (Grivennikov *et.al.,* 2010; Greten and Grivennikov; 2019).

Inflammation and cancer may relate to two different pathways (Fig. 5). The intrinsic pathway is initiated by genetic alterations caused by the tumor. These alterations may involve activation of oncogenes or, the other way around, inactivation of tumor suppressors. On the other hand, the extrinsic pathway creates inflammatory conditions, that can increase the possibility of subsequent tumor formation (Mantovani *et.al.,* 2008). Both pathways then intersect within the cancer cells and activate transcription factors such as nuclear factor- κB (NF- κB), an activator of transcription 3 (STAT3), or hypoxia-induced factor-1α (HIF-1α). These factors can initiate the production of chemokines, cytokines (IL-1β, IL-6, TNF-α), and cyclooxygenase 2 (COX2). Chemokines may attract monocytes and dendritic cells (CCL2, CCL20) or promote angiogenesis (CXCL8). Interleukin 1β is one of the most important cytokines in the inflammation process. Cancer cells often express COX2, which is responsible for prostaglandin synthesis. This whole process activates the same inflammatory mediators in stromal cells, tumor cells, and inflammatory cells, resulting in mediator amplification. Emerged tumor microenvironment with inflammation supports proliferation, cell survival, angiogenesis, migration, or metastasis. (Mantovani *et.al.,* 2008; Grivennikov *et.al.,* 2010; Bottazzi *et.al.,* 2018; Greten and Grivennikov; 2019).

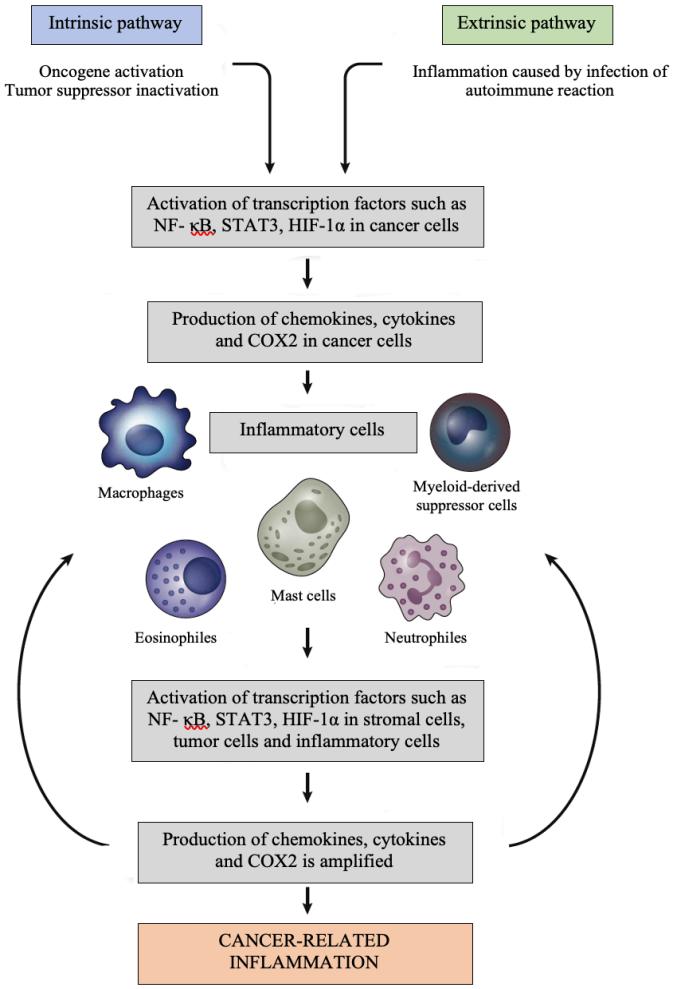


Fig. 5: Pathways that connect inflammation and cancer. Cancer and inflammation are connected by two pathways: the intrinsic pathway and the extrinsic pathway. The intrinsic pathway is activated by genetic events that cause neoplasia. These events include the activation of various types of oncogene mutation, chromosomal rearrangement or amplification, and the inactivation of tumor-suppressor genes. Cells that are transformed in this manner produce inflammatory mediators, thereby generating an inflammatory microenvironment in tumors for which there is no underlying inflammatory condition (for example, breast tumors). By contrast, in the extrinsic pathway, inflammatory or infectious conditions augment the risk of developing cancer at certain anatomical sites (for example, the colon, prostate and pancreas). The two pathways converge, resulting in the activation of transcription factors, mainly NF- κB, STAT3 and HIF-1α, in tumor cells. These transcription factors coordinate the production of inflammatory mediators, including cytokines and chemokines, as well as the production of cyclooxygenase 2 (COX2) (which, in turn, results in the production of prostaglandins). These factors recruit and activate various leukocytes, most notably cells of the myelomonocytic lineage. The cytokines activate the same key transcription factors in inflammatory cells, stromal cells, and tumor cells, resulting in more inflammatory mediators being produced and a cancer-related inflammatory microenvironment being generated. Smouldering cancer-related inflammation has many tumor-promoting effects (After Mantovani *et.al.,* 2008).

3.1.1.10 Mutation and genome instability

The cancer cell genome accumulates a huge number of mutations. All of them can take place during different stages of the cell cycle. Nevertheless, the most common mutations occur during DNA replication since it is the most vulnerable stage of the cycle (Aguilera and García-Muse, 2013). Many different factors might be responsible for mutations within the genome, and they are classified as biological, chemical, physical, and inherited ones (Tab. 3). Generally, tumors have very frequently mutated either p53 or pRb tumor suppressor. Protein p53 is called "the guardian of the genome", as already mentioned in chapter *3.1.1.2*., and its inactivation is one of the most common traits seen in sporadic cancer diseases (Lane, 1992; Andor *et.al*., 2017).

The instability of the genome is one of the conditions facilitating the process of carcinogenesis. In normal cells, changes during DNA repair or chromosome duplication are a natural source of genetic variation. However, defects and mutations in these cells are kept on very low levels, that are secured by cell-cycle checkpoints, double-strand breaks (DSB) repair, or nucleotide/base excision repair (NER/BER) (Aguilera and García-Muse, 2013; Andor *et.al*., 2017). Genome instability results in the accumulation of many mutations leading to the transformation of normal cells into cancer ones. The instability refers to a range of genetic alterations starting with point mutations leading up to chromosome rearrangements and aneuploidy (Aguilera and Gómez-Gónzales, 2008).

Tab. 3.: Overview of common factors causing mutations (After Slabý *et al.,* 2015).

Mutagenic agents	
Biological	Viruses (retroviruses, DNA viruses, HPV, EBV) Bacteria (*Helicobacter pylori*)
Chemical	Aromatic amines, amides (4-aminobyphenyl) Polycyclic aromatic hydrocarbons (benzopyrene) Toxins (aflatoxin) Other (benzene, asbestos, arsenic)
Physical	Ionizing radiation (α, β, γ, X-ray) Ultraviolet (UV)
Inherited	Li-Fraumeni syndrome – *TP53* mutation Cowden syndrome – *PTEN* mutation Lynch syndrome – *MLH1/MSH2/MSH6* or *PMS2* mutation

3.1.1.11 Heat shock proteins

All prokaryotic and eukaryotic cells can express heat shock proteins (HSPs), which are a family of proteins that acts as molecular chaperons. The HSPs are in the cytoplasm, mitochondria, endoplasmic reticulum, or in the nucleus. They participate in cell response to stress conditions as well as in processes such as protein assembly, protein degradation, or regulation of transcriptional factors. Generally, they are divided according to their molecular weight size into small HSPs, HSP40, HSP60, HSP70, HSP90, and HSP100 (Feder and Hofmann; 1999). Cells under normal conditions have low expression of HSPs. On the other hand, it was shown, that HSPs are overexpressed in various types of cancer cells as they are exposed to stress conditions. They are also actively engaged in tumor cell invasion, proliferation, metastasis, or death (Joly *et.al.,* 2010).

HSP27, a member of small HSPs, resides mainly in the cytosol and is involved in refolding damaged proteins, cell migration, or tumor progression. Its expression is reported to be increased in types of cancer such as prostate, ovarian, breast, and brain (Calderwood, 2010).

HSP70 family participates in the survival of stressed cells via the inhibition of lysosomal membrane permeabilization (Nylandsted *et.al.,* 2004). HSP70 can inhibit apoptosis by either downregulation of Bax in the intrinsic pathway or by inhibiting the assembly of DISC in the extrinsic pathway. The overexpression of the HSP70 family is in general linked with poor prognosis in colon, breast, or bladder cancers (Guo *et.al.,* 2005; Stankiewicz *et.al.,* 2005; Murphy, 2013).

The best characterized is the HSP90 protein that is involved in the folding, degradation, and stability of more than 200 proteins. The set of proteins, that require the assistance of HSP90 during their maturation, are referred to as client proteins. Usually, these proteins involve a variety of oncogenic kinases. HSP90 usually works together with HSP70 as their positive correlation in overexpression was find in some types of tumors. The tumor cells are highly dependent on the HSP overexpression and is one of their characteristic traits (Trepel *et.al.,* 2010; Wayne *et.al.,* 2011; Parimi and Tsang; 2014). Inhibitors of HSP90 are used as potential therapeutics since they can decrease the activity of oncoproteins in cancer cells. Since the tumor cells are more dependent on the expression of HSP90, they are more sensitive to the HSP90 inhibitors than the normal cells. The inhibition of HSP90 results in G2/M cell cycle arrest and the cells then may undergo apoptosis (Sharp and Workman, 2006; Parimi and Tsang; 2014).

3.1.2 Histological classification of tumors

As already mentioned in the chapter *3.1.1.6.,* tumors can be either malign or benign based on their potential to invade their surroundings or to form metastasis. This division is based on the behavior of the tumors and belongs into tumor classification called typing (Talmadge and Fidler, 2010). Nevertheless, typing can be also based on histology of the tumor and its placement in the tissues. Overview of the tumor names based on its position is summarized in Table 4 (Tab. 4) (Klauschen *et.al.,* 2015).

Tab. 4.: Tumor classification based on histology (After Mačák *et.al.,* 2012).

Tissue		Benign tumor	Malign tumor
Epithelial tissue	Stratified squamous	Papilloma	Carcinoma
	Glandular	Adenoma	Adenocarcinoma
Connective tissue tumors	Fat	Lipoma	Liposarcoma
	Cartilage	Chondroma	Chondrosarcoma
	Bone	Osteoma	Osteosarcoma
	Muscular	Leiomyoma	Myosarcoma
	Adult fibrous	Fibroma	Fibrosarcoma
	Vascular	Angioma	Angiosarcoma
	Hematopoetic	"Preleukemias", "myeloproliferative disorders"	Leukemia, Lymfoma
Neural tissue	Central	Glioma	Glioblastoma
	Peripheral	Ganglioneuroma, Paraganglioma	Neuroblastoma
Germinal tissue	Ovaries, testis	Gonadoblastoma, Teratoma (mature)	Saminom dysgerminoma, Teratoma (immature)

Typing is not the only way of tumor classification. Another way is called grading and is based on the microscopical differentiation of tumors. In this case, tumors are labeled by the letter G and a specific number (G1-G4). Another system that labels tumors by letters and numbers is staging,

which uses mainly the TNM (tumor-nodus-metastasis) system (Derwinger *et.al.,* 2010; Mačák *et.al.,* 2012).

3.1.3 Cancer therapy

The history of cancer therapy dates to the early 20th century. The term "chemotherapy" was first used by German chemist Paul Ehrlich. He used this term to describe the therapy for infectious diseases using chemicals. However, up until the 1960s, it was radiotherapy and surgery that were the most used methods for cancer treatment. Chemotherapy, radiotherapy, and surgery are the most used treatments up till nowadays. Nonetheless, the spectrum of different methods for curing cancer has grown largely throughout history (DeVita and Chu., 2008; Arruebo *et.al.,* 2011).

3.1.3.1 Chemotherapy

As already mentioned, chemotherapy started developing already in the early 20th century. However, it was not until the 1940s that the first successful treatment with chemotherapy took place. During the times of the First and Second World Wars, there was mustard gas used against the military soldiers. Mustard gas is found to be highly carcinogenic and mutagenic, and therefore the soldiers experienced low levels of leukocytes at these times (Gilman A., 1963; Arruebo *et.al.,* 2011). These observations led to the first cytostatic agent, nitrogen mustard, which was used to treat lymphomas. Nitrogen mustard can form interstrand cross-links (ICLs), which later force the cancer cells to undergo apoptosis initiated by the p53 pathway. One of the milestones in chemotherapy was the introduction of new cytostatic, the cisplatin, which has together with mitomycin C or psoralen, the same ability to form ICLs as nitrogen mustard (Nygren and SBU-group, 2001).

Nowadays, there are around 20 approved cytostatic used for the treatment of chronic myeloid leukemia (CML). This list involves busulfan, cyclophosphamide, or hydroxyurea. Busulfan in combination with mentioned cyclophosphamide (BuCy) was used as a conditioning therapy for hematopoietic stem cell transplantation in patients with acute and chronic myeloid leukemia (Ciurea and AndersSon, 2009). In the case of glioblastoma multiforme (GBM), temozolomide (TMZ) as an alkylating agent, was approved for the treatment of newly diagnosed glioblastoma and against other malignant gliomas. However, many GBMs show resistance to temozolomide treatment mainly because of the methylation of the *MGMT* promoter. The O^6-methylguanine-DNA-methyltransferase (MGMT) is an enzyme that participates in a process of DNA reparation. The methylation causes silencing and downregulation of this gene. Methylation of *MGMT* is may be present in 30 – 60% of all GBMs. Nevertheless, the GBMs therapy is

a combination of temozolomide, radiotherapy and surgical resection (Chinot *et.al.,* 2001; Hegi *et. al.,* 2005; Alifieris and Trafalis, 2015).

3.1.3.2 Radiotherapy

Radiotherapy is based on the usage of radioactive substances and energy rays to destroy cancer cells. Huge progress in radiotherapy is linked with the discovery of X-rays in 1895. One year after its discovery, X-rays were already used for the first breast cancer treatment (Gianfaldoni *et.al.,* 2017). Radiotherapy itself is based on using either corpuscular (protons, neutrons, electrons) or electromagnetic (X-rays, gamma-rays) radiation. Cancer cells can be destroyed by either direct damage of their DNA or by indirect damage within the cells caused for example by free radicals that emerged during the radiation (Cognetta *et.al.,* 2012; Gianfaldoni *et.al.,* 2017).

Even though chemotherapy is commonly used for GBM treatment, radiotherapy remains the standard approach for GBM therapy as well. Most importantly it is the first choice for unresectable GBM (Walker *et.al.,* 1980; Barani and Larson, 2015). Nevertheless, radiotherapy and chemotherapy are commonly used together. In a certain study, patients with glioblastoma were treated either by radiotherapy itself, or radiotherapy with continuous application of temozolomide. The results showed that the combination of TMZ and radiotherapy prolonged the average survival by two and half months (Stupp *et.al.,* 2009; Nachbichler *et.al.,* 2017). Considering the treatment of chronic myeloid leukemia, radiotherapy may be just used in certain situations. If splenomegaly causes pressure on other internal organs, radiotherapy is used for spleen reduction (Zaorsky *et.al,* 2017).

3.1.3.3 Targeted therapy

The history of targeted therapy dates back to the 1970s when the first targeted drug, tamoxifen, was approved. Generally, targeted therapy aims against either specific genes or proteins that are involved in cancer cell survival and proliferation based on the knowledge of the pathophysiology of individual diseases. Tamoxifen was developed as a targeted therapy for breast cancer treatment. It binds to the estrogen receptor (ER) and prevents estrogen to form a bond with this receptor. Therefore, tamoxifen regulates the activity of ER and is effective for patients with ER-positive breast cancer. During the time, many different types of targeted therapy started to emerge. Nevertheless, the two most used types are monoclonal antibodies and low molecular weight inhibitors including the tyrosine kinase inhibitors (TKIs) (Yan *et.al.,* 2011; Buss *et.al.,* 2012).

3.1.3.4 Monoclonal antibodies

Monoclonal antibodies (mAb) are structurally glycoproteins belonging to the immunoglobulins produced by B lymphocytes. The most common subclass of immunoglobulins used for targeted therapy is IgG1 (Scott *et.al.,* 2012). In contrast to polyclonal antibodies, monoclonal ones are identical in their protein sequences. Therefore, they are expected to have not only the same antigen-binding epitope but also the same binding affinity and the ability to downstream biologic effects. The most used host cell line to produce monoclonal antibodies is Chinese hamster ovary cells. (Kunert and Reinhart, 2016; Posner *et.al.,* 2019).

Monoclonal antibodies can interfere with cellular pathways that are necessary for tumor progression. Some of them may block growth surface receptors (e.g., cetuximab, trastuzumab) or can direct the immunological activity against cells with the specific surface target (e.g., rituximab) (Posner *et.al.,* 2019). Trastuzumab binds to human epidermal growth factor receptor 2 (HER2) causing G1-phase arrest and downregulates the PI3K/Akt pathway. In contrast, cetuximab binds to either epidermal growth factor receptor (EGFR) or human epidermal growth factor receptor 1 (HER1) (Hudis, 2007; Messershmith and Ahnen, 2008). On the other hand, the Fab domain of rituximab can bind to antigen CD20 located in the membrane of normal and malignant B-lymphocytes. By this bond formation, it may trigger B-cell lysis (Maloney, 2012; Posner *et.al.,* 2019). Targeted therapy using mAbs is largely used in the treatment of glioblastoma as well. Ipilimumab, which targets the immune checkpoint regulator CTLA-4 on T-lymphocytes, showed in a clinical trial dramatic improvement in survival of patients with metastatic melanoma, also including those with brain metastases (Prieto *et.al.,* 2012; Wilcox *et.al.,* 2018). Nivolumab and pembrolizumab are mAbs that are targeting the second T-lymphomas checkpoint regulator PD-1 Nivolumab is approved as a treatment for advanced melanoma and in combination with ipilimumab, it is used for BRAF V600 wild-type melanoma (Larkin *et.al.,* 2015; Wilcox *et.al.,* 2018). Pembrolizumab, on the other hand, is recommended for the treatment of non-small cell lung cancer (NSCLC) or advanced melanoma. Pembrolizumab is also included in ongoing phase II trial for glioblastoma therapy (Reardon *et.al.,* 2016).

3.1.3.5 Low molecular weight inhibitors

Mutations in protooncogenes and tumor-suppressors that regulate signal transduction connected with cell proliferation or apoptosis are some of the most common mutations found in cancer cells. Somatic mutations are frequently found in pathways regulated by protein kinases. Therefore, TKs are nowadays one of the crucial targets for human cancer treatment (Yan *et.al.,* 2011; Fabbro, 2014). TKs are enzymes that generally catalyze the intramolecular transfer of the phosphate

group. They can be represented in two forms, either as intracellular TKs (Ras, Raf) or as membrane receptors such as EGRF, VEGFR, or HER2/neu (Yan *et.al.,* 2011).

