Effects of combination therapy of PDE5 inhibitor sildenafil and multikinase inhibitors sorafenib and sunitinib in NSCLC

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Widmung

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1 Introduction

Lung cancer is the most common form of cancer worldwide. Statistics show an annual increase of 0.5% in fatalities associated with lung cancer. It is the most common form of death from cancer in Germany (Becker 2010 – [Epidemiology of lung cancer.]). Many experts predict that by the year 2020 lung cancer will account for 50% of cancer-related deaths.

One problem is the late diagnosis, in order that the prognosis for most patients remains poor. In most of the cases the five-year survival rate amounts to 15%. Nevertheless, advances are being made in the battle to combat lung cancer such as research focused on molecular-targeted therapies.

1.1 Lung cancer

1.1.1 Lung cancer pathology

Tumors arising from respiratory epithelium bronchi, bronchioles and alveoli are called lung cancer. According to the World Health Organization (WHO), lung cancer is the leading cause of cancer death in men and woman.

There are two classes of lung cell carcinoma: small cell lung carcinoma (SCLC) and Non-small cell lung carcinoma (NSCLC) with its varieties of squamous, adenocarcinoma and large cell carcinoma. It is important to determine whether a tumor is SCLC or NSCLC for both prognostic and therapeutic reasons. The correct histologic diagnosis needs to be done by an experienced pathologist.

The odds of surviving lung cancer are low, particulary as SCLC and NSCLC require different treatments. The choice of treatment depends on the stage of the disease; that is, on whether it is just commencing, or is already mature.

Diagnosis of lung cancer and staging comprises the following: pulmonary X-rays, CT scans of the thorax and upper abdomen, bronchoscopy and; in selected cases, mediastinoscopy. The classification is based primarily on the results of these examinations. The TNM International Staging System is frequently used for NSCLC classification. TNM stands for tumor size (T), regional node involvement (N), and presence or absence of distant metastasis (M). The different stage groups are defined by combining those three factors.

The TNM classification is not used for SCLC. There is a two-stage system including limited-stage disease and extensive-stage disease.
Limited-stage is defined as a disease confined to regional lymph nodes, one hemithorax and mediastinum.
Extensive-stage disease is used for all other SCLC stadiums exceeding those bounds.

1.1.2 Classification

Cancer can occur in all kinds of tissue, i.e. soft tissue, mesothelium, lymphatics, epithelium or miscellaneous. If there are mixed tumors they need to be named according to the cell form that is most exposed.
Epithelial tumors are classified into two major groups: SCLC and NSCLS.
15% - 20% of the cases are SCLC. This form mainly starts in the central bronchus and grows rapidly.
NSCLC is classified into three types: adenocarcinoma, squamous cell cancer, and large-cell carcinoma. NSCLC accounts for 75% - 80% of the epithelial lung cancer cases.

Adenocarcinoma:

> This is usually localised in the periphery of the lung; it is most commonly found amongst non-smokers. Adenocarcinoma usually accounts for 35% - 40 % of lung cancer cases.

Squamous cell cancer:

> These have a growth rate slower than that of adenocarcinoma. They start in larger airways like SCLC. Because of their slow growth, the size of these tumors varies when exposed.

Large-cell carcinoma:

> These account for about 10%-15% of lung cancers. They are characterised by fast growth and their occurrence near the surface of the lungs.

There are other types of lung cancers, but these only cause less than 5% of all the lung cancer cases. This group of rarely represented cancers includes the following: carcinoid

tumor, sarcomatoid carcinoma and other soft tissue tumor, adenoid cystic carcinoma, mucoepidermoid carcinoma and other unspecified carcinomas (Harrisons Principles of Internal Medicine, 17th edition, p. 551, table 85-1 according to WHO classification).

For research purpose cell lines like A549 or H1437 (human adenocarcinoma), H226 (squamous cell carcinoma) and H460 or H661 (large-cell carcinoma) are used. In this experimental work we decided to take A549 cells to represent the largest subgroup of NSCLC.

1.1.3 Risk factors

Patients with lung cancer of all histologic types are current or former smokers in 75% - 90% of men and 30% - 60% of women. (Becker 2010 – [Epidemiology of lung cancer.]) Even passive smoking is considered a common cause of cancer. (IARC Working Group 2007- Smokeless tobacco and some tobacco-specific)

In general cancer is the final step in a sequence of structural and morphological changes that transform normal cells into malignant cells and emerge over several years. There are, potentially, numerous causes for the occurrence of lung cancer: genetic lesions, including the activation of dominant oncogenes and inactivation of tumor-suppressor or tumor-recessive oncogenes; the immune system, cellular irritation caused by asbestos, radon and many other environmental factors (DGAUM). Proto-oncogenes are responsible for controlling cell cycle control, programming cell death, regulating growth, and terminal differentiation. Faulty expression of the genetic translocations would cause mutations or DNA amplification, which would, consequently, result in tumorigenesis. The best known tumor-supressor gene is p53. In usually modified types of cancer, it is downregulated or defective, like most of tumor suppressor genes and unlike the classical oncogenes. Tumor development continues by obtaining support from surrounding stromal cells, and attracting new blood vessels to bring essentials like oxygen and nutrients, avoiding immune detection and thus paveing the way for metastasis.

By determining the stage and type of cancer, it is possible to treat the disease with certain chemotherapeutic agents, surgery, radiation therapy, or a combination of these treatments.

1.2 Therapy

1.2.1 Surgery

The treatment of NSCLC and SCLC includes the following three surgical procedures for pulmonary resection: lobectomy, wedge resection and pneumonectomy.

Surgical resection is only used to treat SCLC, when the patients have neither a locally advanced disease nor distant metastases.

Pulmonary resection is the treatment of choice in patients with NSCLC stages IA, IB, IIA and IIB provided that they can tolerate the operation (Prasse, Waller et al. 2010 – Lung cancer from the perspective).

1.2.2 Chemotherapy

Multimodal therapy has become the norm for regionally advanced disease, and patients with advanced and metastatic disease are candidates for palliative chemotherapy, for which there is documented evidence of improvements in survival and quality of life measures.

Cisplatin, a strong and widely used chemotherapy drug, is commonly used in both NSCLC and SCLC. In NCSLC cisplatin in combination with vinorelbine provided a marginally better benefit than other drug combinations. (Arriagada, Bergman et al. 2004 - Cisplatin-based adjuvant chemotherapy in patients)

The combination of cisplatin plus etopsid or topotecan is used for the treatment of SCLC, for NSCLC, cisplatin plus etopsid or vinorelbine is used.

1.2.3 Radiation therapy

As described, surgery is not an option when patients cannot tolerate the operation. In that case radiation therapy is adapted to the tumor tissue. 3D conformal radiation therapy is a method to get the highest dose of radiation clearly defined at tumor tissue without damaging much of the surrounding healthy tissue. Even if the tumor is too widespread for surgery and at this stage no distant metastases were found, patients profit from this treatment (Zierhut, Bettscheider et al. 2001 – Radiation therapy of stage I).

1.2.4 Targeted therapy

Commonly used procedures: chemotherapy, radiation therapy, and surgery, all have limited success. Consequently, researchers have been focusing on alternative

approaches that attack molecular mechanisms in tumorgenesis, such as mechanisms related to the cell cycle, angiogenesis, and protein degradation.

Currently, cetuximab, an anti-EGFR monoclonal antibody, and EGFR tyrosine kinase inhibitors such as gefitinib and erlotinib are used in NSCLC therapies. Bevacizumab, an inhibitor targeted specifically at angiogenesis, is also used for treating this kind of cancer. Furthermore there are powerful multitargeted agents to treat non-small cell lung cancer, i.e. sunitinib and sorafenib. Sunitinib and sorafenib are multi-tyrosine kinase inhibitors with antitumor and angiogenetic activities (Katzel, Fanucchi et al. 2009 – Recent advances of novel targeted).