In the late 1990s the first tyrosine kinase inhibitor (TKI), imatinib (Fig. 8), was used in clinical trials, and in 2001 it was approved for the treatment of chronic myeloid leukemia (CML) (Deininger *et.al.,* 1997; Iqbal and Iqbal, 2014). Imatinib binds to the canonical BCR-ABL kinase domain found in CML. By this bond, imatinib prevents the transfer of phosphate group onto tyrosine which is necessary for BCR-ABL activation (Fig.6). Proliferative signals are therefore blocked from reaching the nucleus and apoptosis of leukemic cells is induced (Schindler *et.al.,* 2000; Sacha, 2014). In the historical perspective, it was showed that imatinib is not specific only towards the BCR-ABL kinase but is also able to inhibit other kinases such as KIT that is associated with gastrointestinal stromal tumors (GISTs). In 2002, FDA approved imatinib for the treatment of malignant and/or unresectable GISTs as well (Dagher *et.al.,* 2002). In some cases, resistance, or intolerance to imatinib during GIST therapy may emerge. As an alternative for imatinib, another TKI called sunitinib can be used (Demetri *et.al.,* 2006).

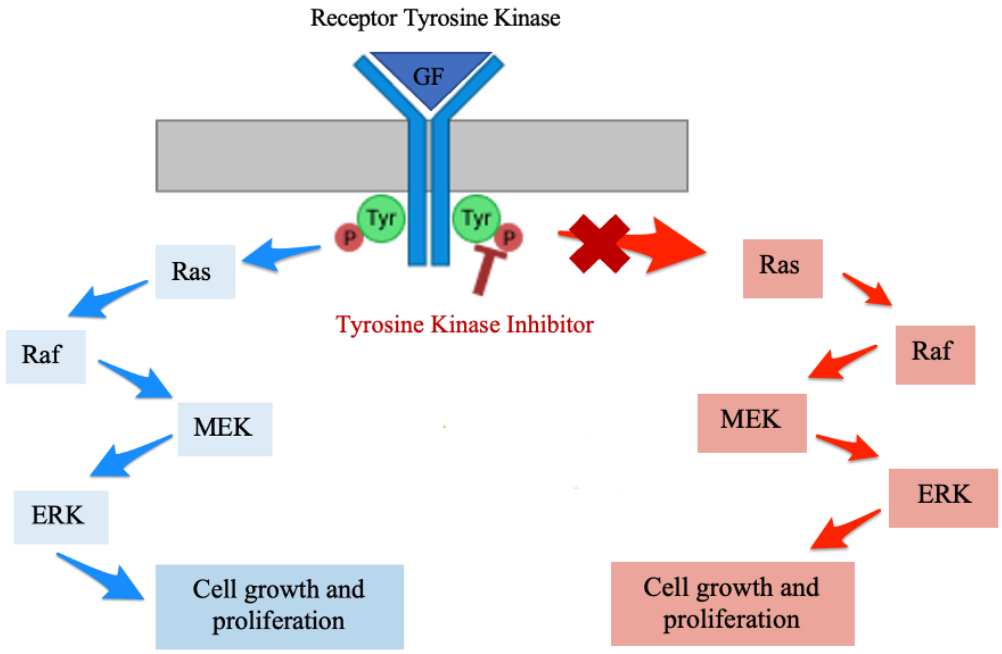


Fig. 6: Receptor tyrosine kinase signaling pathway. Tyrosine kinase receptor with its ligand (growth factor, GF) bonded. Tyrosine kinase inhibitor preventing the transfer of phosphate group onto tyrosine (After Kim and Ko, 2020).

Sunitinib (Fig. 7), the indolinone-derivative, is a multi-kinase inhibitor targeting for example receptors of VEGFR1, VEGFR2, PDGFR, FGFR, FLT3, and KIT. In comparison to imatinib, sunitinib represents a more polyspecific inhibitor. In 2006, sunitinib was approved for the treatment of renal cell carcinoma (RCC), as well as for therapy of imatinib-resistant or imatinib-intolerant GIST. Therefore, sunitinib was the first anticancer drug approved for two

different diseases simultaneously (Fabbro *et.al.,* 2012; Kim and Ko, 2020). Sunitinib may also be used for the treatment of well-differentiated and progressive pancreatic neuroendocrine tumors (pNETs) since they express high levels of VEGFR. In clinical trials, sunitinib prolonged the progression-free survival time from 5.5 to 11.4 months (Raymond *et.al.,* 2011). Antiangiogenic activity of sunitinib was in the past tested on human GBM cell lines. It was shown that certain concentrations of sunitinib reduced microvessel density (MVD) and even resulted in total inhibition of angiogenesis in tested specimens (de Boüard *et.al.,* 2007). Nevertheless, sunitinib and imatinib are only two examples of many other existing inhibitors.

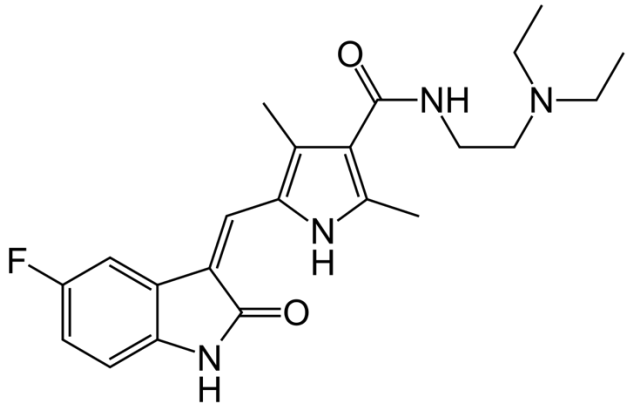


Fig. 7: Chemical structure of sunitinib ([*N*-[2-(diethylamino)ethyl]-5-[(*Z*)-(5-fluoro-2-oxo-1*H*-indol-3-ylidene)methyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxamide]).

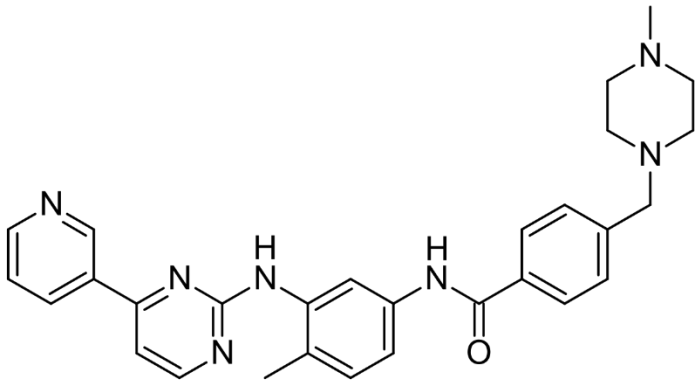


Fig. 8: Chemical structure of imatinib (4-[(4-methylpiperazin-1-yl)methyl]-*N*-[4-methyl-3-[(4-pyridin-3-ylpyrimidin-2-yl)amino]phenyl]benzamide).

3.2 Multidrug resistance in cancer cells

All cell types may behave differently when exposed to a specific drug for the first time. Some of them may exhibit so-called primary (natural, intrinsic) resistance which is often based on the localization of the cell within the organism or its differentiation. It results in the cells not responding to the drug treatment from the beginning. On the other hand, the secondary (acquired) resistance arises from the chemotherapy since the cells may respond at first but slowly gain resistance later. Acquired resistance is often caused by elevated expression of certain proteins in cell-membrane acting as the transporters which can increase the efflux of the particular drugs. Therefore, in the beginning the tumor responds to the treatment but with time the response disappears. Even increasing the dose of the drug is then insufficient to overcome the resistance and the higher concentrations of certain the drug can have a toxic effect on the patient as well (Stavrovskaya, 2000; Gottesman *et al*., 2002; Thomas et.al., 2003; Choi, 2005).

One of the main causes of drug treatment failure is so-called multidrug resistance (MDR). It describes the process of cross-resistance between two or more drugs that are not necessarily related to one another. Cancer cells, therefore, become resistant to a whole class of drugs that are neither structurally nor functionally different (Skovsgaards, 1978; Gottesman *et al*., 2002).

Both primary and secondary resistance can develop against any anticancer drug and can be developed by various mechanisms (Fig. 9). The classic mechanisms that are described are:

- Mutation in targeted sites (inhibitors of tyrosine kinases);
- Defects in apoptotic pathways (mutation of p53, increased expression of *Bcl-2* protein family);
- Activation of genes responsible for the regulation of detoxifying systems (cytochrome P450, glutathione *S*-transferase);
- Alterations in cell cycle with increased DNA repair
- Increased expression of ATP-binding cassette (ABC) transporters resulting in higher drug efflux from the cell (resulting in lowering intracellular drug concentration)
- Reduction in drug uptake (for example 5-fluorouracil or methotrexate).

Nonetheless, other new mechanisms such as the tumor microenvironment, senescence, or increased lysosomal sequestration, can participate in the development of resistance as well.

In the case of increased expression of ABC transporters, there are groups of drugs that are mainly affected by the efflux such as anthracyclines (daunorubicin, doxorubicin), vinca alkaloids (vincristine, vinblastine), paclitaxel, and more. These mechanisms can occur simultaneously resulting in so-called multifactorial multidrug resistance (Gottesman *et al*., 2002; Gillet and Gottesman, 2010).

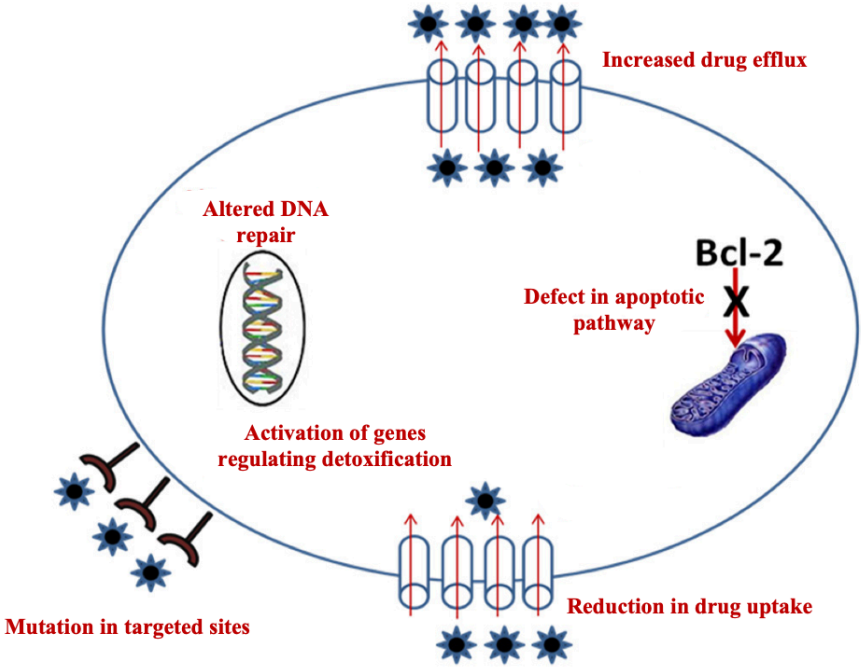


Fig. 9: Mechanisms of multidrug resistance in tumor cells. The main cause of the resistance to anticancer drugs are the mutations in targeted sites. Primary and secondary resistance is developed by increased drug efflux, defects in apoptotic pathway, reduction in drug uptake, altered DNA repair and activation of genes responsible for regulation of detoxifying systems (After Kapse-Mistry *et.al.,* 2014).

3.2.1 Structure and function of lysosomes

Lysosomes are membrane-bound organelles that were discovered already in the 1950s by Belgian scientist Christian de Duve (de Duve, 1974). In the past, lysosomes were mainly connected with the catabolic processes within the cells. With expanding research, the lysosomes were linked to more metabolic processes such as endocytosis, autophagy, apoptosis, reparation of the plasma membrane, and immune or homeostatic maintenance (Kirkegaard and Jäättelä, 2009).

In terms of morphology, lysosomes appear as spheric or ovoid bodies in all mammalian cells except for red blood cells. Their size may vary regarding the cell type that they are located in. In neurons or hepatocytes, they may reach the size of 1 μm, in macrophages up to several microns. However, their size and even the frequency may increase in relation to different pathological states (Lüllmann-Rauh and Saftig, 2007; Saftig and Klumperman, 2009).

The correct and precise function of lysosomes is dependent on the inner soluble and the membrane-bound proteins. The soluble protein class involves dozens different hydrolytic proteins with a pH optimum below 6. These acid hydrolases include lipases, nucleases, proteases, glycosidases, or phosphorylases. The highly acidic inner environment ($pH \leq 5$) is necessary for their optimal activity. These inner conditions are maintained by one of the membrane-bound proteins, vacuolar-type H^+-ATPase (v-ATPase), which by its activity secures a high concentration

of protons inside of the lysosome. Together with v-ATPase, structural proteins and ion channels belong to the membrane-bound proteins. The most essential structural ones are lysosome-associated membrane proteins 1 and 2 (LAMP1, LAMP2) together with lysosome integral membrane protein 2 (LIMP2) ((Lüllmann-Rauh and Saftig, 2007; Saftig and Klumperman, 2009). These proteins are heavily glycosylated as their molecular weight changes from 40 – 45 kDa to approximately 120 kDa after binding of the glycans. With this molecular weight, LAMP1 and LAMP2 contribute to about 50% of all lysosomal proteins integrated into the membrane. It was assumed that these proteins are in the membrane mainly to protect the lysosomal membrane against the hydrolytic enzymes. However, they participate even in the interaction and fusion between lysosomes themselves or between lysosomes with phagosomes, endosomes, or plasma membrane of the cell (Eskelinen, 2006). The third group of membrane-bound proteins is the ion channels, that enable ion transport in and out of the lysosomes. Maintenance of ion concentrations (H^+, K^+, Na^+, Cl^-, Fe^{2+}, Mg^{2+}, Ca^{2+},...) is not only important for the process of digestion products removal but the proper function of exporters as well (Xu and Ren, 2015). Two-pore channels (TPC) play a crucial role while releasing Ca^{2+} and Na^+ into the cytosol. They are not only located on lysosomes but on endosomes as well, and they involve members TPC1, TPC2, and TPC3 (Kintzer and Stroud, 2018). Another channel family is the transient receptor potential (TRP) channels. They are largely non-selective to cations and are located in different places within the cell. However, only TRPML1 and TRPM2 are located within the lysosomes and considering their function in ion transport, they play a role even during lysosomal biogenesis (Lange *et.al.,* 2009; Sterea *et.al.,* 2018).

3.2.1.1 Multidrug Resistance associated with Lysosomal Sequestration

Lysosomal sequestration, sometimes called lysosomal trapping, is now believed to be one of the mechanisms of multidrug resistance. Since the lysosomal inner environment is highly acidic, the hydrophobic weak bases such as sunitinib, daunorubicin or mitoxantrone, move freely across the lysosomal membrane. However, after the entry, they become protonated and start accumulating within the lysosome as they cannot cross the membrane anymore (MacIntyre and Cutler, 1988). Therefore, it is expected that the sequestration of these drugs into the lysosomes causes decreased cytotoxicity since the drugs cannot reach their targeted site in the cells (Duvvuri and Krise, 2005; Zhitomirsky and Assafar, 2015). Two lysosomal mechanisms can be induced by the lysosomal sequestration. One of them is lysosomal biogenesis that can increases back the lysosomal sequestration. The other one is lysosomal exocytosis which is believed to decrease the concentration of the drug in the cells (Raghunand *et.al.,* 1999; Zhitomirsky and Assafar, 2015; Zhitomirsky and Assaraf, 2017).

3.2.1.2 Lysosomal biogenesis

One of the cell stress response to the weak base inhibitors can be lysosomal biogenesis. This process can be regulated by the mammalian target of rapamycin complex 1 (mTORC1), which shows kinase activity on the membrane of lysosomes. mTORC1 can phosphorylate transcription factor EB (TFEB), which is the master gene for lysosomal biogenesis, and inactivate it. However, lysosomal biogenesis is triggered when TFEB is translocated from the cytosol into the nucleus (Martina *et.al.,* 2012; Halaby, 2019). In 2014, Zhitomirsky and Assaraf reported that TFEB-associated lysosomal biogenesis may be triggered by mitoxantrone and doxorubicin. This biogenesis also resulted in increased sunitinib sequestration (Zhitomirsky and Assafar, 2015). Nevertheless, in 2020, it was showed that cell lines exposed to non-toxic concentrations of sunitinib for a limited amount of time increase their TKIs sequestration capacity by lysosomes, however, not based on lysosomal biogenesis. The same article reported the lysosomal fusion as the mechanism for increase sequestration capacity (Skoupa *et.al.,* 2020).

3.2.1.3 Lysosomal exocytosis

Lysosomal exocytosis is a process where lysosomes travel on the periphery of the cells, fuse with the plasma membrane, and release their contents in the extracellular space (Xu and Ren, 2015). Lysosomal exocytosis is a Ca^{2+}-regulated process and, as the lysosomal biogenesis, it is also triggered by activation of TFEB. Usually, most of the lysosomes are located in the perinuclear zone. One of the steps of lysosomal exocytosis is the association of lysosomes with microtubules and their movement towards the plasma membrane. The whole process of lysosomal exocytosis starts when the drugs are sequestrated in lysosomes and therefore, they are unable to reach the intracellular targets. Then the expulsion out of the cell is mediated by lysosomal exocytosis (Groth-Pedersen and Jäätetelä, 2010; Zhitomirsky and Assaraf, 2017). In addition, it was reported, that sarcoma tumor may gain their malignant abilities by lysosomal exocytosis induction. At the same time, overexpression, and secretion of lysosomal enzymes, such as cathepsins B, D, K, and L may influence tumor growth, angiogenesis, and invasion (Machado *et.al.,* 2015).

3.2.2 Senescence and drug resistance

One of the possible mechanisms involved in drug resistance is also the state of senescence. As already mentioned in chapter *3.1.1.4*., normal cells have a finite proliferative capacity. When they reach this proliferative limit, they enter the state called proliferative senescence. In this state, the cells enter the G_0-phase of the cell cycle and do not proliferate anymore. Morphology of the cells changes into being typically enlarged and flattened. A commonly used biomarker for senescent cells is their senescence-associated ß-galactosidase activity (Dimri *et.al.,* 1995; Gewirtz *et.al.,*

2008). Generally, the proliferating cells are more sensitive to different factors than the cells in the senescent state. Since cancer cells are highly proliferating, they are sensitive to chemotherapy. In comparison, the somatic cells are not affected since they are in the G_0-phase of the cell cycle and do not proliferate anymore. Nevertheless, it has been shown that cancer cells can enter a comparable senescent state, called "accelerated senescence", which can be induced by chemotherapeutic drugs or radiation (Gewirtz *et.al.,* 2008). Even though the senescent tumor cells have their growth arrested, they may secrete proteins that have anti-apoptotic, mitogenic, and angiogenic activities (Chang *et.al.,* 2002). In past study it was observed that a senescent phenotype induced by antitumor agents was generated more often in the resistant cells (Achuthan *et.al.,* 2011). The impact of accelerated senescence is not fully described, but it can be considered as one of the mechanisms, that can be involved in the promotion of drug resistance (Gewirtz *et.al.,* 2008; Gordon and Nelson, 2012).

4 EXPERIMENTAL PART

4.1 Material

4.1.1 Cell line K562

Cell line K562 is derived from chronic myeloid leukemia, which is a malignant disorder of pluripotent hematopoietic stem cells. It is characterized by the presence of the Philadelphian chromosome. This chromosome is formed by the fusion of *BCR* and *ABL* genes that are located on chromosomes 22 and 9, respectively. Philadelphian chromosome, therefore, encodes BCR-ABL tyrosine kinase, which is responsible for the malignant transformation of the cells (Sawyer, 1999; Neumann *et.al.,* 2001). This cell culture was obtained from the European Collection of Cell Cultures, Great Britain (ECACC).