It is possible to increase the survival in patients with advanced non-small cell lung cancer by using cetuximab (Pirker, Pereira et al. 2009 – Cetuximab plus chemotherapy in patients).

For patients with advanced or recurrent non-small cell lung cancer, bevacizumab, together with carboplatin and paclitaxel, shows a higher success rate, longer median time to progression and a slightly better increase in survival than in a control group not given bevacizumab in therapy in a randomized phase II study (Johnson, Fehrenbacher et al. 2004 – Randomized phase II trial comparing).

Gefitinib is also a well-tolerated agent in the treatment of NSCLC (Tiseo, Bartolotti et al. 2010 – Emerging role of gefitinib).

1.3 Growth factors

Growth factors include a group of polypeptides which transfer a signal from one cell to another cell by using a specific receptor in the membrane. Some of their principal tasks are differentiation, survival and proliferation. In addition, they induce a variety of intracellular processes. Currently, several of the signal transductions and their associated growth factors platelet-derived growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and transforming growth factor (TGF) are known. They operate by binding to tyrosine kinases receptors.

A possible solution for the treatment of NSCLC is the blocking of the above receptors.

1.3.1 Platelet-derived growth factor

Platelet-derived growth factor receptors (PDGFR) are cell surface tyrosine kinase receptors. There are two important subunits, PDGFR-alpha and PDGFR-beta, for cell

differentiation, cell growth, development and cell proliferation are encoded in different genes. Presently PDGF-C and PDGFR-D are established. There are five isoforms of the PDGF ligand: PDGF-AA, PDGF-AB, PDGF-BB PDGF-CC and PDGF-DD. Some of them can specifically bind to and activate PDGFR-alpha or PDGFR-beta, while others can stimulate heterodimeric PDGFRalpha/beta complexes.

When the ligand binds to the receptor, a dimerization of the intracellular part follows. This is the important requirement for the following transphosphorylation, activation of intracellular proteins and creation of binding sites for downstream well-characterized signaling pathways, e.g. RAS/MAPK, JAK/STAT and P13K (Balottin, Nicoli et al. 2004 – Migraine and tension headache).

According to current research results there is an increased expression of PDGF and its receptors in malignant human tumors such as ovarian, prostate and NSCLC and also in pulmonary hypertension. This proves that PDGF stimulates the angiogenetic process both directly and indirectly. PDGF is also involved in the angiogenetic process, in vitro and in vivo (Nguewa, Calvo et al. 2011 – Tyrosine kinase inhibitors with antiangiogenic).

1.3.2 Vascular endothelial growth factor

The vascular endothelial growth factor (VEGF) is a class V receptor tyrosine kinase (RTK). The transcription of the VEGF gene is physiologically regulated by hypoxia. Alternative splicing and proteolytic processing is involved in the production of different isoforms.

VEGF-A is the most investigative variant and an important mediator of tumor angiogenesis, but presently also VEGF-B to VEGF-E are known. These members of the VEGF-family occupy the three receptor tyrosine kinases VEGFR-1 (also named Flt-1), VEGFR-2 (also named KDR) and VEGFR-3 (also named Flt-4). Flt-1 is expressed on endothelial cells and monocytes. Its ligand-binding domain plays an important role in early embryogenesis by trapping VEGF-A, with the result that there is a lack of proteins for binding on other VEGF-Receptors. Similarly, an absence of the whole Flt-1 receptor in vitro shows an overgrowth of vessels, because KDR primarily induces proliferation and mitogenic activities. The interaction, between these two is necessary for a correctly aligned vascularisation. In both the signaling pathway is a ligand-binding induced autophosphorylation, followed by coupling to intracellular signal transducers. It is expected that there is an interaction between the different intracellular VEGF-receptors.

Redundant KDR plays a significant role in the pathological pathway of angiogenesis. The phosphorylation initiates several intracellular pathways including Ras and the protein kinase C c-Raf-MEK-MAP kinase cascade. VEGFR3 and KDR are involved in lymphatic vessel sprouting, probably by inducing the MAPK pathway.

Previous studies demonstrate an over-expression of VEGFA in most tumors. There is also a correlation between tumor microvessel density and growth. Resumed VEGF ligands and their receptor tyrosine kinases have developed into an important target for cancer therapy, especially because of their variety of action, i.e. apoptosis of pre-existing vessels and inhibition of new vessel formation (Nguewa, Calvo et al. 2011 – Tyrosine kinase inhibitors with antiangiogenic).

Further important growth factors in the study of tumor development have been mentioned.

The ret-protooncogen (RET) is a transmembrane protein and a member of the RTK class XIV. When growth factors bind to these proteins, a cascade of reactions occurs within the cell which instruct the cell to carry out a specific function, for example, dividing or maturing to perform specialized functions.

Flt-3 (Fms-like tyrosine kinase-3) belongs to the class III RTK family to which c-Fms, c-Kit, and the PDGFR (Platelet Derived Growth Factor Receptor) also belong.

Flt-3 is a gene encoding a receptor tyrosine kinase that in this instance time regulates hematopoiesis. The receptor is finally activated by an fms-related tyrosine kinase 3 ligand. The activated receptor kinase subsequently phosphorylates and activates multiple cytoplasmic effector molecules in pathways involved in the apoptosis, proliferation, and differentiation of hematopoetic cells in bone marrow, such as RAS/RAF/MAPK cascades.

CKIT is a protein on the surface of cells that binds to stem cell factor. Thus it is also called stem cell factor receptor. The stem cell factor induces certain types of cells to grow.

CSF-1R is also a cell-surface protein encoded by the CSF-1R gene. It is a receptor for a cytokine called colony-stimulating factor 1. This is a cytokine which controls the differentiation, production and function of macrophages.

The epidermal growth factor receptor (EGFR) is situated on the cell surface to bind on members of the epidermal growth factor family of extracellular protein ligands, which belong to RTK class I. The activation of this receptor includes signal transduction

pathways that are involved in the regulation of cellular proliferation, differentiation, and survival.

Simply put, the MAPK (mitogen-activated protein kinases) or ERK (extracellular signal regulated kinases) pathway is a chain of intracellular proteins whose activation starts with binding of a growth factor to a receptor on the surface of the cell. As a result the tyrosine kinase gets activated; that is, the cytoplasmic domain of the receptor gets phosphorylated. Subsequently a variety of proteins with a typical SH2 domain can bind to the phosphotyrosine residues of the activated receptor. With the help of another group of proteins (Guanine nucleotide exchange factors, GEFs), RAS gets activated by releasing GDP and gaining GTP. After that the kinase cascade continues contributing the serine/threonine-selective protein kinases RAF, MEK and MAPK. At the end of these cascades the activated MAP-kinases accumulate in the cell nucleus and start the phosphorylisation of several transcription factors. As a result of this the transcription and translation of mRNA into protein is possible. Such proteins are needed by a cell, for example, to regulate embryogenesis, apoptosis, cell growth and differentiation (Wilhelm, Adnane et al. 2008 – Preclinical overview of sorafenib).

1.4 Multikinase inhibitors

1.4.1 Sunitinib

Developed by Pfizer sunitinib emerged as a efficacious target to inhibit cell proliferation and migration by modify signaling pathways as outlined above mentioned. This drug targets different growth factor receptors such as VEGFR-1, PDGFR-alpha, PDGFR-beta, FLT3, c-KIT and CSF-1R. Inhibition of those receptors prevents the generation of signals that result in tumor growth, angiogenesis and metastasis. There are experimental studies which show the antitumor activity effect and implicate the reduction of angiogenesis in different xenograft models, such as colon, lung and kidney.

Presently, sunitinib is a possible first-line treatment of patients with advanced or metastatic renal cell carcinoma and gastrointestinal stroma tumor (GIST). It is also being investigated as a possible therapy for other cancers, including breast cancer, colorectal cancer, and non-small cell lung cancer. Sunitinib even functions as a receptor tyrosine kinase inhibitor. In summary there are several studies that conduct research into the efficacy of sunitinib, both on its own and combined with other drugs, in the

treatment of human tumors (Wilhelm, Adnane et al. 2008 – Preclinical overview of sorafenib, Gridelli, Maione et al. 2007 – Sorafenib and sunitinib,).

1.4.2 Sorafenib

The oral multikinase inhibitor sorafenib is currently used for the treatment of patients with advanced renal cell carcinoma and for patients with unresectable hepatocellular carcinoma. Additionally this target is explored in several preclinical cancer models, including human colon, ovarian, thyroid, breast, pancreatic and non-small-cell lung carcinomas. Sorafenib inhibits a variety of receptor tyrosine kinases considered responsible for causing tumorangiogenesis and progression, including VEGFR, PDGFR, Flt3, RET and cKIT. Also it is a potent inhibitor of the RAF kinase pathway. In some xenograft models there was also a reduction in tumor angiogenesis. On the whole it is speculted that sorafinib may inhibit tumor growth by a dual mechanism: it affects the tumor angiogenesis directly by inhibiting RAF and KIT signalling, and it affects the tumor angiogenesis indirectly by inhibiting the signalling of VEGFR and PDGFR (Wilhelm, Adnane et al. 2008 – Preclinical overview of sorafenib, Gridelli, Maione et al. 2007 – Sorafenib and sunitinib).

Sorafenib has been approved for the treatment of HCC (hepatocelllular carcinoma) since 2007. The results of the Phase-III study “Sorafenib HCC Assessment Randomized Protocol” (Sharp-Studie) show demonstrably that sorafenib, compared to a placebo, increases the survival period of patients with HCC by 44 % (journal onkologie, 27.09.2007).

All current therapies have a significant number of side effects. Commonly used chemotherapeutic agents have some systemic effects such as hair loss, anaemia, infections, diarrhea and constipation; nerve, muscle, skin and nail problems are also common. Radiation therapy also causes fatigue and skin problems. These side effects severely debilitate the patients.

It is thus necessary to carry out research on new therapeutic agents and combinations of these agents in order to minimize the side effects and get a better outcome and quality of living.

1.5 Phosphodiesterases

1.5.1 Cyclic nucleotide signalling

Another promising advance in tumor therapy is the modulation of the intracellular cyclic nucleotides cAMP and cGMP. CAMP is typically produced by hormone stimulation of seven-transmembrane G protein-coupled receptors. These receptors stimulate membrane-associated adenylyl cyclase to convert ATP into cAMP. Three effectors of cAMP are known: protein kinase A (PKA), cAMP-gated ion channels, and cAMP-activated exchange factors (EPAC). These effectors are called the core effectors in all cells. Inside the cell cAMP is degraded by phosphodiesterases (PDEs) (Savai, Pullamsetti et al. 2010 – Targeting cancer with phosphodiesterase inhibitors).

CGMP is generated from GTP after stimulation of soluble guanylyl cyclase (sGC) by nitric oxide (NO) or particulate guanylyl cyclase (pGC) by natriuretic peptides (NP). These so-called second messengers can now bind to protein kinase G (PKG) and cGMP-gated ion channels or get deactivated by PDEs. PKG catalyses the phosphorylation of a number of physiologically relevant proteins involved in the contractile activity of smooth muscle cells.

All effectors of cAMP and cGMP induce cellular responses such as proliferation, apoptosis, differentiation, vasodilation or vasoconstriction and inflammation. In breast cancer cells and colon cancer cells the increasing intracellular concentrations of cAMP may induce apoptosis, arrest growth, and rarefy cell migration (Savai, Pullamsetti et al. 2010 – Targeting cancer with phosphodiesterase inhibitors).

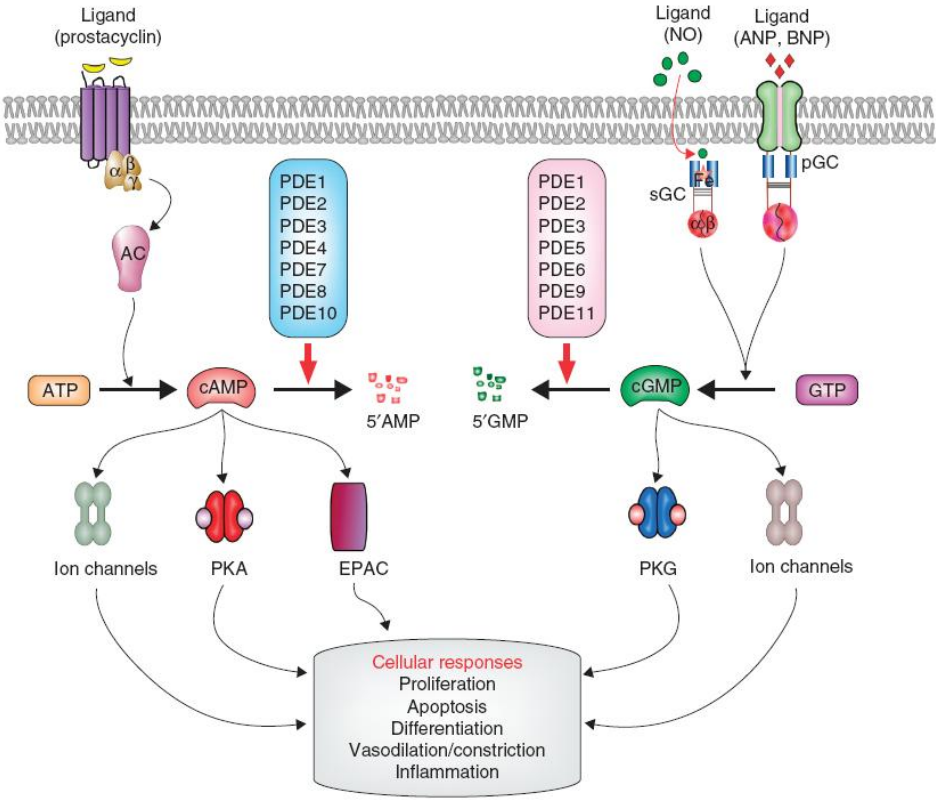


Figure 1: Cyclic nucleotide phosphodiesterase signalling. This graphic shows the basic synthetic, regulatory, and downstream pathways that are involved in the effects of endogenous cyclic adenosine monophosphate (cAMP) and cyclic guanosin monophosphate (cGMP). Cyclic nucleotide phosphodiesterases (PDEs) catalyze the hydrolysis of cAMP and/or cGMP and thereby regulate the amplitude and duration of cell signalling. Mechanisms mediated by cAMP and cGMP (Savai, Pullamsetti et al. 2010 – Targeting cancer with phosphodiesterase inhibitors).