Cell line K562 was cultivated in DMEM medium supplemented with a 10% fetal bovine serum (FBS) and with the addition of penicillin and streptomycin (0.1 mg/ml) in a 5% CO_2 atmosphere at 37 °C. They were stored in cell culture flasks and passaged twice a week.

4.1.2 Cell line T98G

Cell line T98G is derived from human glioblastoma multiforme tumor. The T98G cells are typical for their increased expression of the *MGMT* gene, encoding the methylguanine methyltransferase (MGMT), which is involved in the resistance to temozolomide (Stein, 1979; Braun and Ahluwalia, 2017). This cell culture was obtained from the European Collection of Cell Cultures, Great Britain (ECACC).

Cell line T98G was cultivated in RPMI-1640 medium also supplemented with a 10% fetal bovine serum (FBS) and with the addition of penicillin and streptomycin (0.1 mg/ml) in a 5% CO_2 atmosphere at 37 °C. They were stored in cell culture flasks and passaged twice a week.

4.1.3 Chemicals and Reagents

- 1x phosphate buffered saline (PBS): 130 mmol/l NaCl; 10 mmol/l NaH2PO4; 2,7 mmol/l KCl; 1,8 mmol/l KH2PO4; pH 7,4
- Sunitinib ([N-[2-(diethylamino)ethyl]-5-[(Z)-(5-fluoro-2-oxo-1H-indol-3-ylidene)methyl]-2,4-dimethyl-1H-pyrrole-3-carboxamide].: MedChemExpress (MCE), USA

4.1.4 Devices and Instruments

Analytic scale	ABS 120-4N (KERN, Germany)
Digital scale	DENVER MXX 612 (Merci, Czech Republic)
Magnetic stirrer	RCT basic IKAMAG (Verkon, Czech Republic)
Shaker	Bio RS-24 (BioSan, Latvia)
Cell culture incubator	CelCulture (ESCO, USA)
pH meter	pH meter 3510 (Jenway, Great Britain)
Centrifuge	BOEco M-240 (BioTech, Germany)
Centrifuge	HERMLE Z 326 K (LaborTechnik, Germany)
Minicentrifuge	myFuge^{TM} (Benchmark Scientific, USA)
Thermoblock	Bio-TDB 100 (BioSan, Latvia)
Vortex	BioVortex V1 (BioSan, Latvia)
Laminar flow cabinet	MSC-Advantage (ThermoScientific, USA)
Cell counter and viability analyzer	Vi-CELL ^{TM}XR (Beckman Coulter, USA)
Flow cytometer	Cytomics FC 500 (Beckman Coulter, USA)
Spectrophotometer	Epoch (BioTek, Netherlands)
Vertical electrophoresis chamber	MiniProtean-3 cell (BioRad, USA)
Western blot chamber	TransBlot SD (BioRad, USA)
Voltage source	PowePac HC (BioRad, USA)

4.2 Methods

4.2.1 Determination of count of live and dead cells

The count of live and dead cells in cell suspension was determined using the Vi-CELL ^{TM}XR device. Analyzer evaluates the viability of the cells based on the permeability of the cytoplasmatic membrane for trypan blue (Sigma Aldrich, USA). Trypan blue is a dye that stains dead cells since the stain goes through a disrupted cytoplasmatic membrane. Living cells with the intact membrane will stay stainless. For measurement, there was used 600 µl of cell suspension.

4.2.2 Determination of cell proliferation and viability

For the determination of cell proliferation and viability the standard MTT assay was used (Mosmann, 1983). This test is based on its usage of tetrazolium dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT]. Metabolically active cells can reduce soluble yellow tetrazolium dye MTT to insoluble purple formazan (crystals) by using their products of dehydrogenases. Formazan is extracted from the cells by ethanol acidification and the created amount of formazan is directly proportional to the number of living cells. The disadvantage of

the MTT assay is that there is no distinguishing between cells that stopped proliferating and the dead cells.

One milliliter of cell suspension together with 100 μl of MTT were incubated at the temperature of 37 °C. After 1 hour the cells were centrifugated (5 min, 3000 rpm, laboratory temperature). There was added 500 μl of acidified ethanol for the extraction of formazan followed by another centrifugation (5 min, 14 000 rpm, laboratory temperature). Formed formazan was detected spectrophotometrically at 570 nm.

Used reagents and chemicals:

- MTT solution: 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0,04 mol/l HCl, 96% (v/v) ethanol

4.2.3 Determination of living cells using TMRE

TMRE (tetramethylrhodamine, ethyl ester) is a fluorescent dye that can be detected by using filters for phycoerythrin (PE) such as 575/526 or 582/515 nm. Emerged fluorescence, is in the case of active mitochondria, detected by using flow cytometry to assess apoptosis or mitochondrial depolarization. Non-apoptotic cells or cells with polarized mitochondria emit red fluorescence. Apoptotic cells or cells with low membrane potential of mitochondria have reduced emission of red fluorescence. TMRE has its excitation maximum at 549 nm (blue), 561 nm (green-yellow), and emission maximum at 574 nm.

To 1 ml of cell suspension, there was 1 μl of 100 μM TMRE added. The suspension was incubated for 30 minutes at 37 °C and then the samples were analyzed by using flow cytometry.

4.2.4 Cell cycle analysis

The flow cytometer is used for the estimation of DNA content. Estimation is enabled by fluorescent staining (usually propidium iodide or DAPI), which intercalates into cell DNA and forms stochiometric complexes. This method may be used also for the estimation of apoptotic cells, which have reduced volume of nuclear DNA (hypodiploid DNA). On the other hand, cells in the G1 phase have a diploid number of chromosomes, in the S phase cells double their number of chromosomes, and in the G2/M phase, the number of chromosomes is tetraploid.

One milliliter of cell suspension was centrifugated (5 min, 2 500 rpm, laboratory temperature) and the created supernatant was removed. The created pellet was dissolved in 400 μl of Vindal solution and incubated for 30 minutes in dark. The sample was assessed by flow cytometry at 488/525 nm. The resulting histogram represents the proportion of cells in each phase of the cell cycle.

Used reagents and chemicals:

- Vindal Solution (100 ml): 20 mg/ml RNAse A; 2 mg/ml propidium iodide (Invitrogen, USA); 100 µl Triton X-100 (Serva, Germany), 100 ml 1x PBS

4.2.5 Western blot analysis

The methods of electrophoresis and western blot were used for the overall evaluation of protein expression in the individual cell lines.

4.2.5.1 Cell lysis

Cell suspension (3 x 10^6 cells) was centrifugated (5 min, 1 500 rpm, 4°C) and the pellet was wash by using cold 1x PBS. The sample was then centrifugated once again (5 min, 1 500 rpm, 4°C), the supernatant was removed and to the remaining pellet, there was added 200 µl of 1x lysate solution. Cells were incubated for 30 minutes on ice with being mixed a few times. The cell lysate was purified by centrifugation (10 min, 15 000 rpm, 4°C).

Used reagents and chemicals:

- 1x Lysis buffer (RIPA): 150 mmol/l NaCL, 1% Nonidet P 40, 50 mmol/l Tris, 50 mmol/l NaF, 5 mmol/l EDTA, 5 mmol/l sodium pyrophosphate decahydrate (NAPP) were in sterile conditions dissolved in 100 ml deionized water and the pH of the solution was corrected to value pH 8. Inhibitors of proteases and phosphatases (Roche) were added to the buffer solution before its addition to the samples

4.2.5.2 Determination of protein concentration

Protein concentration was determined by Bradford protein assay. This method uses Coomassie Brilliant Blue G-250 as a coloring agent, which can bind to the molecules of proteins. Blue color intensity is directly proportional to the amount of protein in a sample. This intensity was detected by a spectrophotometer at 595 nm.

One microliter of purified cell extract was added to 1 ml of Bradford reagent. The mixture was then incubated for 10 minutes at laboratory temperature. Emerged colorization was evaluated by a spectrophotometer at 595 nm. Pure Bradford reagent was used as a blank, and calibration was done by FBS.

Used reagents and chemicals:

- Bradford reagent (1 l): 50 mg/l Coomassie Brilliant Blue G-250; 50 ml methanol; 100 ml 85% H3PO4, 850 ml deionized water. FBS was used as a standard.
- FBS as an intern standard

4.2.5.3 Protein separation, Western blot, and visualization

All samples were diluted by water to their final concentration and there was 4x concentrated Loading buffer added as well. Proteins were denatured in ThermoBlock for 5 min at 95°C.

Samples were then separated by using electrophoresis with 7,4% SDS-polyacrylamide separation gel and 3,5% focusing gel (SDS-PAGE; 80V/120V).

Separated proteins were transferred on a nitrocellulose membrane by using wet western blotting (0,15 A for one membrane, 30 min). Membranes were then blocked in 5% blocking milk with 1x PBS 0,1% Tween-20 for 1 hour. Membranes were also washed for 10 minutes in 1x PBS, 3x 10 minutes in 1x PBS 0,1% Tween-20, and at last for 10 minutes in 1x PBS. The primary monoclonal Rabbit antibodies (all by Cell Signaling Technology) were diluted in blocking milk in different ratios:

- LAMP1 (D2D11) XP(R): ratio 1:1000
- LAMP2 (D5C2P): ratio 1:1000
- vATPase (9804S): ratio 1:1000 in K562 and 1:500 in T98G
- HSP90 (4874S): ratio 1:1000
- HSP70 (4872S): ratio 1:500
- HSP27 (D6W5V): ratio 1:1000
- GAPDH (D16H11): ratio: 1:2000

Membranes with antibodies were incubated over night at 4°C. The following day were the membranes washed again for 10 minutes in 1x PBS, 3x 10 minutes in 1x PBS 0,1% Tween-20, and at last for 10 minutes in 1x PBS. During the last washing, the secondary antibodies were diluted in blocking milk in the ratio 1:5000. Antibodies were then applied to the membranes and incubated for 1 hour at the laboratory temperature. Once again, the membranes were washed for 10 minutes in 1x PBS, 3x 10 minutes in 1x PBS 0,1% Tween-20, and at last for 10 minutes in 1x PBS.

Visualization was then carried out by using a chemiluminescent ECL kit containing a substrate for horseradish peroxidase bounded to the secondary antibody. Membranes were incubated with a chemiluminescent substrate for 5 minutes in the dark. The results were black stripes/bands representing individual proteins on photographic film (GE HealthCare). For quantitative densitometric evaluation of protein expression, the ImageJ program was used.

Used reagents and chemicals:

- 1x PBS + 0,1% Tween 20 (1 l): 100 ml 10x PBS, 1 ml Tween 20, 900 ml deionized water
- 10x Running buffer: 30 g TRIS, 145 g glycine, 1 l deionized water

- Transfer buffer (Western blot): 100 ml 10x Running buffer, 200 ml methanol, 700 ml deionized water
- 5% blocking buffer (blocking milk): 5 g of dry non-fat milk, 100 ml 1x PBS + Tween 20
- TANK buffer: 100 ml 10x Running buffer, 10 ml 10% SDS, 900 ml deionized water
- 6% Loading buffer (Laemmli buffer): 12% SDS, 0,3 mol/l TRIS (pH 8,9), 0,3% bromothymol blue, 60% glycerol, 0,75 mol/l DTT
- Molecular weight marker: Spectra Multicolor (Thermo Scientific)
- Secondary antibodies: Polyclonal Swine anti-Rabbit immunoglobulins/HRP (horseradish peroxidase; Dako),
- ECL kit: GE HealthCare, UK
- Photographic fixer: G150 (AGFA)
- Photographic developer: G354 (AGFA)

4.2.6 Morphological analysis of lysosomes

For the monitoring of morphological changes in lysosomes, the immunohistochemical approach was used. It was based on the staining of LAMP1 protein, which is a marker protein of the lysosomes that cannot be found in any other cell organelle.

Cell suspension (10 ml, approx. 300 000/ml) was centrifugated for 5 minutes at 1 600 rpm and afterward washed with 4 ml of cold 1x PBS. After another centrifugation, there was 4 ml of 3,7% formaldehyde in 1x PBS added and left through the night in the fridge.

The other day all the work with the cells was performed on the ice. Cells were washed with cold 1x PBS and centrifugated for 4 minutes at 1 600 rpm. The supernatant was removed, and the created pellet was suspended in 1.5 ml of permeabilization solution. Cells were left with the solution for 10 minutes and centrifugated again. The created pellet was suspended according to its size in the appropriate amount of permeabilization solution so that there is for one sample at least 50 µl of cell suspension. To each sample, there was added 1 µl of the primary antibody (the same LAMP1 primary antibody as in the Western blot analysis). Eppendorf tubes containing the samples with antibody were then left for one hour to spin on the rotor inside of the fridge. Samples were centrifugated once again and the secondary antibody was added. The samples must be washed after 1 hour on the rotor with cold 1x PBS. Secondary antibodies were prepared in the ratio 1:1000 (1 ml of 1x permeabilization solution + 1 µl secondary Ab). Into new Eppendorf tubes there was 80 µl of diluted solution added. Washed cells were then centrifugated and the created pellets were suspended in 20 µl of permeabilization solution. This 20 µl was then added into 80 µl containing the secondary antibody and left for one hour to spin on the rotor in the fridge.

After labeling with primary and secondary antibodies, suspense cells are mixed with 50 µl of DAPI for better visualization, and 15 µl are applied on the slide. In the case of sessile cells, whole the process was carried in moistening chambers. After DAPI application, coverslips were put on the top of labeled cells and sealed by liquid glue. The visualization was carried under the fluorescent microscope and the final editing of the pictures was done in the ImageJ program.
Used reagents and chemicals:

- Permeabilization solution: 1x PBS + 0,1% nonidet + 5% albumin
- Primary antibodies: LAMP1 monoclonal Rabbit antibodies (D2D11) XP(R) (Cell Signaling Technologies)
- Secondary antibodies: FITC polyclonal antibodies labeled with horseradish peroxidase (Dako)

4.2.7 Cellular senescence assay

During senescence, changes in morphology, proliferation and ß-galactosidase activity occur. Senescence assay is based on histochemical staining for ß-galactosidase activity at pH 6. In senescent cells, the ß-galactosidase is detectable, while in quiescent, immortal, or tumor cells it is not. T98G cells were fixed in a 6-well desk plate and incubated with staining mixture for approximately 6 hours bated at 37 °C without CO_2. For the detection of the ß-galactosidase, the senescence cells histochemical staining kit by Sigma-Aldrich was used (CS0030).

5 RESULTS

5.1 Characterization of T98G cells

The first aim of the experimental part was to establish a resistant T98G cells by long-term cultivation with gradually increasing sunitinib concentrations. First, we determined the half-maximal inhibitory concentration (IC_{50}) for the sensitive T98G cells by using the MTT essay. We observed that the IC_{50} was 2.3 ± 0.3 μM (Tab. 5). In the next step we exposed T98G cells to 2 μM sunitinib as it is close to the IC_{50} of sensitive cells. The treated cells cultivated with 2 μM SUN underwent a crisis approximately after two weeks after the initial exposure. Their proliferation slowed down and a moderate amount of them died (data not shown). Nevertheless, their proliferation was restored after one month. Then, we measured their IC_{50} by MTT assay and observed a significant increase to the value of 10.5 ± 0.9 μM (Tab. 5). Since MTT assay is not able to distinguish between living cells that stopped proliferating and dead cells, their viability was measured by TMRE staining and the flow-cytometry. The treated cells with 2 μM showed significant increase in their viability (Fig. 10). These cells were considered as resistant cells and were labeled as T98G 2SUN cells. Because the prolonged cultivation with sunitinib increased the IC_{50} value and the viability of the cells, we used these resistant T98G 2SUN cells for further study. T98G 2SUN cells were exposed to further concentration of 4 μM sunitinib. These cells underwent the same crisis as T98G 2SUN cells with their proliferation restoration after one month. Once again, we determined their IC_{50} by MTT assay resulting in further increase to the value of 14 ± 0.7 μM (Tab. 5). We evaluated their viability by TMRE staining and the flow-cytometry and we observed increased viability these cells as well (Fig. 10). These cells were considered as resistant cells and were labeled as T98G 4SUN cells. Therefore, we exposed these resistant cells to the sunitinib concentration of 8 μM. However, these cells went into the crisis and this concentration was lethal for all of them (data not shown). Therefore, further analysis of resistance mechanisms was performed on T98G 2SUN and T98G 4SUN cells. The IC_{50} in all three cases was evaluated after 78 hours of incubation with rising concentrations of SUN, precisely 0.3, 1, 3, 10, and 30 μM.

Tab.5: Cell sensitivity to sunitinib. The data represent average value ± SE obtained in two independent experiments. Sigma Plot software was used to determine the IC_{50}.

Cell line	Sunitinib [μM]
T98G	2.3 ± 0.3 μM
T98G 2SUN	10.5 ± 0.9 μM
T98G 4SUN	14 ± 0.7 μM

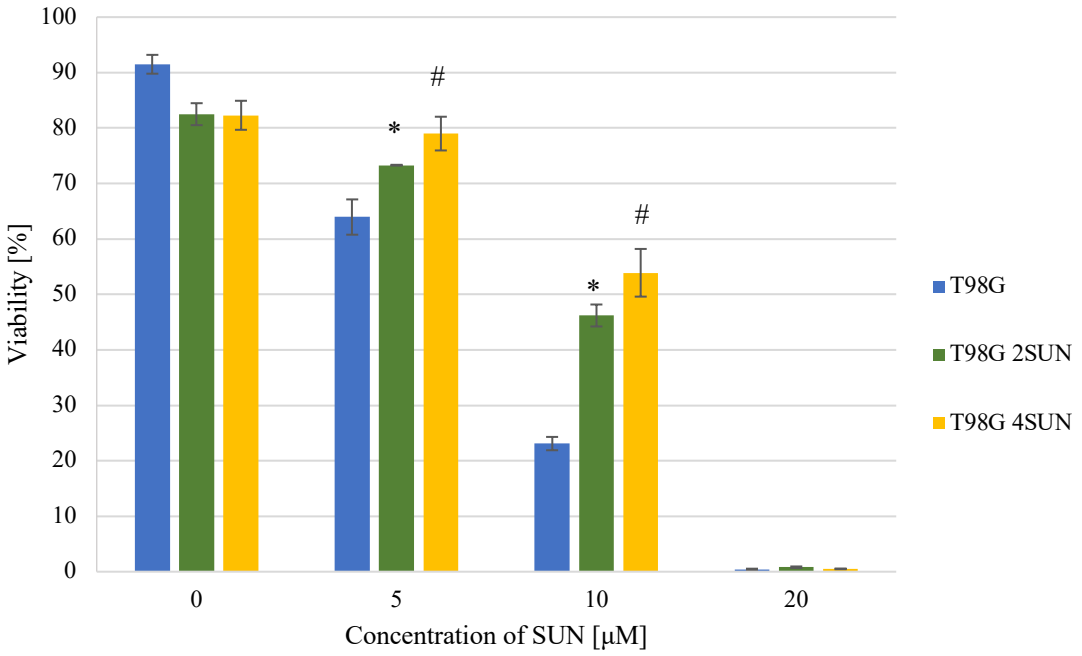


Fig. 10: Effect of sunitinib on the viability of T98G 2SUN cells and T98G 4SUN cells**.** Cells were incubated with sunitinib for 48 hours in the standard medium at 37 °C and 5% CO_2. The data represent average values ± SE obtained in three independent experiments. *, # …statistically significant difference in the viability of T98G 2SUN cells and 4SUN cells after exposure to sunitinib.