1.5.2 Phosphodiesterase family

Phosphodiesterases are key enzymes modulating the existent amount of the second messangers cAMP and cGMP by controlling the degradation. Currently, there are 11 known families of phosphodiesterases (PDE1- PDE11). They are named and classified because of the similarity of their sequences, preference of substrate, and sensitivity to various inhibitors. Some of the PDE gene families have isoform subfamilies produced in different but according to genes. These so-called subfamilies distinguish themselves through their three-dimensional structure, kinetic properties, regulatory mechanisms, intracellular localisation, cellular expession and inhibitor sensitivities. The role of PDEs is determined according to their family-specific inhibiors (Omori, Kotera 2007 – Overview of PDEs and their). Each PDE molecule can be divided into three regions: the N-terminal splicing region, a regulation domain, and a C-terminal catalytic domain (Ke, Wang 2007 – Crystal structures of phosphodiesterases). Within the PDE families there are no big differences concerning the N-terminal splicing region. The conserved

catalytic domain contains about 300 amino acids and shows considerable divergence. The regulatory domains offer variations of structure, i.e. GAF in PDE2, 5, 6, 10 and 11. GAF domains belong to the group of SMBDs (small molecule binding domains). This group consists of the first three protein classes in which these domains have been discovered. These three are the cGMP-specific phosphodiesterases, the Adenylyl cyclase from the cyanobacterium Anabaena and the E.coli transcription factor Fh1A (Hofbauer 2007 – Die GAF-Domänen der humanen Phosphodiesterase). In general the regulation of PDEs is a complex process involving cellular distribution and intracellular compartmentalization (Lugnier 2006 – Cyclic nucleotide phosphodiesterase PDE superfamily).

Table 1: Classification and expression of phosphodiesterases (PDEs)

Family	Members	Substrate	Tissue/cellular expression	Commonly used inhibitors
PDE1	A,B,C	cAMP/cGMP	Lung, heart, brain, testes, sperm, smooth muscle, lymphocytes, macrophages	Vinpocetine, IC224, SCH51866, 8-MeoM-IBMX
PDE2	A	cAMP/cGMP	Lung, liver, heart, brain, macrophages, endothelial cells, adrenal gland	EHNA, BAY60-7550, PDP, IC933
PDE3	A,B	cAMP/cGMP	Lung, heart, kidney, liver immune cells, dipocytes, hepatocytes, platelets	Milrinone, Tolafentrine, Cilostazol, Cilostamide, Trequinsin, OPC-33540, Dihydropyridazinone, Lixazinone
PDE4	A,B,C,D	cAMP	Ubiquitous	Cilomilast, Rolipram, Ro20-1724, Roflumilast, AWD12281, V11294A, SCH35159, Denbufylline, Arofylline
PDE5	A	cGMP	Lung, brain, heart, kidney, skeletal muscle, platelets	Sildenafil, Tadalafil, DA8159, E402, Vardenafil, Zaprinast, DMPPO, Dipyridamole
PDE6	A,B,C,D,G	cGMP	Photoreceptors	Zaprinast
PDE7	A,B	cAMP	Heart, liver, brain, kidney, pancreas, testes, skeletal muscle, immune cells	BRL 50481, IC242, Dipyridamole, BMS-586353, Thiadiazoles
PDE8	A,B	cAMP	Liver, heart, kidney, brain, spleen, ovary, testes, colon, thyroid, skeletal muscle, immune cells	Dipyridamole
PDE9	A	cGMP	Lung, liver, brain, spleen, kidney, prostate	BAY73-669, SCH51866, Zaprinast
PDE10	A	cAMP/cGMP	Heart, brain, thyroid, testes	Papaverine, Dipyridamole, PQ-10
PDE11	A	cAMP/cGMP	Liver, kidney, thyroid, testes, prostate, pituitary glands, skeletal muscle	Dipyridamole

1.5.3 Inhibitors

In general PDE family-specific inhibitors make it possible to get insights into the functional role of specific PDEs and may be useful for predicting the potential therapeutic effects of targeting.

Inhibitors of PDEs can be classified into nonselective phoshodiesterase inhibitors and selective inhibitors. Methylated xanthines and their derivatives belong to the group of nonselectives. The first inhibitor methylxanthine was described in the literature in 1962 (Butcher, Sutherland 1962 – Adenosine 3',5'-phosphate in biological materials). The selective PDE inhibitors are currently being investigated for the treatment of a variety of diseases, as these examplesillustrate.

The PDE2 inhibitor may be of therapeutic interest in pneumococcal pneumonia (Witzenrath, Gutbier et al. 2009 – Phosphodiesterase 2 inhibition diminished acute). The PDE3 inhibitor Milrinone is presently used in the acute treatment of heart failure to diminish long-term risk (Shin, Brandimarte et al. 2007 – Review of current and investigational). The PDE4 inhibitors like roflumilast are generally accepted in the treatment of asthma (Hoymann, Wollin et al. 2009 – Effects of the phosphodiesterase type, Böhmer, Nassr et al. 2009 – The targeted oral) and COPD (Gross, Giembycz et al. 2010 – Treatment of chronic obstructive pulmonary). Research is being conducted in the development of the PDE7 inhibitor ASB16165 and may be useful for the treatment of diseases in which cytotoxic T lymphocyte has a pathogenic role, e.g. autoimmune diseases (Kadoshima-Yamaoka, Murakawa et al. 2009 – Effect of phosphodiesterase 7 inhibitor). Specific inhibitors for PDE8-PDE11 have been detected recently and their design has already begun.

1.5.4 Phosphodiesterase 5 and the specific inhibitor sildenafil

Phoshphodiesterase 5 isozymes were found in several tissue types including lung, brain, heart, kidney, skeletal muscle and platelets. This enzyme causes a decrease of cGMP inside a cell. Consequently a specific PDE5 inhibitor like sildenafil is responsible for an increasing cGMP concentration. Sildenafil was the first orally active, effective therapy for the treatment of erectile dysfunction. The mechanism of action in penile erection is described below:

Nitric Oxide, NO, binds to the receptors of the enzyme guanylate cyclase, which results in increased levels of cyclic guanosine monophosphate (cGMP). This leads to smooth muscle relaxation (vasodilation) of the intimal cushions of the helicine arteries, resulting in increased inflow of blood and an erection. The drug now arrests the degradation of cGMP and prolongs the duration of an erection. However the drug was originally studied for its treatment of hypertension and angina pectoris.

It was discovered that whilst the drug efficacy in treating against angina pectoris was insignificant, it was quite effective in causing erections. In the year 1996 the pharmacological company called Pfizer decided to market it for the treatment of erectile dysfunction.

Sildenafil is also used for the treatment of pulmonary hypertension, which it relieves by inducing vasodilatation. Pulmonary hypertension is an increase in blood pressure in lung vasculature, including pulmonary arteries, pulmonary veines and pulmonary capillaries.

In general the regulation of PDEs in several cancers and the antitumor effect of PDE inhibition is currently investigated and discussed. The overexpression of PDE5 appears in many carcinomas, including adenocarcinomas. These phosphodiesterases are supposed to play a role in the development of carcinogenesis. Data demonstrate differences in PDE and cyclic nucleotide levels between neoplastic and normal tissues, especially in those with increased cGMP PDE activity (Savai, Pullamsetti et al. 2010 – Targeting cancer with phosphodiesterase inhibitors).

2 Aims of the study

- Expression profile of tyrosine kinase receptor in A549 tumor tissue, human donor monocytes, macrophages, lymphocytes and fibroblasts
- In vivo effects of PDE5 inhibitor sildenafil in combination with multikinase inhibitor sunitinib or sorafenib in sn adenocarcinoma mouse model
- Analysis of morphology, angiogenesis and proliferation in tumor tissue
- Analysis of the role of angiogenesis in tumor tissue growth

3 Material

3.1 Cell culture

A549 cells

A549 cells serve as a model to draw inferences about adenocarcinomic cells as a subgroup of NSCLC. The cell lines were obtained from American Type Culture Collection (ATCC).