5.1.1 Analysis of lysosomal membrane proteins expression in resistant T98G 2SUN and T98G 4SUN cells

In the next step, we wanted to evaluate if the acquired resistance in T98G 2SUN cells and T98G 4SUN cells can be related to lysosomal biogenesis. Therefore, the analysis of lysosomal membrane-bound proteins was performed. The studied proteins were LAMP1, LAMP2, and vATPase. The expression of these proteins was analyzed by the method of Western blot.

Increased expression of LAMP1 and LAMP2 was observed not only in T98G 4SUN cells but already at the T98G 2SUN cells (Figs. 11, 12). Considering the expression of vATPase, no change was seen in T98G 2SUN or T98G 4SUN cells (Fig. 13). These results did not completely support the idea of lysosomal biogenesis and further analysis of the resistant cells was performed.

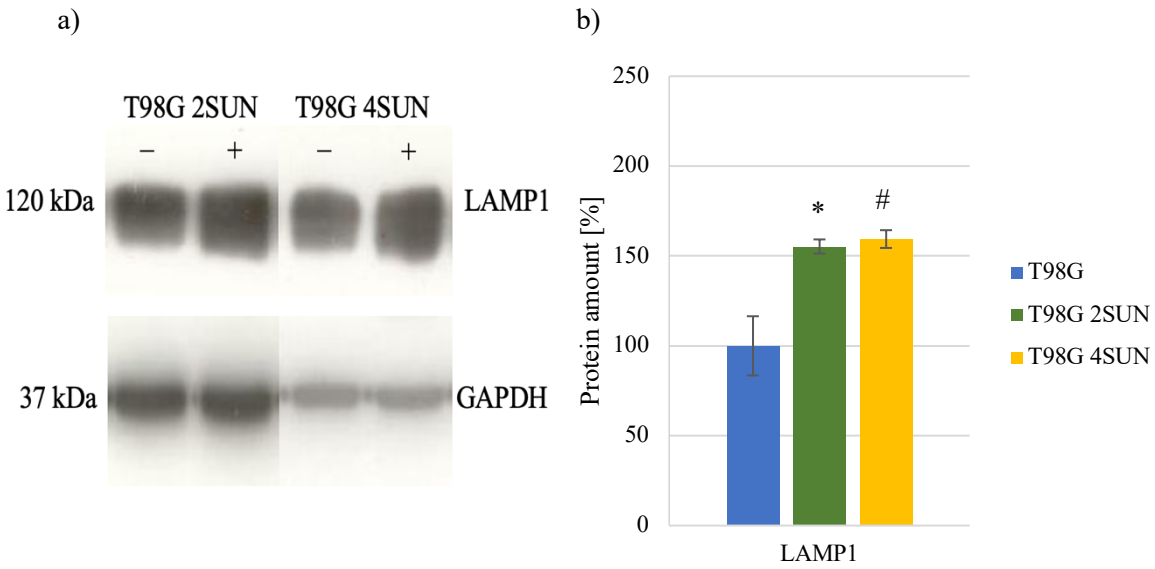


Fig. 11: Effect of sunitinib on the expression of LAMP1 in T98G 2SUN cells and T98G 4SUN cells. a) Typical pictures of western blot: sensitive T98G cells (-), T98G 2SUN/4SUN cells (+). GAPDH was used as the protein loading control. b) Quantitative densitometric evaluation of LAMP1 expression. As the control value, 100% were used in sensitive T98G cells. The evaluation was done with the program ImageJ. The data represent average values ± SE obtained in three independent experiments. *, # …statistically significant difference in LAMP1 expression in T98G 2SUN cells and T98G 4SUN cells (P<0,05).

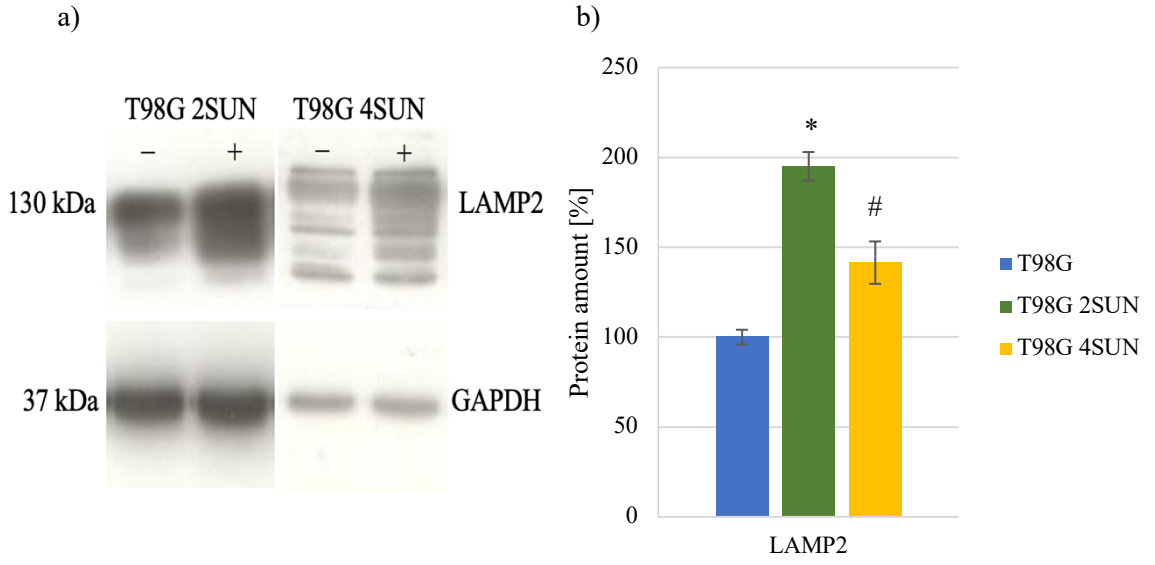


Fig. 12: Effect of sunitinib on the expression of LAMP2 in T98G 2SUN cells and T98G 4SUN cells. a) Typical pictures of western blot: sensitive T98G cells (-), T98G 2SUN/4SUN cells (+). GAPDH was used as the protein loading control. b) Quantitative densitometric evaluation of LAMP2 expression. As the control value, 100% were used in sensitive T98G cells. The evaluation was done with the program ImageJ. The data represent average values ± SE obtained in three independent experiments. *, # … statistically significant difference in LAMP2 expression in T98G 2SUN cells and T98G 4SUN cells (P<0,05).

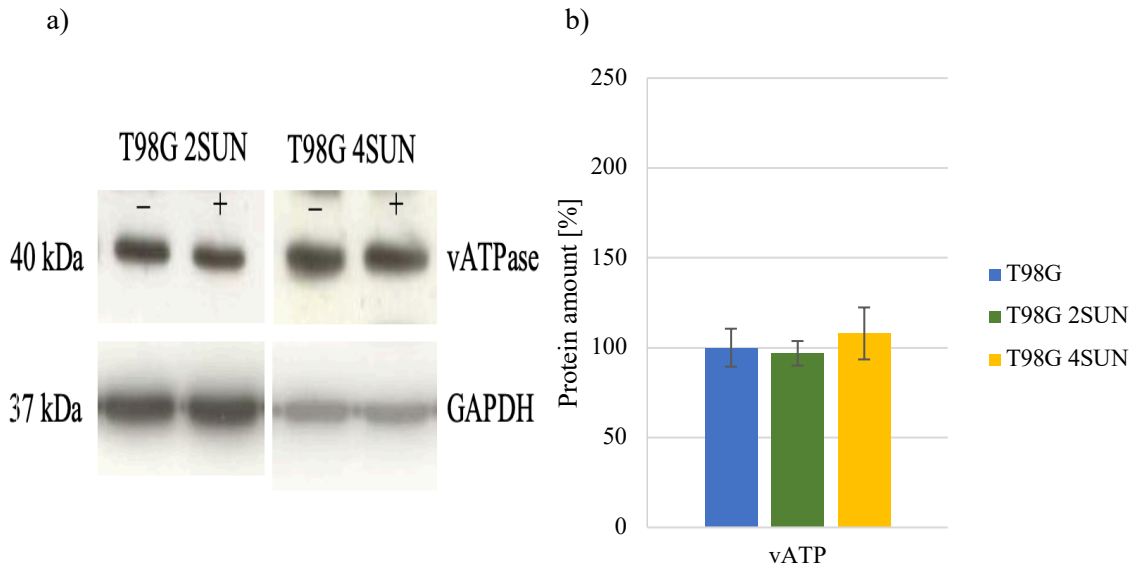


Fig. 13: Effect of sunitinib on the expression of vATPase in T98G 2SUN cells and T98G 4SUN cells. a) Typical pictures of western blot: sensitive T98G cells (-), T98G 2SUN/4SUN cells (+). GAPDH was used as the protein loading control. b) Quantitative densitometric evaluation of vATPase expression. As the control value, 100% were used in sensitive T98G cells. The evaluation was done with the program ImageJ. The data represent average values ± SE obtained in three independent experiments.

5.1.2 Morphological analysis of lysosomes in resistant T98G 2SUN and T98G 4SUN cells

Since the results of lysosome-membrane proteins' expression did not completely support the idea of lysosomal biogenesis, we further analyzed the morphological changes in the lysosomes of resistant cells. Immunohistochemical staining of LAMP1 with primary and secondary antibodies was done and the mounts were observed under the fluorescent microscope. There was a significant difference between lysosomes of sensitive T98G cells and T98G 4SUN cells (Figs. 14a, 14d). Since SUN emits fluorescence on the visible spectrum, it interferes with the signal of LAMP1 protein and, therefore, interferes with the evaluation of the lysosomal morphology. The sensitive T98G cells were incubated with 4 μM SUN for one hour before the staining of LAMP1. With this incubation we observed the fluorescence of SUN in sensitive T98G cells (Fig. 14b). Number of lysosomes in T98G 4SUN cells increased significantly (Fig. 15a). After the measurement of the average lysosome size we observed, that even though their average number in T98G 4SUN cells increased, their size significantly decreased in comparison to the sensitive cells (Fig. 15). The changes in T98G 2SUN cells were comparable to those in T98G 4SUN (Fig. 14c). However, because of the time demands, only results for lysosomal number and size of T98G 4SUN cells are shown.

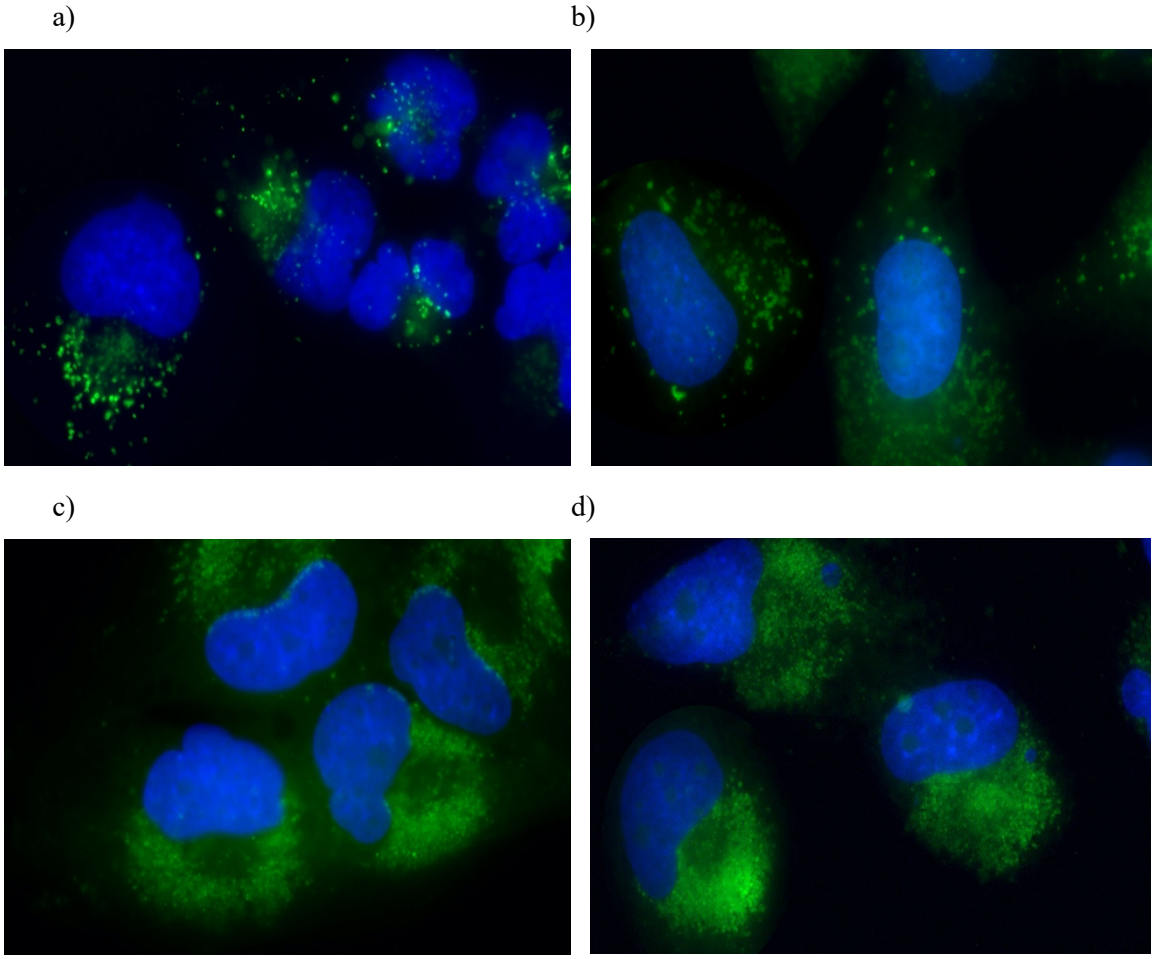
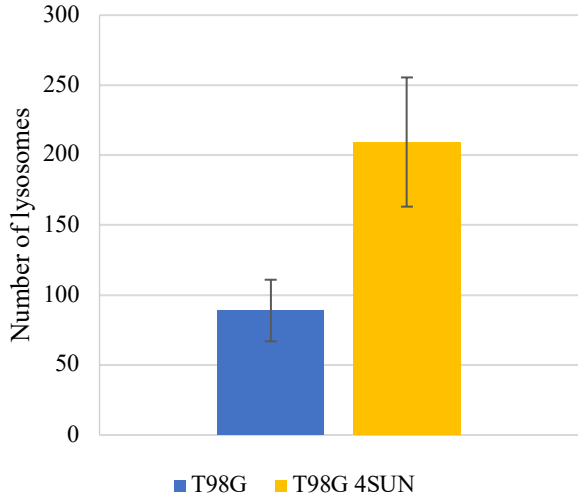


Fig. 14: Effect of sunitinib treatment on lysosomal morphology in T98G 4SUN. Typical pictures of a) sensitive T98G cells with LAMP1 stained by primary and secondary antibodies and the nucleus stained by DAPI for better visualization. b) sensitive T98G cells incubated for one hour with 4 µM SUN before being stained for LAMP1 and nucleus. c) T98G 2SUN cells with LAMP1 stained by primary and secondary antibodies and the nucleus stained by DAPI. d) T98G 4SUN cells with LAMP1 stained by primary and secondary antibodies and the nucleus stained by DAPI.

a)



b)

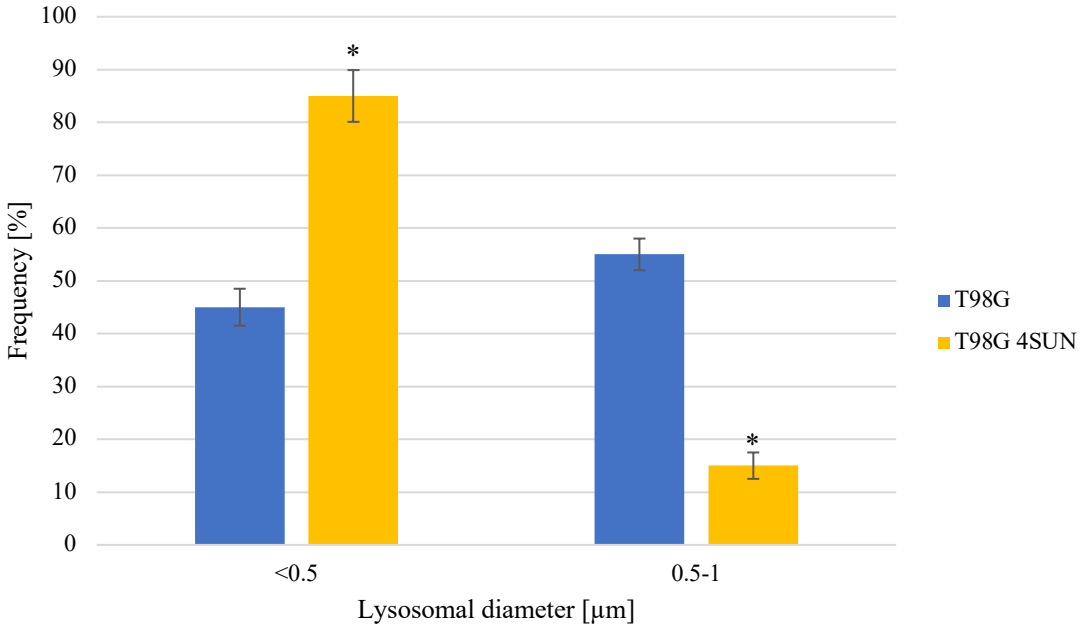


Fig. 15: Quantitative analysis of lysosomes in T98G cells. a) The average number of lysosomes in 30 counted cells of sensitive T98G cells and T98G 4SUN cells. b) The average lysosomal diameter of sensitive T98G cells and T98G 4SUN cells. *… significantly decreased size of the lysosomes in T98G 4SUN in comparison to the untreated T98G cells.

5.1.3 Evaluation of senescence phenotype in resistant T98G 2SUN and T98G 4SUN cells

Since the previous results do not support the theory about lysosomal biogenesis, other mechanisms that could be responsible for the resistance in T98G 2SUN and T98G 4SUN cells were observed. One of them is the theory about the cells acquiring the senescence phenotype.

Three most important features of the senescence were evaluated, including the morphological changes, proliferation potential and the ß-galactosidase activity (Gewirtz *et.al.,* 2008).

The senescence phenotype is typical for their enlarged and flattened morphology. When comparing the morphology of sensitive T98G cells and resistant T98G 4SUN, there was no difference in the morphology seen (Figs.16a, 16b). We also analyzed the cell cycles of the resistant cells by the method of TMRE staining and flow cytometry. Using these results, we did the analysis of cell cycles to get the distribution of living cells in each cell cycle phase. It was shown, that both T98G 2SUN and 4SUN presented a higher number of living cells in the G1-phase in comparison to the sensitive T98G cells (Fig. 17). This fact can indicate their decreased proliferation rate.

a) b)

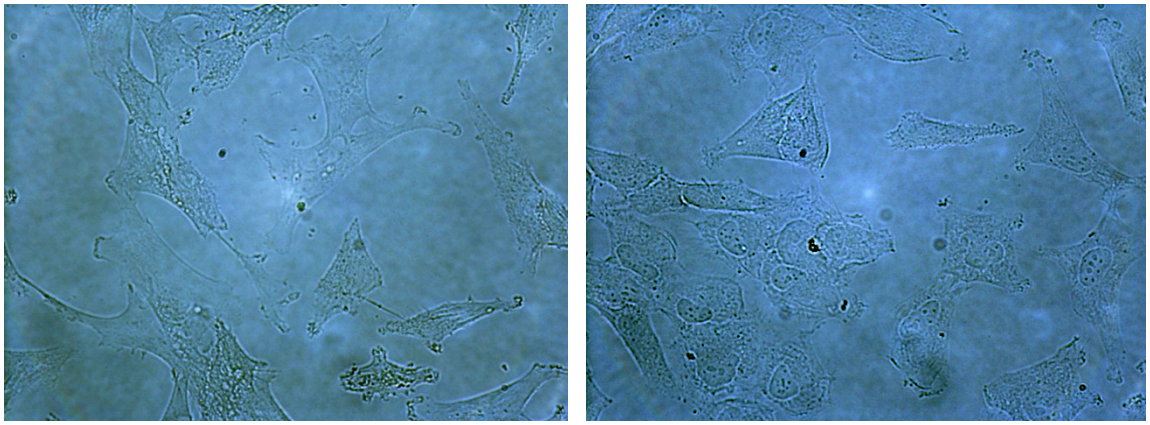
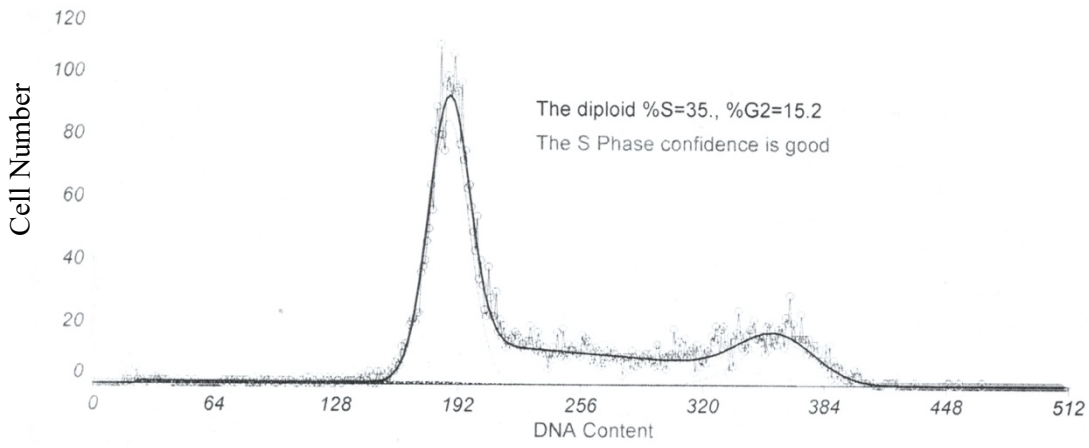


Fig. 16: Morphology of T98G cells. a) Morphology of sensitive T98G cells. b) Morphology of T98G 4SUN cells exposed to SUN for 5 months.

a)



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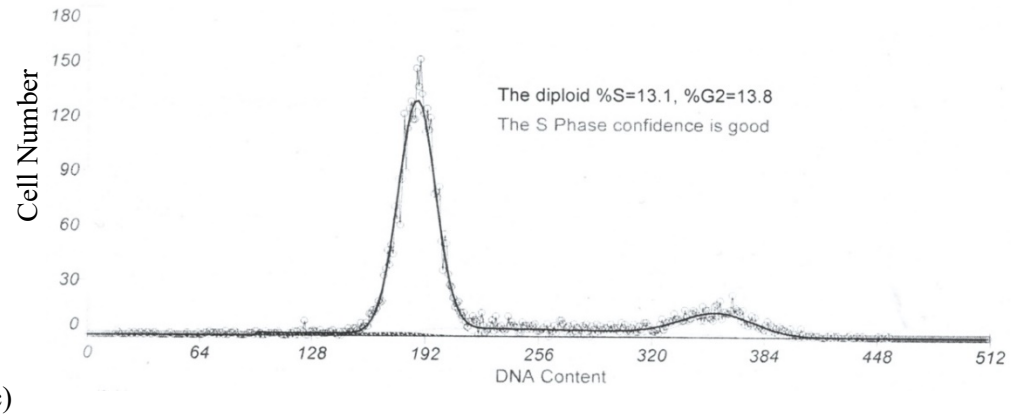
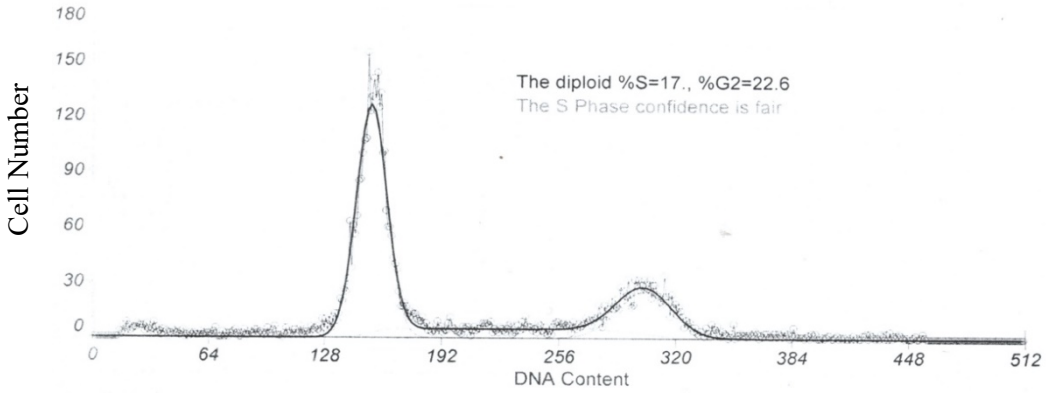


c)



d)

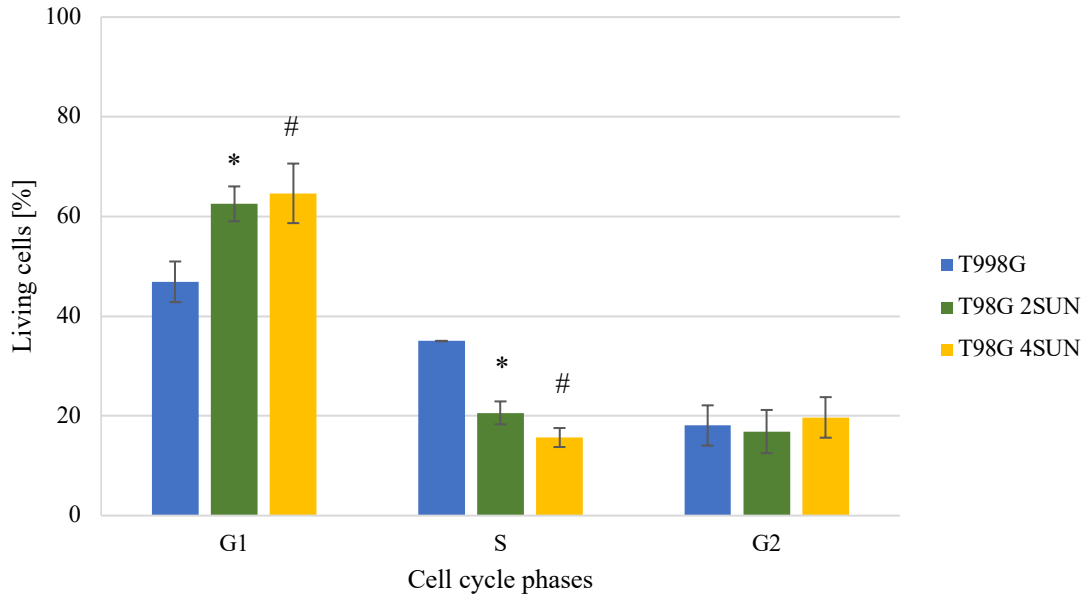
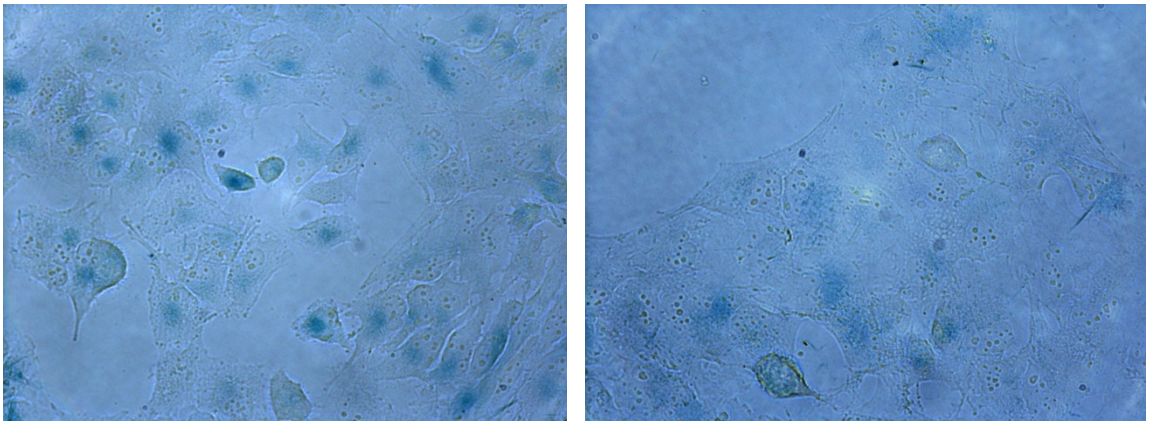


Fig. 17: Cell cycle analysis of T98G cells. Typical histograms of a) sensitive T98G cells, b) T98G 2SUN cells, c) T98G 4SUN cells, d) quantitative analysis of living cells in the cell cycle phases. The cell cycle was measured by using flow cytometry every week for past three months. *, # …statistically significant difference in cell cycle distribution T98G, T98G 2SUN, and T98G 4SUN cells (P<0,05).

Since the evaluation of morphology and proliferation did not completely show the senescent phenotype of the resistant cells, we evaluated the ß-galactosidase activity in sensitive cells and T98G 4SUN cells using the senescent cell histochemical staining kit. In the case of senescent cells, the ß-galactosidase activity would be detected by their blue color staining. On the other hand, cells that are not in the state of senescent would not be stained by the blue color. As the positive control, sensitive T98G cells treated by 100 nM doxorubicin for 24 hours and then left in a new medium for 4 days were used. The results shown that in case of sensitive T98G cells and T98G 4SUN cells, there were only few cells stained by the blue color. The cells after the treatment with doxorubicin were significantly bluer, therefore, indicating the senescent phenotype (Fig.18).

a) b)



c)

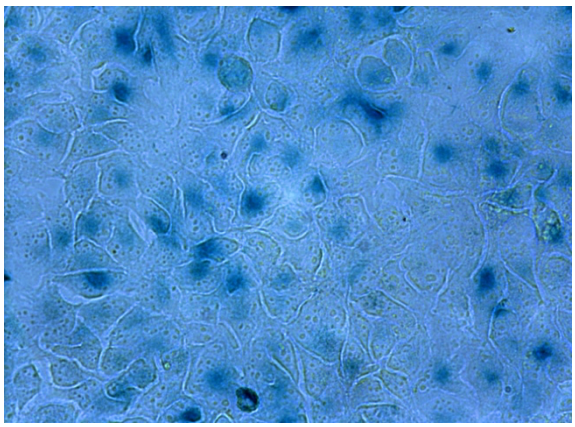


Fig.18: Evaluation of senescence phenotype using the evaluation of ß-galactosidase activity of a) sensitive T98G cells, b) resistant T98G 4SUN cells, c) sensitive T98G cells used as a positive control after the treatment of 100 nM doxorubicin for 24 hours.

5.1.4 Analysis of heat shock proteins expression in resistant T98G 2SUN and 4SUN cells

Since not all the features of senescence were observed, we tried to analyze other mechanisms that could be involved in the acquired resistance in T98G 2SUN and T98G 4SUN cells. The expression of heat shock proteins (HSPs) was evaluated since they are generally involved in the stress response in the cells. Many of the HSPs have increased expression in the cancer cells as there is an increased requirement for stabilization of over-expressed and mutant oncoprotein found in each tumor. HSP70 is involved in the survival of stressed cells mainly by inhibiting permeabilization of the lysosomal membrane. HSP70 can stabilize the lysosomal membrane (Kirkegaard *et.al.,* 2010; Murshid *et.al.,* 2012).

There was no significant change in the expression of HSP90 or HSP27 proteins in both T98G 2SUN and T98G 4SUN cells (Figs. 19, 20). However, in long-term observation of HSP70 protein, there was a significant decrease in its expression in T98G 4SUN cells, while in T98G 2SUN cells there was a slight increase in the expression observed (Fig. 21). The T98G 2SUN cells with the increase of HSP70 were exposed to the concentration of 2 µM SUN for three months. T98G 4SUN were cultivated with the concentration of 4 µM SUN for five months with the fact, that the most significant decrease in HSP70 expression could be observed after four and half month's exposition to sunitinib (Fig. 22a). Nevertheless, the increased expression of the HSP proteins as described in the literature was not observed in any other cases than in the HSP70 in T98G 2SUN (Fig. 22).

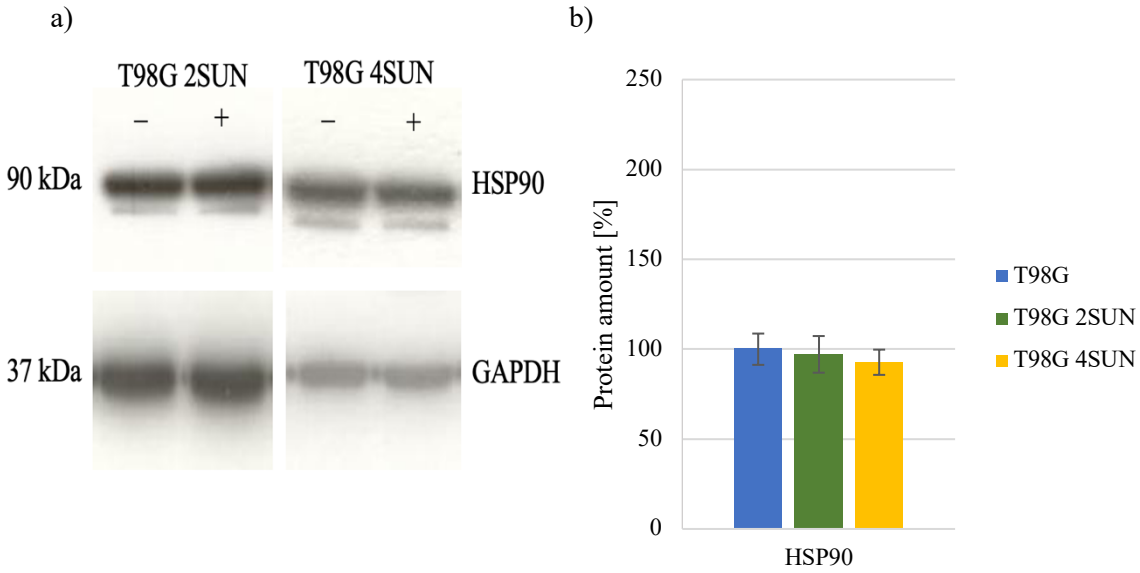


Fig. 19: Effect of sunitinib on the expression of HSP90 in T98G 2SUN cells and T98G 4SUN cells. a) Typical pictures of western blot: sensitive T98G cells (-), T98G 2SUN/4SUN cells (+). GAPDH was used as the protein loading control. b) Quantitative densitometric evaluation of HSP90 expression. As the control value, 100% were used in sensitive T98G

cells. The evaluation was done with the program ImageJ. The data represent average values ± SE obtained in three independent experiments.

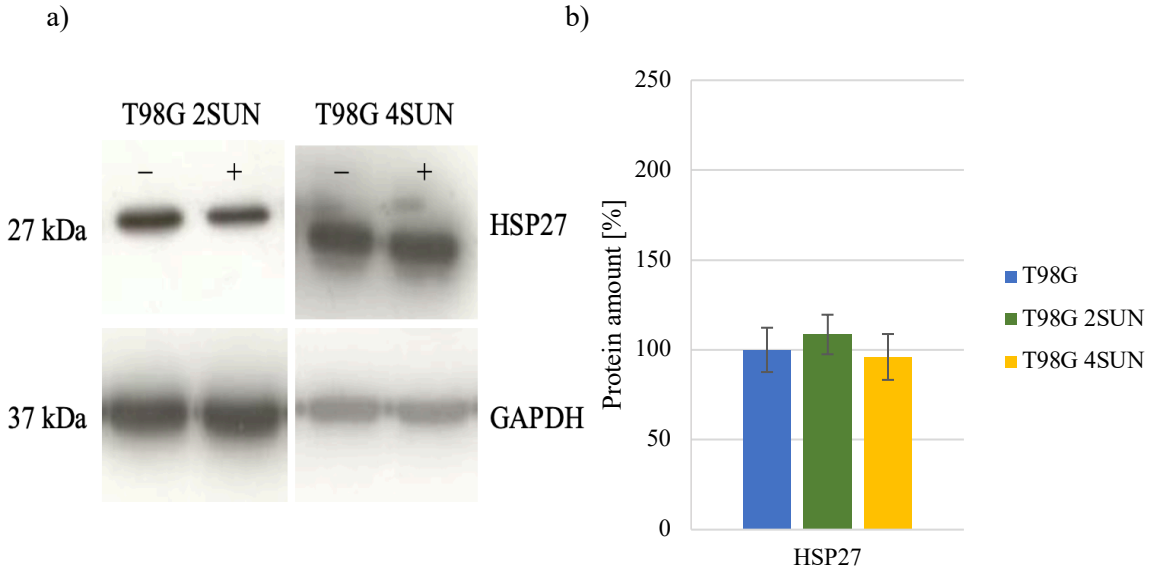


Fig. 20: Effect of sunitinib on the expression of HSP27 in T98G 2SUN cells and T98G 4SUN cells. a) Typical pictures of western blot: sensitive T98G cells (-), T98G 2SUN/4SUN cells (+). GAPDH was used as the protein loading control. b) Quantitative densitometric evaluation of HSP27 expression. As the control value, 100% were used in sensitive T98G cells. The evaluation was done with the program ImageJ. The data represent average values ± SE obtained in three independent experiments.