A549 medium

A549 cells were grown in DMEM plus 10% FCS, 1% penicillin-streptomycin, 1% vitamines, 1% glutamin, 1% non-essential aminoacid.

1% trypsin/PBS

Freezing medium

RPMI-1640 70%, FCS (filtered) 20%, DMSO 10%

3.2 Immunohistochemistry

PBS 10x buffer

NaCl 80.0 g, KCl 2.0 g, $Na_2HPO_4x2H_2O$ 11.5 g, KH_2PO_4 2.0 g

dissolve in 900 ml aqua dest. and then fill up to 1 L.

For using mix one part 10x buffer with 10 parts aqua dest. for getting 1x buffer concentration.

For staining 10% BSA in PBS plus 0.013% NaN3

Recipes for Goldner - Masson - Staining

Ponceau acid fuchsin solution

Ponceau de Xylidine 0.2 g, Acid fuchsin 0.1 g, Acetic acid 1% 0.6ml

aqua dest. 300 ml

Orange G - Solution

Phosphorwolframacid 8 g, Orange G 4 g, aqua dest. 200 ml

Light green - Solution

Light green SF 0.4 g, acetic acid 1% 0.4 ml, aqua dest. 200 ml

3.3 Reagents

Table 2: Reagents for immunohistochemistry and PCR

Product name	**Company**
immunohistolochemistry	
Acetic acid, Rotipuran 100%	Roth, Germany
Albumin bovine fraction V	Serva, Germany
Citrate buffer pH 6, 20x concentration	Zytomed, Germany
Eosin-y-alcoholic	Thermo Fisher Scientific, UK
Ethanol 70 %, denatured with Methylethylketon	Fischar, Germany
Ethanol 96 %, denatured with Methylethylketon	Fischar, Germany
Ethanol 99.6%, denatured with Methylethylketon	Fischar, Germany
Fe-Hematoxylin A-solution Weigert	Chroma, Germany
Fe-Hematoxylin B-solution Weigert	Chroma, Germany
Fetal calf serum (FCS)	Invitrogen Gibco, Germany
H_2O_2, puriss. p.a. 30%	Merck, Germany
Hämalaun solution, acid	Chroma, Germany
Hematoxylin ready to use	Invitrogen, Germany
Hydrogen Peroxide 30%	Merck, Germany
ImmPRESS kit anti-rabbit Ig, peroxidase	Vector Laboratories, USA
Isopropylalkohol (2-Propanol)	Sigma-Aldrich, Germany
Light green	Sigma-Aldrich, Germany
Methanol, reinst, p.a.	Riedel-de-Häen, Germany
NovaRED substrate kit for peroxidase	Vector Laboratories, USA
Orange G	Chroma, Germany
Pertex	Medite, Germany
Ponceau de Xylidine	Chroma, Germany

Potassium dihydrogen phosphate	Merck, Germany
Potassium chloride	Roth, Germany
Proteinase K	Dako, USA
Sodium chloride	Roth, Germany
Sodium hydrogen phosphate dihydrate	Merck, Germany
Trypsin, Digest All 2	Zytomed, Germany
Wolframphosphoracid Hydrat, p.a.	Merck, Germany
Xylol	Roth, Germany
Zymed Digest-All Trypsin Kit	Zytomed, Germany
PCR	
ImProm-II™ Reverse Transcriptase	Promega, Germany
Magnesium	Promega, Germany
Oligo(dT15) Primer	Promega, Germany
PCR Nucleotide Mix	Promega, Germany
SYBER® Green PCR Kit	Qiagen, Germany

3.4 Antibodies

3.4.1 Immunohistochemistry

Table 3:Antibodies

Product name	**Company**	**Dilution**
Alpha-actin	Sigma	1:900
C-Kit (C-19): sc-168	Santa Cruz Biotechnology	1:1500
EGFR	Abcam	1:500
Flt-1 / VEGFR1 Ab-1	Thermo Scientific	1:1
PCNA	Santa Cruz	1:100
PDE5a	Scottish Biometrics	1:50
PDGFR, alpha	Thermo Scientific	1:50
PDGFR, beta Ab-1	Thermo Scientific	1:100
vWF	Dako	1:600

3.5 Primer

Table 4: PCR primer

Gene (human)	Accession	Primer sequence forward	Primer sequence reverse
colony stimulating factor 1 receptor (CSF-1R)	NM_005211	5'-GTG GCT GTG AAG ATG CTG AA-3'	5'-CCT TCC TTC GCA GAA AGT TG-3'
epidermal growth factor receptor (EGFR)	NM_005228	5'-CTC AGC CAC CCA TAT GTA CC-3'	5'-CGT CCA TGT CTT CTT CAT CC-3'
Fms-related tyrosine kinase 1 (FLT-1)	NM_002019	5'-TCG TGT AAG GAG TGG ACC AT-3'	5'-CGA GTA GCC ACG AGT CAA AT-3'
Fms-related tyrosine kinase 3 (FLT-3)	NM_004119	5'-AGA GCA CGT GTG CTT TTA CC-3'	5'-TAC TTC TGA CTG GCC CTG AG-3'
Fms-related tyrosine kinase 4 (VEGFR3, FLT4)	NM_002020	5'-GGT TAA GGC ATA CGA GAG CA-3'	5'-TCT GTG TTG CCA GCG TAT AA-3'
kinase insert domain receptor (KDR)	NM_002253	5'-GTT CTT GGC TGT GCA AAA GT-3'	5'-GTC TTC AGT TCC CCT CCA TT-3'
phosphodiesterase 2A, cGMP-stimulated (PDE2A)	NM_002599	5'-CCT ATA TCC CTG AGC TGC AA-3'	5'-GGA AGT CCA GCG AGT TGT TA-3'
phosphodiesterase 5A, cGMP-specific (PDE5A)	NM_033437	5'-AAG CAA ATG GTC ACA TTG GA-3'	5'-TCT GGA AGT TCT GCA CAA GG-3'
platelet-derived growth factor receptor, alpha polypeptide (PDGFRalpha)	NM_006206	5'-CCA GCA GTT TCC AGT CCT AA-3'	5'-ACA GAT TGG CAG ACC ACA TT-3'
platelet-derived growth factor receptor, beta polypeptide (PDGFRbeta)	NM_002609	5'-CCC AGC AAG TCT CAA GAA CA-3'	5'-GGT GAT CTC ATT TGC CCT CT-3'

ret proto-oncogene (RET)	NM_020630	5'-TGT CCT CTT CTC CTT CAT CG-3'	5'- AAT TCC CAC TTT GGA TCC TC-3'
V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (KIT) (c-KIT)	NM_000222	5'-CCG TGG TAG ACC ATT CTG TG-3'	5'-GTG CCC ACT ATC CTG GAG TT-3'

3.6 Equipment Table

Table 5: Equipment

Bench Hera safe HS18	Heraeus Instruments, Germany
Centrifuge 3 S-R	Heraeus Instruments, Germany
Combi-Tips	Eppendorf, Germany
Culture dishes	BD Falcon, Germany
Incubator Function line BB16	Heraeus Instruments, Germany
Incubator histology	Memmert, Germany
Microscope	Leica, Germany
Light Microscope in Cell Culture, DM IL	Leica, Germany
Multipette plus	Eppendorf, Germany
Pipette 10 ml, 25 ml, 50 ml	BD Falcon, Germany
Pipette P20, P100, P200, P1000	Eppendorf, Germany
Pipette peaks	Sarstedt, Germany
Pipette peaks PCR	Sorenson, USA
Vortex REAX control	Heidolph, Germany
Waterbath GFL 1083	Gesellschaft für Labortechnik, Germany
Centrifuge in Cellculture	Thermo Fisher Scientific, UK
Cell Scraper, BD Falcon 18 cm	BD Biosciences, USA
Falcon Tubes 50 ml, 15 ml	Greiner Bio-One, Germany
Freezer +4 ˚C	Bosch, Germany
Freezer -20 ˚C	Bosch, Germany
Frezer -80 ˚C	Thermo Fisher Scientific, UK
Freezing Box for Cryotubes	Sarstedt, Germany
PCR Plates, ABgene Thermo-Fast	Thermo Fisher Scientific, UK
T3000 Thermocycler	Biometra, Germany

pH-Meter HISTO	Knick, Germany
qPCR Device, Mx3000P	Stratagene, USA
Tubes 1,0 ml; 1,5 ml; 2 ml	Eppendorf, Germany

4 Methods

4.1 Animals

Immunodeficient balb/c nude male mice aged 7 - 8 weeks were acquired from Charles River, kept under specific pathogen-free conditions in an animal house with free access to food and water.

Animals were handled in accordance with the European Community recommendations for experimentation. RP Darmstadt No: V54-19c20/15-B2/290

4.1.1 Anaesthesia for subcutaneous infiltration

For the subcutaneous infiltration of A549 cells into the right backside of the mice the following composition was used in a ratio of 1:1:2.

Ketavet 100 mg : Rompun 2% : NaCl 0.9%

Per mice 40 - 100 µl of anaesthesia-mix was given.

4.1.2 Tumor growth in nude mice

Tumor genesis was induced by subcutaneous injection of A549 cells (2.5 x 106 cells/200 µl PBS) in 7 - 8 week-old balb/c nude male mice. The size of the tumor was measured using digital calipers (Mitutoyo., UK). The tumor volume (TV) was calculated by the formula: TV (mm^3) = (L x W^2)/2. L means the longest dimension of the tumor (in mm), and W is the shortest dimension of the tumor (in mm). Every day each mouse got the treatment with the specific group therapy, every fourth day of a total 24 days of therapy, the tumor growth was measured. After 24 days of measuring the mice were sacrificed and tumors were prepared.

4.1.3 Treatment groups

There were seven groups: control group (n = 10) no treatment; placebo group (n = 7) treated with methylcellulose; sildenafil group (n = 7) treated with 100 mg/kg bw; sutent group (n = 7) treated with 40 mg/kg bw; sildenafil + sutent group (n = 7) treated with

100 mg/kg bw + 40 mg/kg bw; sorafenib group (n =7) treated with 50 mg/kg bw; sildenafil + sorafenib group (n = 7) treated with 100 mg/kg bw.

4.2 Cell culture

A549 cells were grown in a prepared A549 medium at 37 °C, 5% CO_2 in humid atmosphere.

For further processing the cells were cultivated in 100 mm dishes.

All liquid materials had to be warmed up in the warm water bath to 37 °C excepting the freezing medium. Which had to be 0 °C when used. This temperature was reached by laying the tube with the freezing medium on crushed ice.

Trypsinization and splitting

After sucking off the supernatant 6 ml of trypsin were admitted to the adherent cells.

The dish was put back into incubator for 1-3 minutes to detach the cells.

During that time a 50 ml tube was prepared with 2 ml of FCS and after that the cells were put in by using a pipette. The dish was spoiled with 10 ml of standard medium and the solution was although poured in.

Subsequently the tube was centrifuged at 1600 rpm for 8 minutes.

The supernatant was disposed and the cell clot was resuspended with 6 ml A549 medium and put back to 2-4 new dishes which had been before been prepared with 7ml medium. Splitting mostly 1:3 or 1:4 when the cells were 80% - 90 % confluency before.

Freezing of cells

The cell clot from one dish resulting from centrifugation was mixed with 900 µl of freezing medium. Then resuspended and put into freezing tubes 450 µl of each.

Next the tubes were kept for 24 hours in -80 °C before being froan at -130 °C.

All patients whose tissue was used in this study gave their written informed consent.

4.3 RNA isolation

RNA was isolated from cells and tissue using TRIzolTM reagent. For the isolation of RNA from cultured cells, the culture medium was removed and cells were washed with PBS, than resuspended in TRIzol. TRIzol-cell-mix was collected and transferred to a

1.5 ml tube using cell scraper and pipette. In order to get RNA from tumor or lung tissue 30 mg of tissue were crushed with 700 µl of TRIzol on ice. Samples were incubated at room temperature (r.t.) for 10 min. After that 200 µl of chloroform were added then centrifuged at 12000 rpm, 4 ºC for 15 min. The aqueous phase was transferred to a new tube, mixed with 500 µl of isopropanol (per ml TRIzol) inverted and incubated at r. t. for 10 min. Samples were centrifuged for 10 min at 12000 rpm at 4 °C. The supernatant was discurded. Then the pellet was washed with 1 ml 75% EtOH and centrifuged at 7500 rpm at 4 °C. The RNA pellet was finally redissolved in 10-30 µl of DEPC-treated water for 10 min at 55 ºC. Samples were placed on ice and quantified by NanoDrop device. Samples were stored at -80 ºC immediately after RNA isolation.

4.4 cDNA synthesis

The RNA isolated in this way was then used to synthesize complementary DNA (cDNA) using the ImProm II reverse transcription system. 1 µl of RNA and 1 µl of oligo(dt) primer were filled to a final volume of 5 µl with nuclease-free water in order to equalize the RNA concentration.

Tubes were placed into the thermocycler and denatured at 70 °C for 6 min and then cooled at 4 °C for 5 min. The second step involved DNA synthesis itself, the reverse transcription reaction mix (1 µl of RT, 1 µl of dNTP-Mix [10 mM], 4 µl of reaction buffer, 2 µl of $MgCl_2$ [25 mM], 0.5 µl of ribonuclease inhibitor [40 U/µl] and 6.5 µl of DEPC-treated water) was added. Again tubes were placed into the thermocycler now programmed as follows: 25 °C for 5 min, 42 °C for 60 min, followed by 70 °C for 15 min to inactivate the RT. Directly after synthesis cDNA was frozen immediately and stored at -20 °C.

4.5 Quantitative real time polymerase chain reaction

Quantitative real time polymerase chain reaction (qPCR) is a method based on the principles of PCR allowing not only to amplify specific DNA sequences but also to get information about their quantity by measurement of fluorescence dye attachment to the product during amplification. The amplification is performed by repetition of the three key steps of PCR: denaturation, primer annealing and elongation.

The qPCR was performed in Mx3000P® device from Stratagene, the reaction mix was prepared with Platinum® SYBR® Green qPCR SuperMix-UDG. 25 µl of the reaction mix includes 12,5 µl Platinum® Syber® Green qPCR SuperMix-UDG (end conc. 1x), 1 µl $MgCl_2$ 50 mM (end conc. 5 mM (3 mM issued in SuperMix)), 0.5 µl forward [10 mM] and 0.5 µl backward [10 mM] primer (end conc. 0.2 µM each), ROX Reference Dye [25 µM] (end conc. 0.1 µM), 2 µl cDNA [0.25 µg/µl] (end conc. 0.5 µg) and 8.4 µl DEPC-treated water as components. Then the RT-PCR machine was programmed as follows: activation 95 °C for 10 min, 40 cycles with denaturation at 95 °C, annealing 58 °C, extension 72 °C each 30 s, denaturation 95 °C for 1 min. For the negative control, the cDNA was omitted.

The relative transcript rate of the genes of interest was compared to the same samples rate of the house keeping gene HRPT presented as ΔCt values ($\Delta Ct = Ct_{reference} - Ct_{target}$); Ct standing for cycle threshold, representing the point where fluorescence exceeds background.