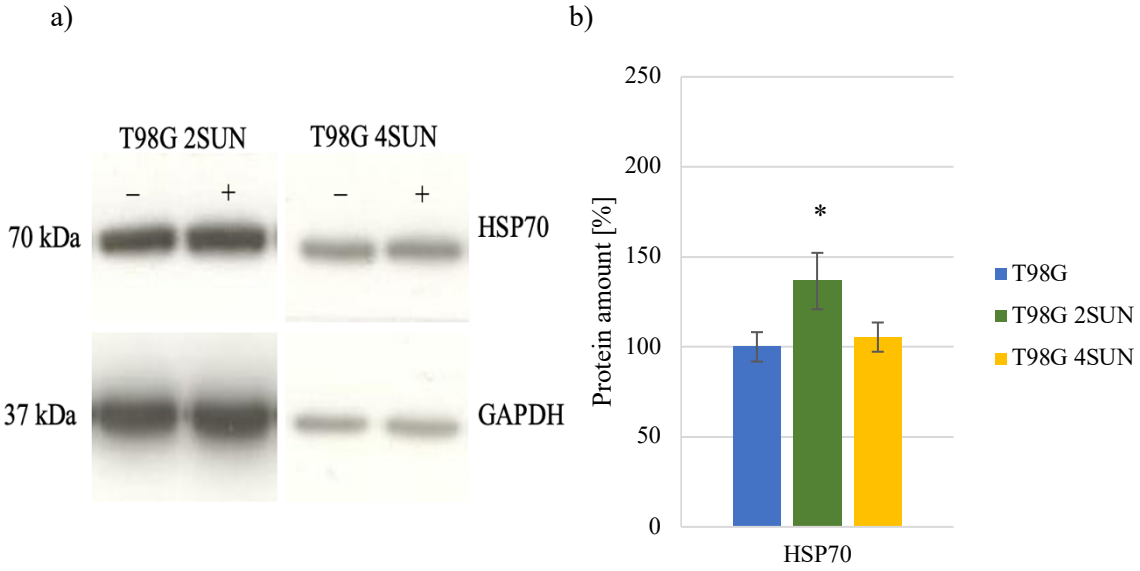


Fig. 21: Effect of sunitinib on the expression of HSP70 in T98G 2SUN cells and T98G 4SUN cells. a) Typical pictures of western blot: sensitive T98G cells (-), T98G 2SUN/4SUN cells (+). GAPDH was used as the protein loading control. b) Quantitative densitometric evaluation of HSP70 expression. As the control value, 100% were used in sensitive T98G cells. The evaluation was done with the program ImageJ. The data represent average values ± SE obtained in three independent experiments. * … statistically significant difference in HSP70 expression in T98G 2SUN cells (P<0,05).

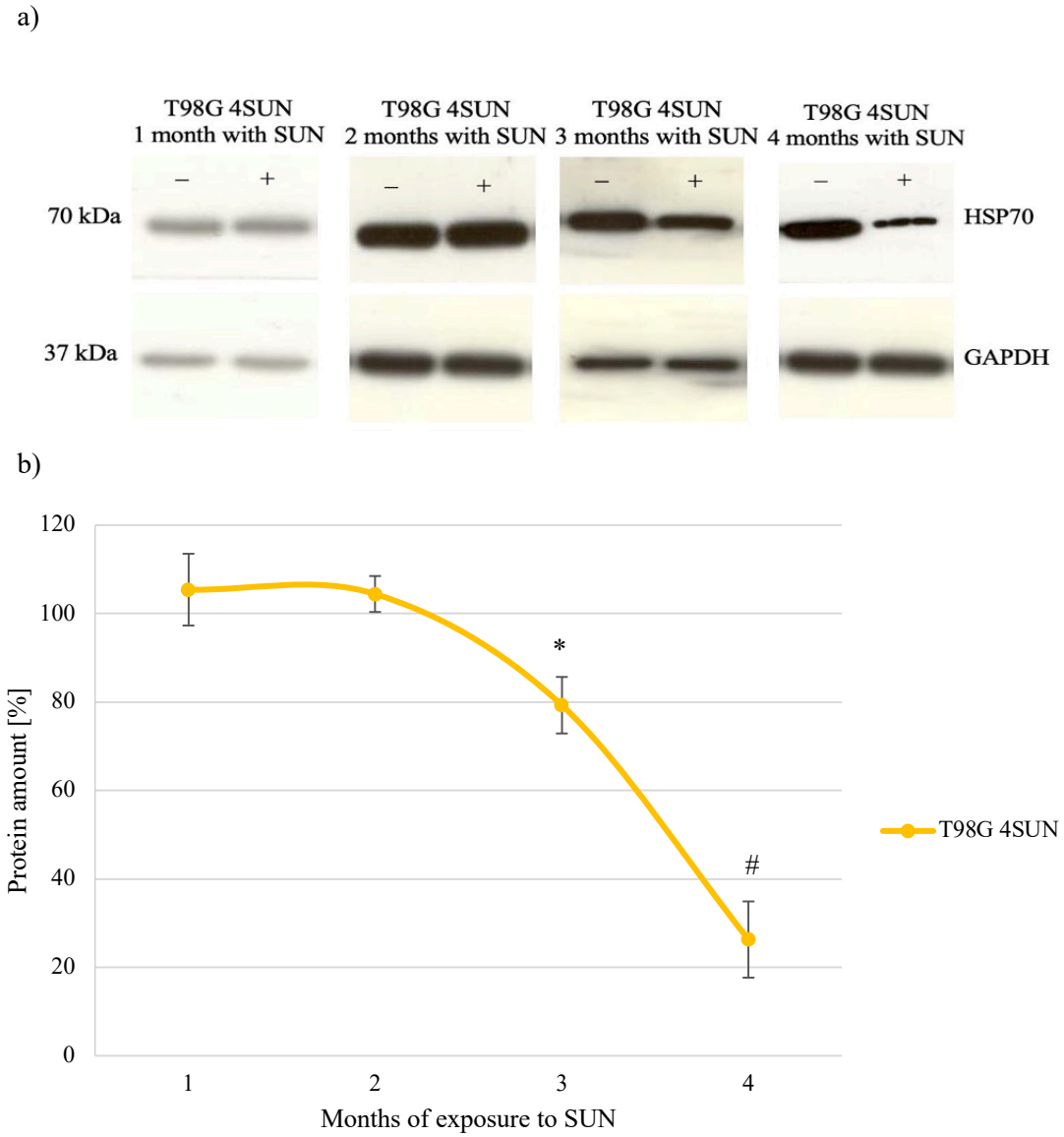


Fig. 22: Effect of sunitinib on the expression of HSP70 in T98G 4SUN. a) Typical pictures of western blot: sensitive T98G cells (-) and T98G 4SUN cells (+). GAPDH was used as the protein loading control. b) Quantitative densitometric evaluation of HSP70 expression in time. The evaluation was done with the program ImageJ. The data represent average values ± SE obtained in four independent experiments. *, # … statistically significant difference in HSP70 expression in T98G 4SUN cells (P<0,05).

5.2 Characterization of K562 cells

The characterization of K562 cells was carried similarly as in the case of T98G cells. As already mentioned, the first aim was to obtain a resistant cell line. First, the IC_{50} of sensitive K562 cells was obtained by the MTT test. The IC_{50} value for these cells was 2.4 ± 0.4 µM. Therefore, the first used concentration of SUN was 2 µM. The cells were cultivated with this concentration for a month, also experiencing the crisis and the subsequent stabilization of proliferation (data not

shown). After the stabilization, the viability was measured by TMRE staining and flow cytometry. The viability of cells exposed to SUN was comparable to the one of sensitive K562 cells (Fig. 23). We took the resistant cells for further study and labeled them as K562 2SUN. Then the IC_{50} of these cells was evaluated in the same way using the MTT test. In K562 2SUN cells there was just a slight difference from the sensitive cells since the value obtained was 3.5. ± 0.5 µM (Tab. 6). The IC_{50} in both cases was evaluated after 78 hours of incubation with rising concentrations of SUN, precisely 0.3, 1, 3, 10, and 30 µM.

Later, the concentration of SUN in K562 2SUN cells was increased to 4 µM. Nevertheless, this concentration was lethal for all K562 2SUN cells. Therefore, we analyzed and studied the mechanisms of resistance only in K562 2SUN cells.

Tab. 6: Cell sensitivity to sunitinib. The data represent average value ± SE obtained in two independent experiments. Sigma Plot software was used to determine the IC_{50}.

Cell line	Sunitinib [µM]
K562	2.4 ± 0.4 µM
K562 2SUN	3.5 ± 0.5 µM

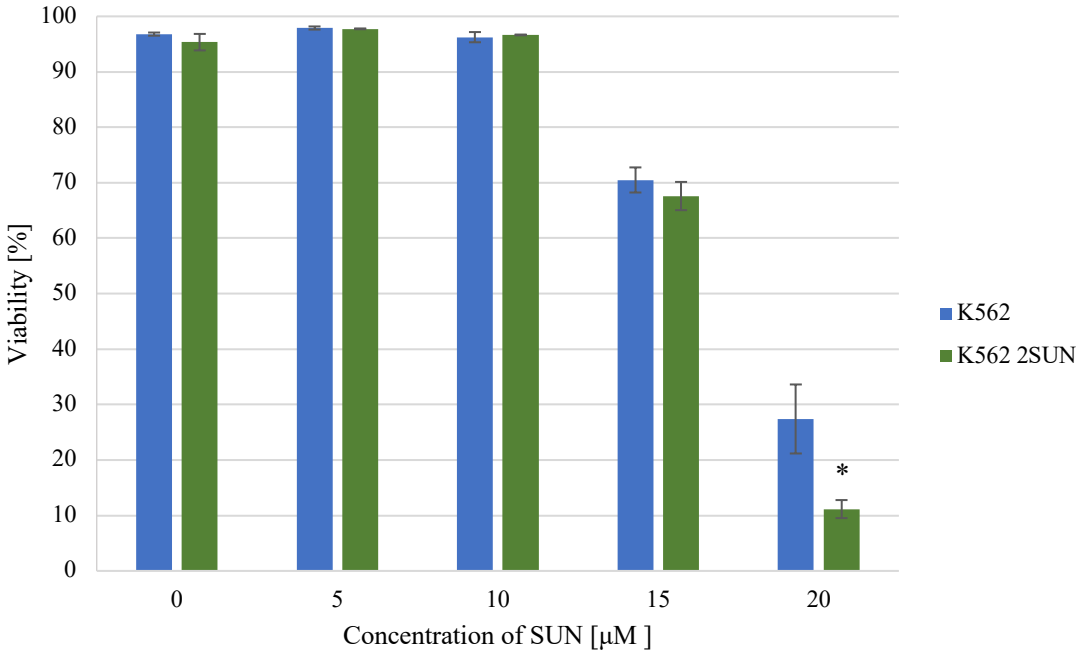


Fig. 23: Effect of sunitinib on the viability of K562 2SUN cells**.** Cells were incubated with sunitinib for 48 hours in the standard medium at 37 °C and 5% CO_2. The data represent average values ± SE obtained in three independent experiments. *…statistically significant difference in the viability of K562 2SUN cells after exposure to sunitinib.

5.2.1 Analysis of lysosomal membrane proteins expression in resistant K562 2SUN cells

In K562 2SUN and its sensitive K562 cells, the determination of lysosome membrane proteins was performed as well. The studied proteins were LAMP1, LAMP2, vATPase, and their expression was analyzed by the method of Western blot.

In the sensitive K562 cells, as well as in the resistant K562 2SUN cells, there was only an increase in LAMP1 detected (Fig. 24). In the case of LAMP2, the expression was slightly decreased in K562 2SUN cells in comparison to the sensitive K562 cells (Fig. 25). We observed a constant amount of vATPase during all the experiments (Fig. 26). Since these observations do not show the significant increase in all three proteins simultaneously, the theory about lysosomal biogenesis was not supported and we evaluated other resistance mechanisms.

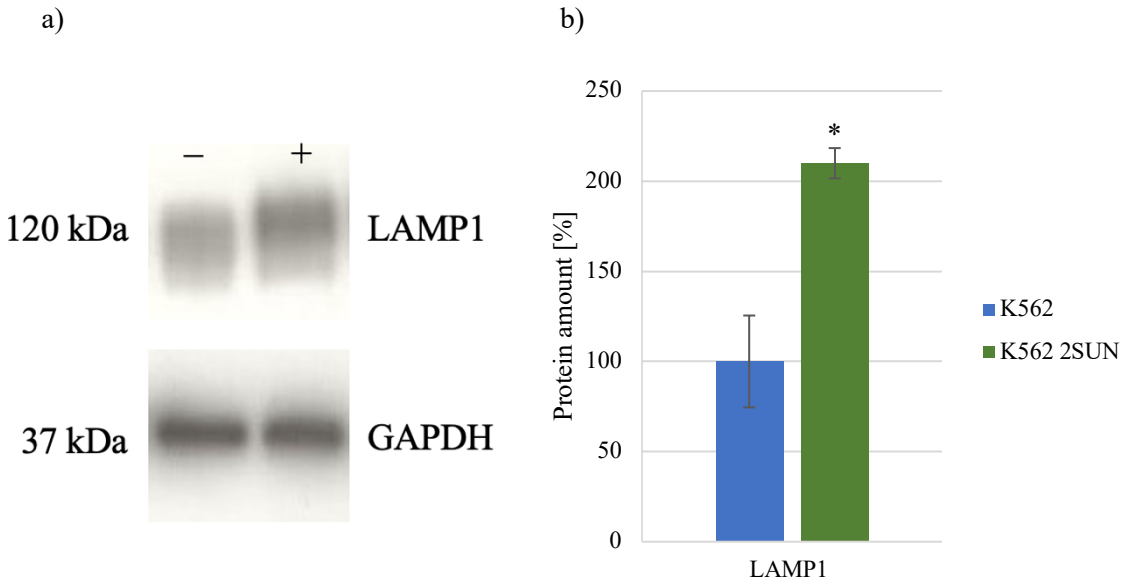


Fig. 24: Effect of sunitinib on the expression of LAMP1 in K562 2SUN cells. a) Typical pictures of western blot: sensitive K562 cells (-), K562 2SUN cells (+). GAPDH was used as the protein loading control. b) Quantitative densitometric evaluation of LAMP1 expression. As the control value, 100% were used in sensitive K562 cells. The evaluation was done with the program ImageJ. The data represent average values ± SE obtained in four independent experiments. *…statistically significant difference in LAMP1 expression between K562 cells and K562 2SUN cells ($P<0,05$).

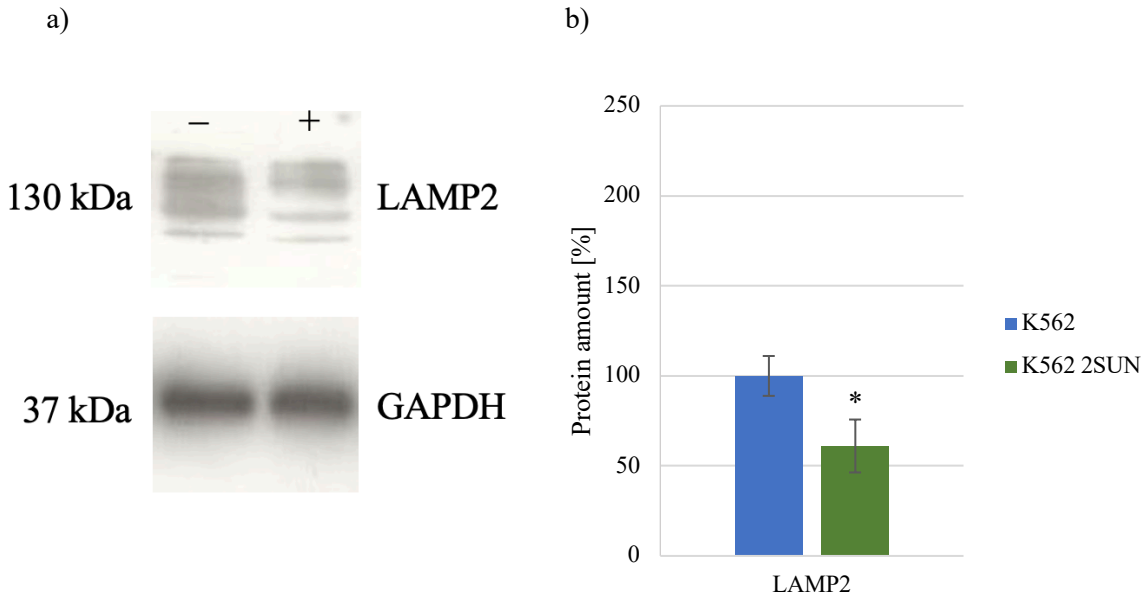


Fig. 25: Effect of sunitinib on the expression of LAMP2 in K562 2SUN cells. a) Typical pictures of western blot: sensitive K562 cells (-), K562 2SUN cells (+). GAPDH was used as the protein loading control. b) Quantitative densitometric evaluation of LAMP2 expression. As the control value, 100% were used in sensitive K562 cells. The evaluation was done with the program ImageJ. The data represent average values ± SE obtained in four independent experiments. *…statistically significant difference in LAMP2 expression between K562 cells and K562 2SUN cells (P<0,05).

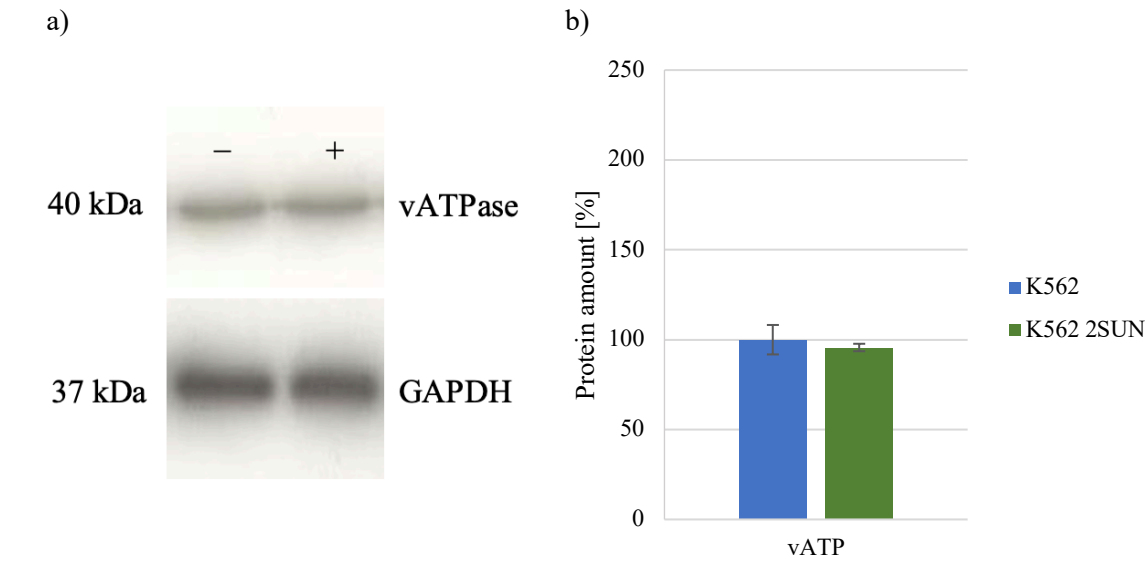


Fig. 26: Effect of sunitinib on the expression of vATPase in K562 2SUN cells. a) Typical pictures of western blot: sensitive K562 cells (-), K562 2SUN cells (+). GAPDH was used as the protein loading control. b) Quantitative densitometric evaluation of vATPase expression. As the control value, 100% were used in K562 control cells. The evaluation was done with the program ImageJ. The data represent average values ± SE obtained in four independent experiments.