4.6 Immunohistochemistry

4.6.1 Staining methods

For both methods (protein kinase K and trypsin) the slides had to be prepared in the same way. They were stored over night at 37 °C and before starting at 59 °C for 60 min. Then they were immersed in series into the following solutions: 3 times in xylene for 10 min, 2 times in absolute 99.6% ethanol for 5 min, 96% ethanol for 5 min, 70% ethanol for 5 min.

For using protein kinase K the slides had to be cooked in citrate buffer pH 6 for 20 min, kept warm in buffer for 10 min, then 30 min at r. t., cleaned with aqua dest. 2 times for 5 min.

Next steps for both methods: 100 ml of methanol plus 100 ml 30% H_2O_2 for 20 min, aqua dest. for 5 min, 2 times in PBS for 5 min.

According to the manufacturer's instruction each slide was threated with either protein kinase K and stored at r.t. or trypsin and stored at 37 °C for 15 min, washed in aqua dest., 2 times in PBS for 5 min followed by 180 µl of 10% BSA and stored 60 min at r.t. Subsequent to this the slides were washed again two times with PBS for 5 min and ongoing 2.5% normal horse serum were put drop by drop on the slides for 20 min. After

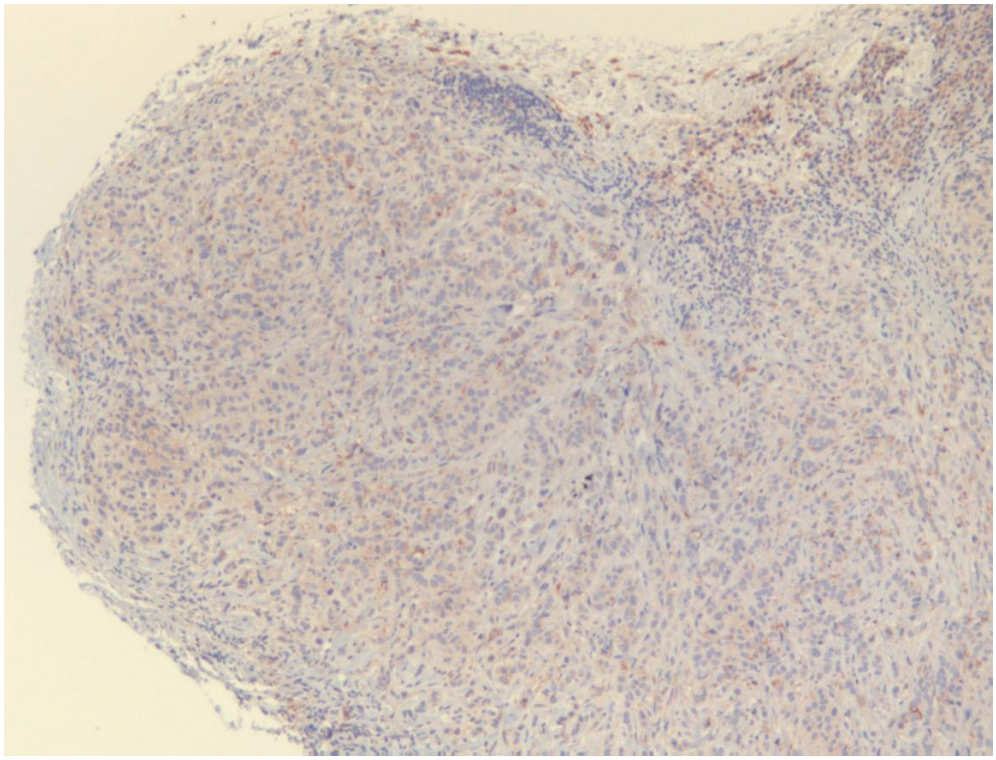
removing the supernatant, slides were incubated with primary antibody over night at 4 °C.

The next day, slides were spoiled for 2 h in PBS, buffer change every 15 min. Then secondary-antibody was put on each slide, after which slides were stored for 30 min at r.t., then PBS 4 times for 5 min. Subsequently the NovaRED – substrate (5 ml aqua dest + 3 drops Reag. 1, mix, plus 2 drops Reag.2, mix, plus 2 drops Reag.3, mix, plus 2 drops Hydrogen Peroxide, mix) was put on the slides while developing under the microscope for sec. to 15 min according to the antibody. Following the final alcohol line: 96% ethanol for twice 2 min, isopropanol for twice 5 min, xylene for three times 5 min. Last step covering with pertex and glass.

For HE-staining the slides were dehydrated, put into aqua dest. for 2 min, followed by 20 min of incubation in haematoxylin solution (mayer), 5 min tap water, 1 min 96% Ethanol, 4 min eosin-y-alcoholic and washing in aqua dest. Followed by the final alcohol line as decribed before.

For trichrome staining the first steps were the same. After 5 min in 70% ethanol the slides were put directly in Fe hematoxylin Weigert A und B (1:1) for 10 min followed by 5min blueing under tap water and put into ponceau-acid fuchsin for 10 min. Then 4-5 times dip in 1% acetic acid till no more streaks of colour were visible and stayed in orange g-solution for 5 min before washing with 1 % acetic acid again. Finally the slides had to stay in lightgreen for 20 min until the alcoholic line started.

4.7 Quantification



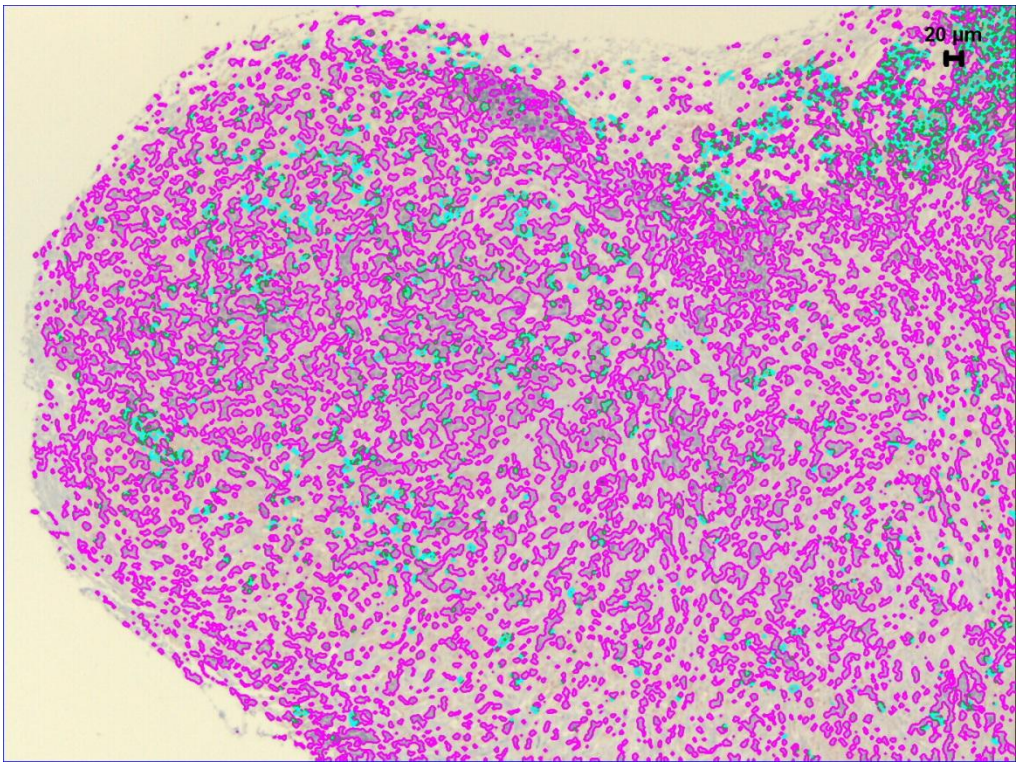


Figure 2: Quantification by Leica Qwin, Example picture

Figure 2 shows an example for quantification. The upper image shows a histological slide of a tumor after staining procedure. Cells were marked blue, in this case specific PDE5 receptors were marked red. Due to the staining procedures tumor stroma also got colored red. To distinguish between the PDE5 receptors and the tumor stroma Leica Microsystems have developed a quantification program called Leica QWin.This program just marked the specific red color before selected on the whole slide. The same procedure followed for the blue stained areas. At the end the tumor slide was scanned, and the percentage of color was put in relation to the tumor size and to the two colors as illustrated in the screenshot below.

Dualstaining Analyse

Leica QWin

Eingabe gemessene Gesamtfläche Schnitt [mm²] :	10,190	Gemessene Gesamtfläche [mm²] :	2,000
		Bildanzahl :	1

	Färbung 1				Färbung 2			
Hochgerechnet pro Färbung [mm²] :		4,759	74912			16,010	175207	
Flächensumme :	-	0,93401	14703,00000		-	3,14221	34388,00000	
Mittelwert :	5,85	0,07185	1131,00000	920,16707	19,67	0,24171	2645,23077	2152,12575
Einzelergebnisse:								
Bild	Fläche [%]	Fläche [mm²]	Anzahl	Anzahl / mm²	Fläche [%]	Fläche [mm²]	Anzahl	Anzahl / mm²
1	2,982	0,03665700	1014	824,97735600	29,475	0,36228700	2946	2396,827637
2	0,862	0,01059500	409	332,75714100	8,971	0,11025900	927	754,195313
3	1,188	0,01460700	547	445,03216600	11,609	0,14268500	1314	1069,053467
4	6,671	0,08199300	1291	1050,34106400	16,981	0,20871900	3590	2920,777832
5	8,730	0,10730600	1802	1466,08398400	18,519	0,22762600	3910	3181,125732
6	4,218	0,05184200	579	471,06695600	10,409	0,12793600	2288	1861,487427
7	5,607	0,06891600	1546	1257,80578600	17,468	0,21470500	3907	3178,685059
8	8,163	0,10033900	1726	1404,25146500	52,713	0,64791400	1688	1373,335083
9	10,260	0,12610300	1765	1435,98132300	29,327	0,36046200	3762	3060,714844
10	15,363	0,18882700	970	789,17956500	16,733	0,20566700	2998	2439,134277
11	4,059	0,04989200	892	725,71972700	11,692	0,14371300	1917	1559,646606
12	5,445	0,06693100	1130	919,35345500	15,200	0,18682300	2994	2435,879883
13	2,441	0,03000000	1032	839,62194800	16,549	0,20340900	2147	1746,771606

Figure 3: Dualstanding Analyse, Screenshot example

4.8 Statistics

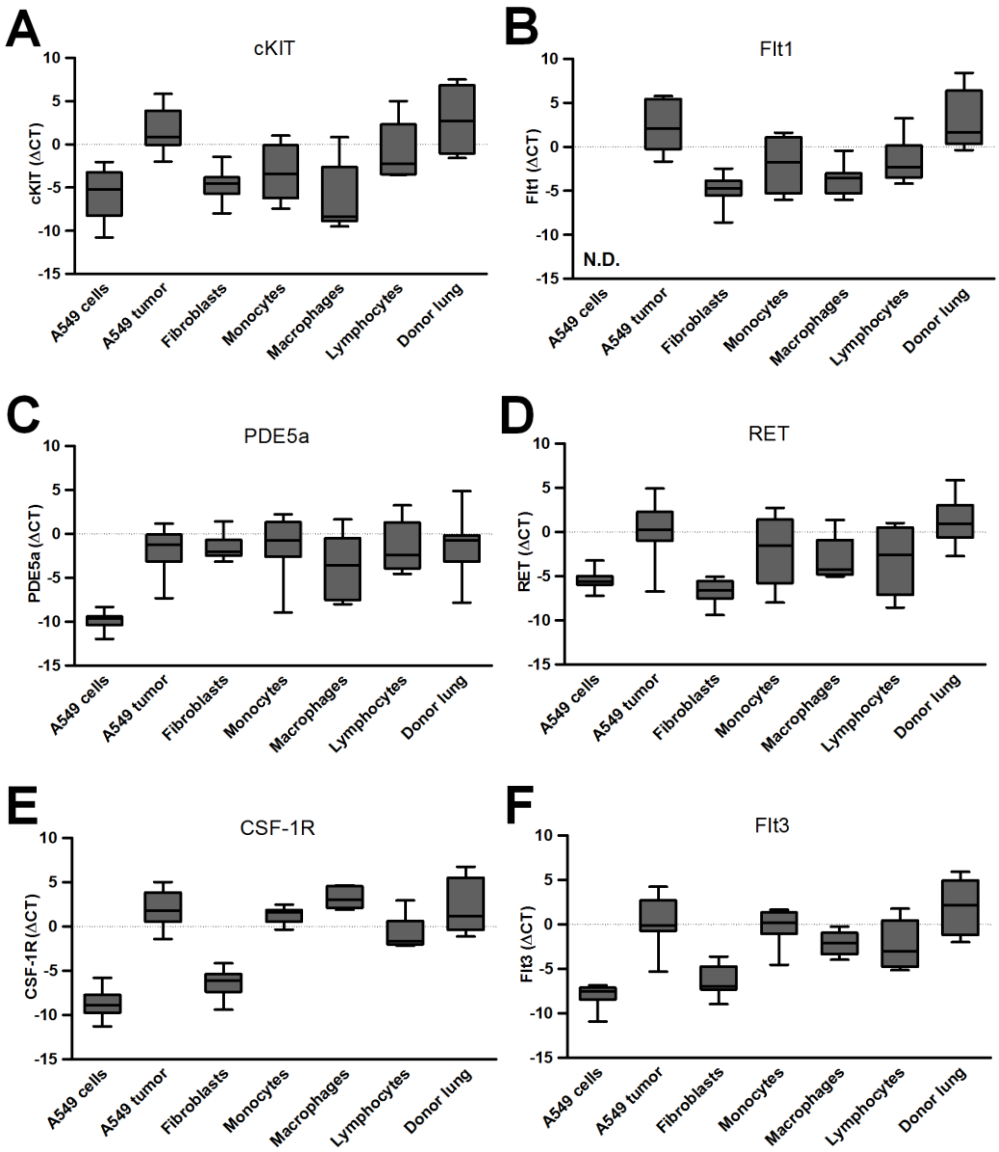
All data are given as the mean +/- SEM. Graphical and statistical analyses were performed using commercial computer software (GraphPad Prism 5, San Diego, CA, USA). Statistical significance of differences between two groups was established as $p < 0,05$ using the unpaired t test with Welchs correction.

5 Results

5.1 Profiling of receptor expression

By RT-PCR the expression profile of the following TKR were verified in control A549 cells, in A549 tumor tissue, in human fibroblasts, monocytes, macrophages, lymphocytes and in human donor lung tissue (figure 4 A-L).

The mRNA expression levels were normalized to housekeeping gene (HRPT). CKIT, PDE5a, RET, CSF-1R, Flt3, PDGFR-alpha and PDGFR-beta, EGFR and VEGFR3 were expressed in all kinds of used tissues. Flt1 and KDR were expressed in A549 tumor tissue, in human fibroblasts, monocytes, macrophages, lymphocytes and human donor lung tissue, but not in A549 cells. The results show that in figure 4 all genes were differently expressed. We do not have an exact control cell to make a fold change.