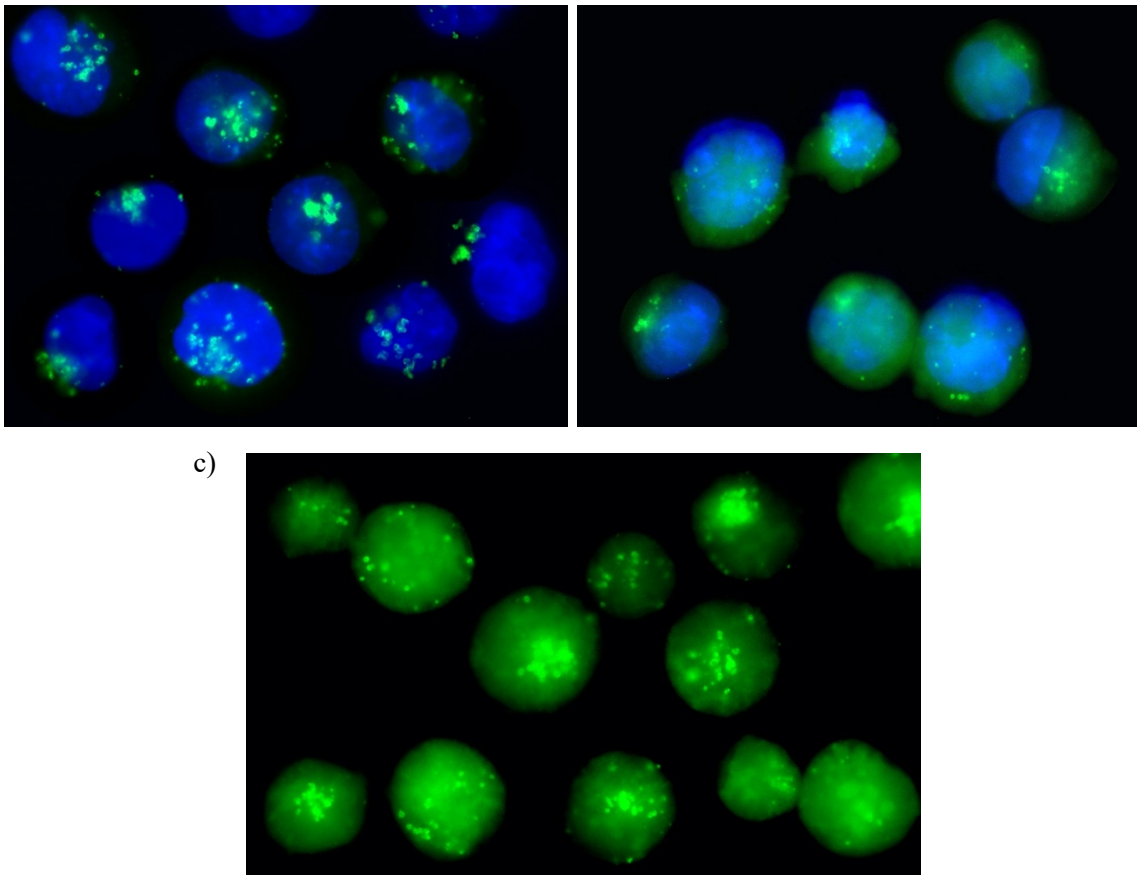
5.2.2 Morphological analysis of lysosomes in resistant K562 2SUN cells

As the results of marker lysosomal proteins LAMP1, LAMP2 and vATPase expression show, there should be no change in the number of lysosomes.

Immunohistochemical staining of LAMP1 with primary and secondary antibodies was done and the mounts were observed under the fluorescent microscope. There was no change between the number or size of the lysosomes in both sensitive K562 cells and K562 2SUN cells (Fig. 27). The sensitive K562 cells were also incubated with 2 μM SUN for one hour before the staining process. The number of lysosomes in both sensitive K562 cells and K562 2SUN cells was obtained by counting lysosomes in 100 cells and then making the average amount together with SE (Fig. 28a).

In terms of measuring the lysosomal diameter, ImageJ program was used. In both sensitive K562 cells and K562 2SUN cells, there were 20 lysosomes measured and divided into two groups according to their size. These two intervals involved cells smaller than 1 μm and cells between 1 – 2 μm (Fig. 28b).

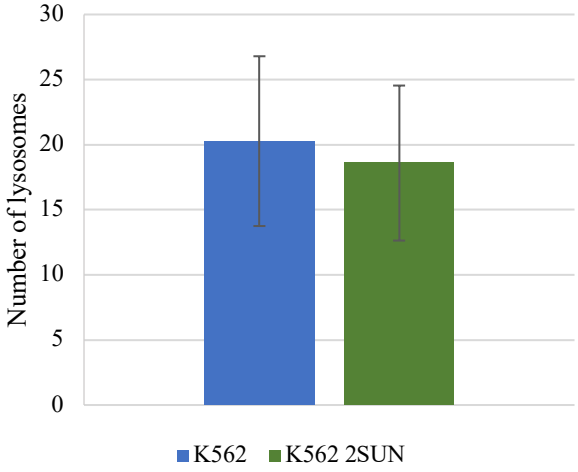
a) b)



c)

Fig. 27: Effect of sunitinib treatment on lysosomal morphology in K562 2SUN cells. Typical pictures of a) sensitive K562 cells with LAMP1 stained by primary and secondary antibodies and the nucleus stained by DAPI for better visualization. b) sensitive K562 cells incubated for one hour with 2 μM SUN before being stained for LAMP1 and nucleus. c) K562 2SUN cells with LAMP1 stained by primary and secondary antibodies.

a)



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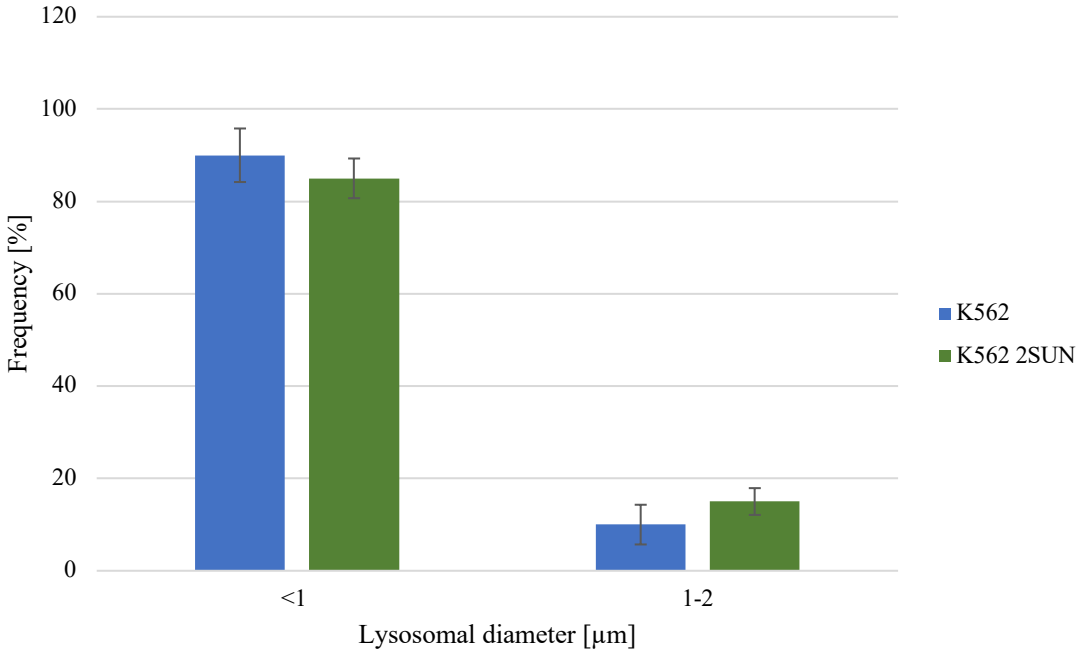


Fig. 28: Quantitative analysis of lysosomes in K562 cells. a) The average number of lysosomes in 100 counted cells of sensitive K562 cells and K562 2SUN cells. b) The average lysosomal diameter of sensitive K562 cells and K562 2SUN cells.

5.2.3 Evaluation of senescence phenotype in resistant K562 2SUN cells

Since the previous results do not support the theory about lysosomal biogenesis, we determined whether the resistant K562 2SUN cells did not acquire the senescence phenotype. We observed three most important features of the senescence including the morphological changes, proliferation potential and the ß-galactosidase activity.

First, the morphology of sensitive K562 cells and K562 2SUN cells was compared. There was no difference in the morphology seen when comparing the sensitive and resistant cells (Fig. 29). When analyzing the living cell distribution in the cell cycles, there was no significant difference between the untreated and treated cells. Even though we could see a higher number of the cells in G1-phase in T98G 2SUN and 4SUN cells, in the case of K562 2SUN there was no G1-phase arrest seen (Fig. 30).

a) b)

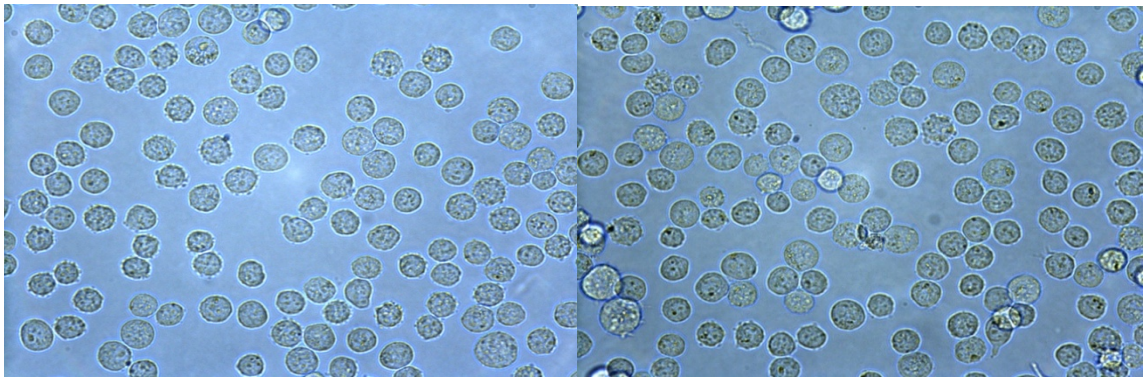
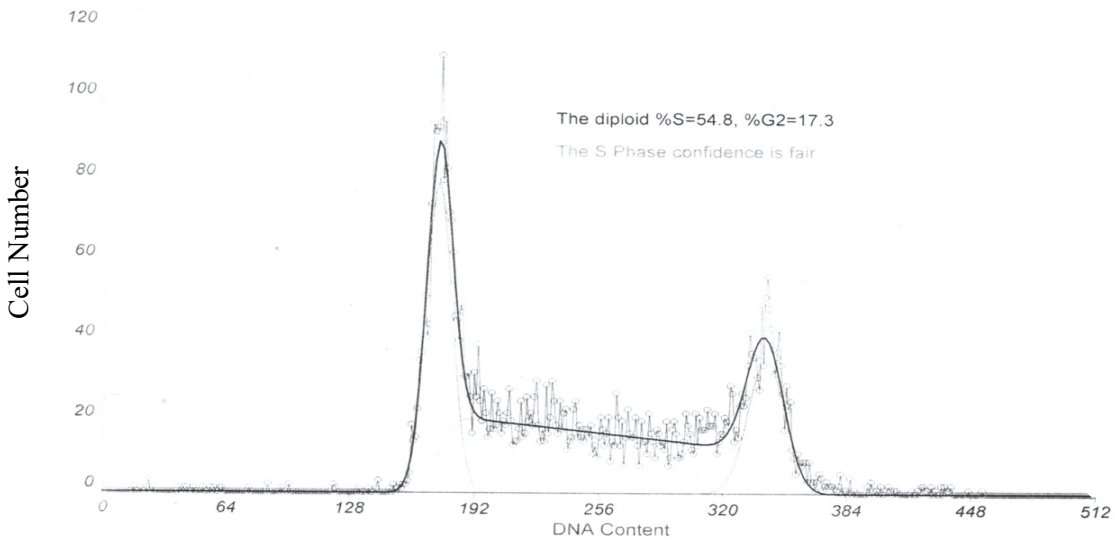
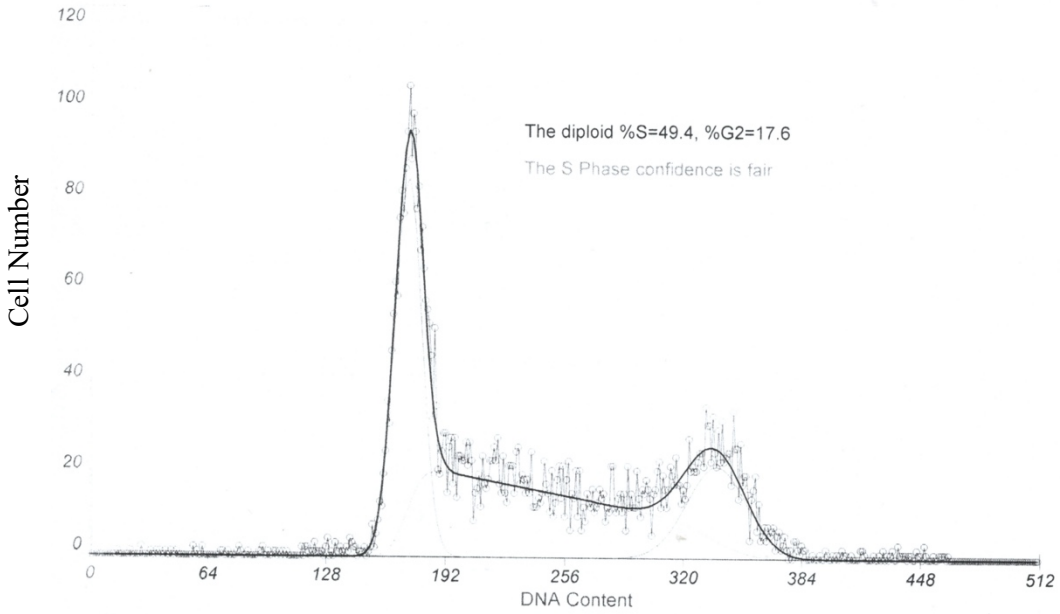


Fig. 29: Morphology of K562 cells. a) Morphology of sensitive K562 cells. b) Morphology of K562 2SUN cells exposed to SUN for 4 months.

a)



b)



c)

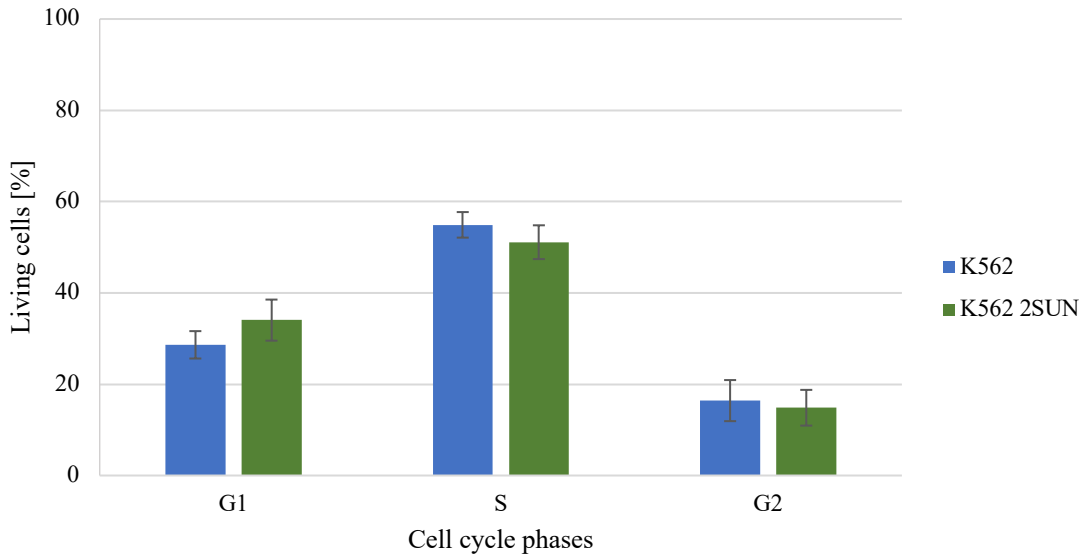


Fig. 30: Cell cycle analysis of K562 cells. Typical histograms of a) sensitive K562 cells, b) K562 2SUN cells, c) quantitative analysis of living cells in the cell cycle phases. The cell cycle was measured by using flow cytometry every week for past three months.

As we could see in figure 30., the proliferation of the cells was not arrested in the G1 phase as in the T98G 2SUN or T98G 4SUN cells. Since there is no significant change in morphology or in the cell cycle analysis, the last senescence feature, the ß-galactosidase activity, was not furthermore tested.

5.2.4 Analysis of heat shock proteins expression in resistant K562 2SUN cells

The last mechanism tested in the experimental part was the expression of HSPs. Since there was no significant difference in all the evaluations of the K562 2SUN cells so far (other than increased LAMP1), we tried to evaluate the expression of these proteins as well.

There was no significant difference in the expression of any of the HSPs observed in sensitive K562 cells or resistant K562 SUN (Figs. 31, 33). There was only a slight increase in the HSP70 protein that is involved in the stabilization of the lysosomal membrane. However, taking into consideration the standard deviation, the increase in the HSP70 was not significant in the evaluation of the expression of this protein (Fig. 32). Therefore, the HSPs also do not show the involvement in the acquired resistance of K562 2SUN cells.

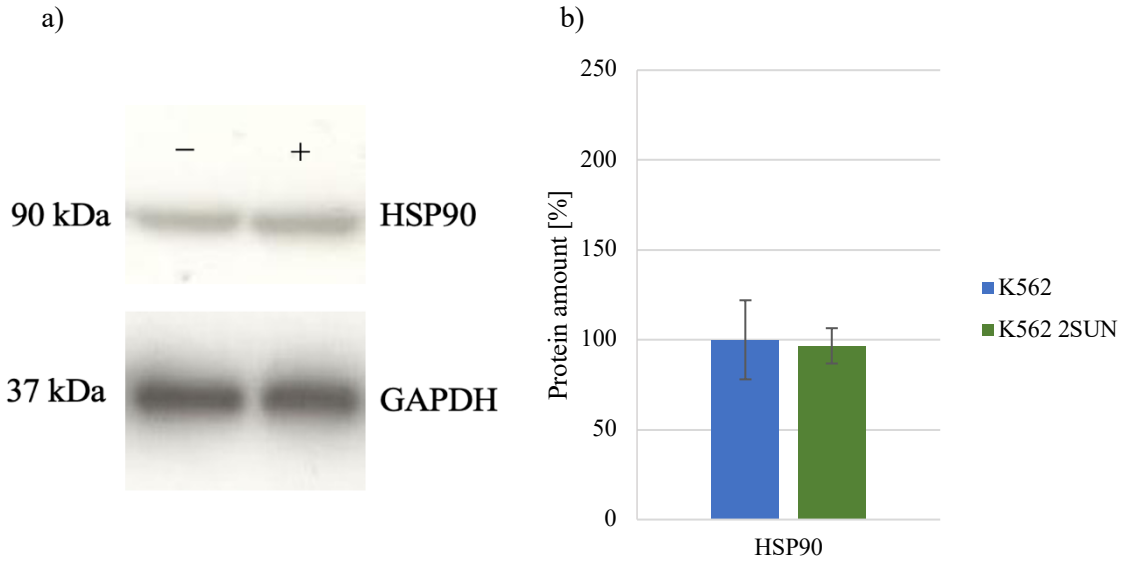


Fig. 31: Effect of sunitinib on the expression of HSP90 in T98G 2SUN cells. a) Typical pictures of western blot: sensitive K562 cells (-), K562 2SUN cells (+). GAPDH was used as the protein loading control. b) Quantitative densitometric evaluation of HSP90 expression. As the control value, 100% were used in sensitive K562 cells. The evaluation was done with the program ImageJ. The data represent average values ± SE obtained in three independent experiments.

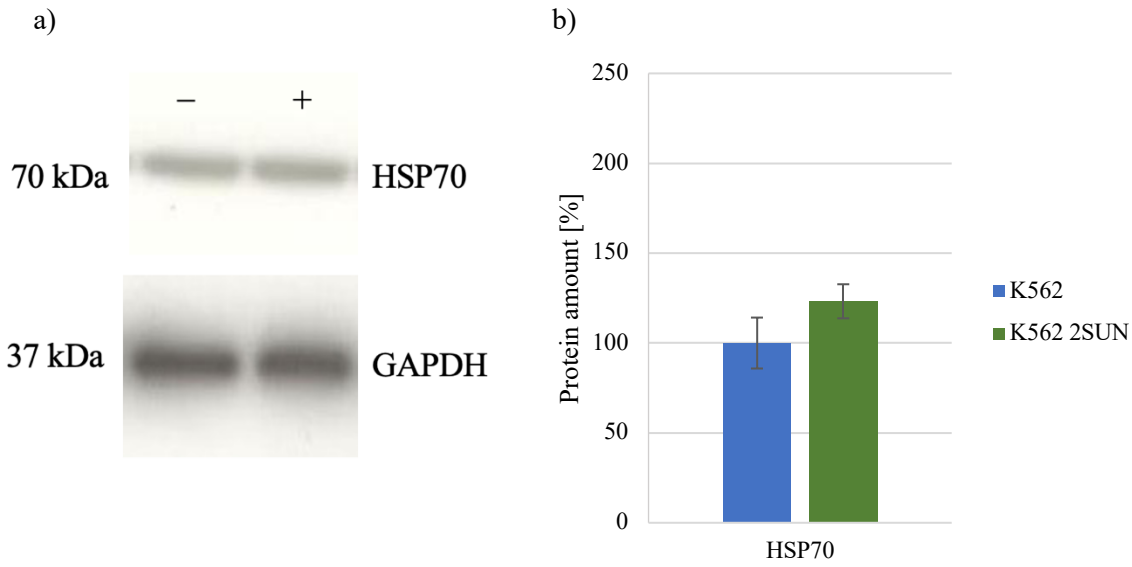


Fig. 32: Effect of sunitinib on the expression of HSP70 in K562 2SUN cells. a) Typical pictures of western blot: sensitive K562 cells (-), K562 2SUN cells (+). GAPDH was used as the protein loading control. b) Quantitative densitometric evaluation of HSP70 expression. As the control value, 100% were used in sensitive K562 cells. The evaluation was done with the program ImageJ. The data represent average values ± SE obtained in three independent experiments.

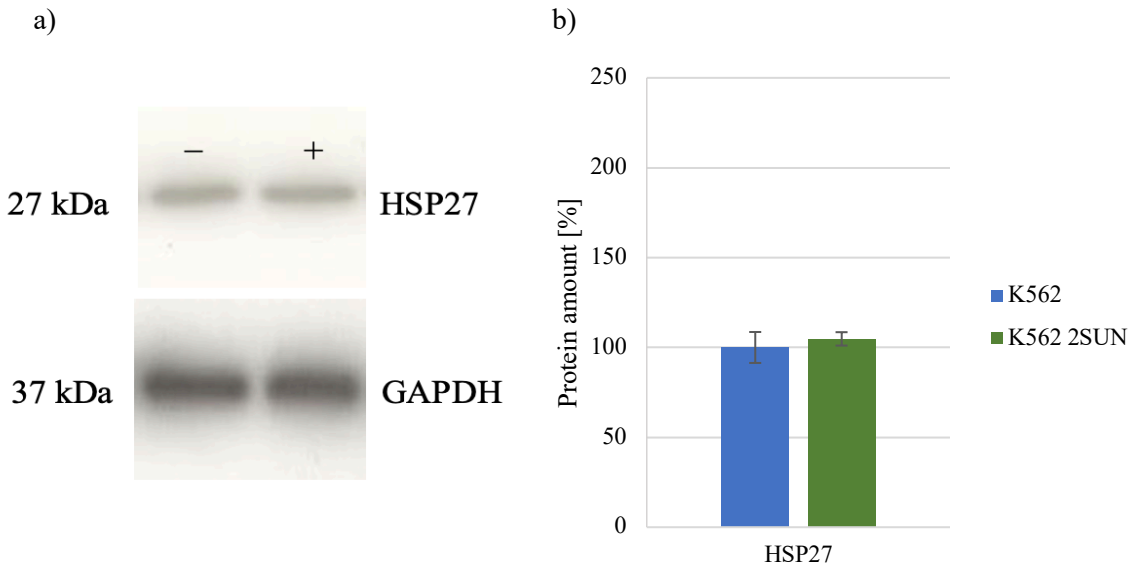


Fig. 33: Effect of sunitinib on the expression of HSP27 in K562 2SUN cells. a) Typical pictures of western blot: sensitive K562 cells (-), K562 2SUN cells (+). GAPDH was used as the protein loading control. b) Quantitative densitometric evaluation of HSP27 expression. As the control value, 100% were used in sensitive K562 cells. The evaluation was done with the program ImageJ. The data represent average values ± SE obtained in three independent experiments.

6 DISCUSSION

The theoretical part of this diploma thesis was focused on hallmarks of tumors summarized by Hanahan and Weinberg. Then the contemporary cancer treatments were described together with the low molecular weight inhibitors including SUN. Subsequently, the "classic" mechanisms of acquired multidrug resistance according to Gottesman were listed (Gottesman *et al*., 2002). Apart from these classic mechanisms, the theory was also focused on the less typical resistance mechanisms such as resistance mediated by the lysosomes or senescence phenotype. At last, the role of heat shock proteins in drug resistance was described as they are included in every aspect of tumorigenesis and cancer resistance.

Multidrug resistance is the most crucial reason for drug treatment failure. It is characterized by many mechanisms, such as a mutation in the targeted site, defects in the apoptotic pathway, or altered DNA repair (Gottesman *et al*., 2002) Nevertheless, other mechanisms are involved in the development of drug resistance, one of them being in the past years the lysosomal sequestration (Zhitomirsky and Assaraf, 2015; Halaby, 2019). This process is linked with the accumulation of hydrophobic weak bases inside of the lysosomes. According to the theory, these anticancer drugs are then unable to reach their targeted sites and it is believed that sequestration, therefore, decreases their cytotoxic effects (MacIntyre and Cutler, 1988; Duvvuri and Krise, 2005). The initial idea of lysosomal sequestration being involved in cancer cell resistance came up when the accumulation of anthracyclines (doxorubicin and daunorubicin) was studied. In this study, the drug uptake by lysosomes was evaluated by fluorescence microscopy (Hurwitz *et al.,* 1997). However, many different reports in the past years provided results contradictory to these findings. A recent study from our laboratory proposed that the lysosomal sequestration of TKIs is dependent on their concentration. Higher concentrations of TKIs result in increased lysosomal sequestration. Nevertheless, despite the high lysosomal sequestration, it was not sufficient to decrease the extracellular concentrations of TKIs which are necessary for the manifestation of resistance (Ruzickova *et.al.,* 2019). Zhitomirsky and Assaraf observed that lysosomal biogenesis triggered by weak base drugs, such as mitoxantrone, SUN, or doxorubicin, can increase their lysosomal sequestration and, therefore, enhance the resistance of the cells (Zhitomirsky and Assaraf, 2015). Another recent study from our laboratory questioned whether the lysosomal biogenesis is the only mechanism responsible for increased lysosomal capacity. It was shown that certain cell lines were able to increase their lysosomal sequestration of TKIs without any prove of lysosomal biogenesis. In the same article, the mechanism of lysosomal fusion was proposed instead (Skoupa *et.al.,* 2020).

According to the literature, SUN is a tyrosine-kinase inhibitor sequestrated in the lysosomes. The accumulation of SUN inside of the lysosomes can be involved in the lysosome-mediated resistance (Azijli *et.al.,* 2015; Gotink *et.al.,* 2015). As already mentioned, the lysosomal sequestration can be enhanced by lysosomal biogenesis and can also increase cancer cell resistance (Zhitomirsky and Assaraf, 2015). Based on these findings we established SUN resistant cells by exposing them to low concentrations of SUN. The resistance that is acquired by gradually increasing concentration of drugs is generally used for the establishment of resistant cells. The established resistant cells K562 2SUN, T98G 2SUN, and T98G 4SUN were characterized. We successfully established significantly resistant cells T98G 2SUN ($IC_{50} = 10.5 \pm 0.9$ μM) and T98G 4SUN cells ($IC_{50} = 14 \pm 0.7$ μM), which were 4.5 times and 6 times more resistant than the paternal T98G cells, respectively (Tab. 5). In K562 cells we established very low but significant resistant K562 2SUN cells ($IC_{50} = 3.5 \pm 0.5$ μM), which were only 1.5 times more resistant than the paternal K562 cells (Tab. 6). We then studied the possible mechanisms involved in the established resistance.

Lysosomal biogenesis is linked with positive TFEB regulated transcription of genes, that are responsible for the expression of all lysosomal proteins. Therefore, we first studied the expression of lysosome-membrane proteins LAMP1, LAMP2, and vATPase since the expression of all three of them is regulated by TFEB. If the lysosomal biogenesis-enhanced sequestration was involved, all these three proteins would have increased expression. In T98G 2SUN and T98G 4SUN cells, the expression of both LAMP1 and LAMP2 proteins significantly increased (Figs. 11, 12). However, the expression of vATPase did not show any changes throughout all the experiments (Fig. 13). On the other hand, in K562 2SUN cells, we could observe only significantly increased expression of LAMP1 protein, with a simultaneous slight decrease in LAMP2 protein (Figs. 22, 25). As in the resistant T98G cells, the expression of vATPase did not change even in K562 2SUN cells (Fig. 26). Lysosomes do not contain only structural membrane proteins, but also soluble ones. Nevertheless, we did not focus our attention on the analysis of the soluble proteins because their concentration can be influenced by lysosomal exocytosis. Since the protein expression characterization did not show unequivocal overall expression of these three proteins, we evaluated their morphology in the resistant cells. LAMP1 protein was immunohistochemically stained as it is one of the typical markers for lysosomes. At first, it seemed that in T98G 2SUN and T98G 4SUN cells the lysosomal compartment increased. However, since SUN emits fluorescence on the visible spectrum, it interferes with the signal of LAMP1 protein. We tried to decrease the SUN interference by its extraction before the LAMP1 staining. Nevertheless, our used extraction solutions did not successfully remove the sunitinib from the cells and cause changes in the lysosomal morphology (data not shown). In T98G 4SUN cells we observed a significant increase in the number of lysosomes and at the same time a

significant decrease in their size (Figs. 14, 15). On the other hand, in K562 2SUN cells, there was no significant change in the number, or size of the lysosomes observed (Figs. 27, 28). Therefore, the lysosomal biogenesis as a mechanism mediating the resistance was not supported by either expression of lysosomal proteins or morphological changes in the lysosomes of resistant cells.

Based on the previous results of the lysosomal evaluation, we examined another possible mechanism of resistance. During the establishment of resistant cells, we observed their proliferation by the evaluation of cell cycle by flow cytometry. In T98G 2SUN and T98G 4SUN we observed a decrease in proliferation as the frequency of the living cells was increased in the G1-phase of the cell cycle (Fig. 17d). Even though there was no change in the proliferation of resistant K562 2SUN cells observed (Fig. 30c), we evaluated whether the senescent phenotype can be involved in the mechanism of cell resistance as it was previously suggested in the literature. We studied another two most typical features of senescence in resistant T98G 2SUN, T98G 4SUN, and K562 2SUN cells (Gewirtz *et.al.,* 2008; Achuthan *et.al.,* 2011). We evaluated changes in the morphology of the cells. In both T98G 4SUN and K562 2SUN cells, there were no changes in comparison to the sensitive cells of each cell line observed (Figs. 16, 29). As we did not observe any changes in the morphology, we evaluated another feature of senescent cells, which was their ß-galactosidase activity. We evaluated the activity only in T98G 4SUN cells, as in the case of K562 there was no change in morphology or proliferation observed. In this test, the senescent cells were stained with blue color. However, when comparing the sensitive T98G cells, resistant T98G 4SUN cells, and the positive control, we observed no significant increase in the senescent cells in T98G 4SUN cells (Figs. 18a, 18b, 18c). These results suggested that the senescence phenotype is not involved in the acquired resistance of our established resistant cells. Nevertheless, decreased proliferation in resistant T98G 2SUN and T98G 4SUN cells could contribute to the acquired resistance. However, without further study, we cannot confirm this hypothesis.

Since none of the mechanisms that we evaluated did suggest its involvement in the resistance, we decided to study the expression of HSPs. These proteins are involved in different cell stress responses and are usually overexpressed in the cancer cells. The major focus was on three HSPs including HSP90, HSP70, and HSP27. Among them, HSP70 plays important role in the stabilization of the lysosomal membrane. Its depletion causes the permeabilization of lysosomal membrane resulting in the release of lysosomal hydrolases into the cytosol. The overexpression of HSP70 in cancer cells can act like the survival protein based on the stabilization of the lysosomal membrane (Gyrd-Hansen *et.al.,* 2004; Nylandsted *et.al.,* 2004). We expected a significantly increased expression of HSPs in the resistant cells. Nevertheless, HSP90 and HSP27 did not change in any of the resistant cells that we established (Figs. 19, 20, 31, 33). A slight increase in expression of HSP70 protein was observed in T98G 2SUN and K562 2SUN cells

(Figs. 21,32). However, in T98G 4SUN cells the expression of HSP70 started to decrease with the time of exposure to SUN. In the first two months with this concentration, the HSP70 did not show any changes in its expression. After three months with SUN, the expression decreased and after four-month exposure, the expression of HSP70 decreased by more than 50% (Figs. 22a, 22b). There is no exact explanation for this decrease in expression. However, as already mentioned, SUN is a very cytotoxic agent and can induce lysosomal membrane permeabilization (Ellegaard *et.al.,* 2013). Therefore, we speculated that this SUN ability can be a reason why we were not able to establish resistant T98G cells to 8 μM SUN. If the expression of HSP70 decreased in the T98G 4SUN cells, then when increasing the concentration to 8 μM SUN the stabilization of the lysosomal membrane would not be possible. We also exposed the sensitive T98G cells to the concentration of 6 μM SUN with the same lethal effect as with the concentration of 8 μM SUN. In the case of K562 cells, we were not able to obtain cells resistant to a higher concentration of SUN than to 2 μM. Therefore, SUN had even more cytotoxic effects on K562 cells than on T98G cells. These results suggest that even the HSPs are not directly involved in the acquired resistance seen in our T98G 2SUN, T98G 4SUN, and K562 2SUN cells.

In conclusion, we did not uncover the mechanism which was responsible for the acquired resistance in SUN resistant K562 2SUN, T98G 2SUN, and T98G 4SUN cells. None of the studied mechanisms did unequivocally suggested its involvement in SUN resistance. We only observed a decreased proliferation potential in SUN resistant T98G 2SUN and T98G 4SUN cells. Therefore, the decreased proliferation can be involved in the acquired SUN-resistance, but the real mechanism must be further studied.

7 CONCLUSION

1. The theoretical part summaries the hallmarks of tumor cells and current treatment methods such as chemotherapy, radiotherapy, and targeted therapy. Apart from the classic mechanisms of resistance, mechanisms of lysosomal sequestration, senescence phenotype and the involvement of HSPs is described.
2. The SUN resistant K562 2SUN, T98G 2SUN, and T98G 4SUN cells were established and characterized.
3. Based on the expression analysis of lysosomal-membrane proteins and examination of lysosomal morphology we concluded that the lysosomal biogenesis was not involved in the acquired resistance to SUN in K562 2SUN, T98G 2SUN, and T98G 4SUN cells.
4. The senescence phenotype was not observed in SUN resistant K562 2SUN, T98G 2SUN, and T98G 4SUN cells. Therefore, we do not assume its involvement in acquired resistance.
5. Studied HSPs (HSP27, HSP70, and HSP90) were not involved in the acquired resistance to SUN in K562 2SUN, T98G 2SUN and T98G 4SUN cells as we did not find their increased expression. In T98G 4SUN, the expression of HSP70 decreased with the time of exposure to SUN.
6. The mechanism of acquired resistance in established SUN resistant cells remains unknown since none of the studied mechanisms unequivocally contributed to the acquired resistance.

8 LITERATURE

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9 ABBREVIATIONS

Apaf1	apoptotic protease activating factor-1
ATP	adenosine triphosphate
BuCy	busulfan-cyclophosphamide
CCL2	CC-chemokine ligand 2
CCL20	CC-chemokine ligand 20
CD36	cluster of differentiation 36
CDK4	cyklin-dependent kinase 4
CDK6	cyklin-dependent kinase 6
CIL	contact inhibition of locomotion
CIP	contact inhibition of proliferation
CML	chronic myeloid leukemia
Co-SMAD	common mediator SMAD
COX2	cyclooxygenase 2
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CXCL8	CXC-chemokine ligand 8
DAPI	4',6-diamidin-2-fenylindol
DD	death domain
DISC	death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle Medium
DSB	double strand breaks
DTT	dithiotreitol
EBV	Epstein-Barr virus
ECACC	European Collection of Cell Cultures
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ERK1/2	extracellular signal-regulated kinase ½
FA	formic acid
Fab	antigen-binding fragment
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FLT3	fms like tyrosine kinase 3

GBM	glioblastoma multiforma
GF	gefitinib
GIST	gastrointestinal stromal tumor
GLUT	glucose transporter
HER1	human epidermal growth factor receptor 1
HER2	human epidermal growth factor receptor 2
HIF-1α	hypoxia induced factor 1a
HK	hexokinase
HPV	human papillomavirus
HRP	horseradish peroxidase
IC_{50}	half maximal inhibitory concentration
ICLs	interstrand cross-links
IL-1β	interleukin 1β
IL-6	interleukin 6
IL-10	interleukin 10
IM	imatinib
LAMP1	lysosome-associated membrane protein 1
LAMP2	lysosome-associated membrane protein 2
LIMP2	lysosome integral membrane protein 2
LOH	loss of heterozygosity
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MDR	multi drug resistance
MGMT	O^6-methylguanine-DNA-methyltransferase
MLH1	MutL homolog 1
MSH2	MutS homolog 2
MSH6	MutS homolog 6
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVD	microvessel density
NAADP	nicotinic acid adenine dinucleotide phosphate
NAPP	sodium pyrophosphate decahydrate
NF- κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NSCLC	non-small cell lung cancer
p21	CDK-interacting protein 1

p53	tumor protein 53
PD-1	programmed cell death protein 1
PDGF	platelet-derived growth factor
PDGF-B	platelet-derived growth factor subunit B
PDGFR	platelet-derived growth factor receptor
PE	phycoerytrin
PFK	phosphofructokinase
PMS2	PMS1 homolog 2
pNETs	pancreatic neuroendocrine tumors
pRB	retinoblastoma 1 protein
PTEN	phosphatase and tensin homolog
RIPA	radioimmunoprecipitation assay buffer
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
STAT3	signal transducer and activator of transcription 3
SUN	sunitinib
TERT	telomerase reverse transcriptase
TFEB	transcription factor EB
TGF-β	transforming growth factor beta
TGF-βR	transforming growth factor beta receptor
TK	tyrosine kinase
TKI	tyrosine kinase inhibitor
TME	tumor microenvironment
TMRE	tetramethylrhodamine, ethyl ester
TNF-α	tumor necrosis factor α
TNRF	tumor necrosis factor receptor
TPC	two-pore channel
TRIS	tris(hydroxymethyl)aminomethane
TRP	transient receptor potential channel
TRPM2	transient receptor potential melastatin 2
TRPML1	transient receptor potential mucolipin 1
TSP-1	thrombospondin-1
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor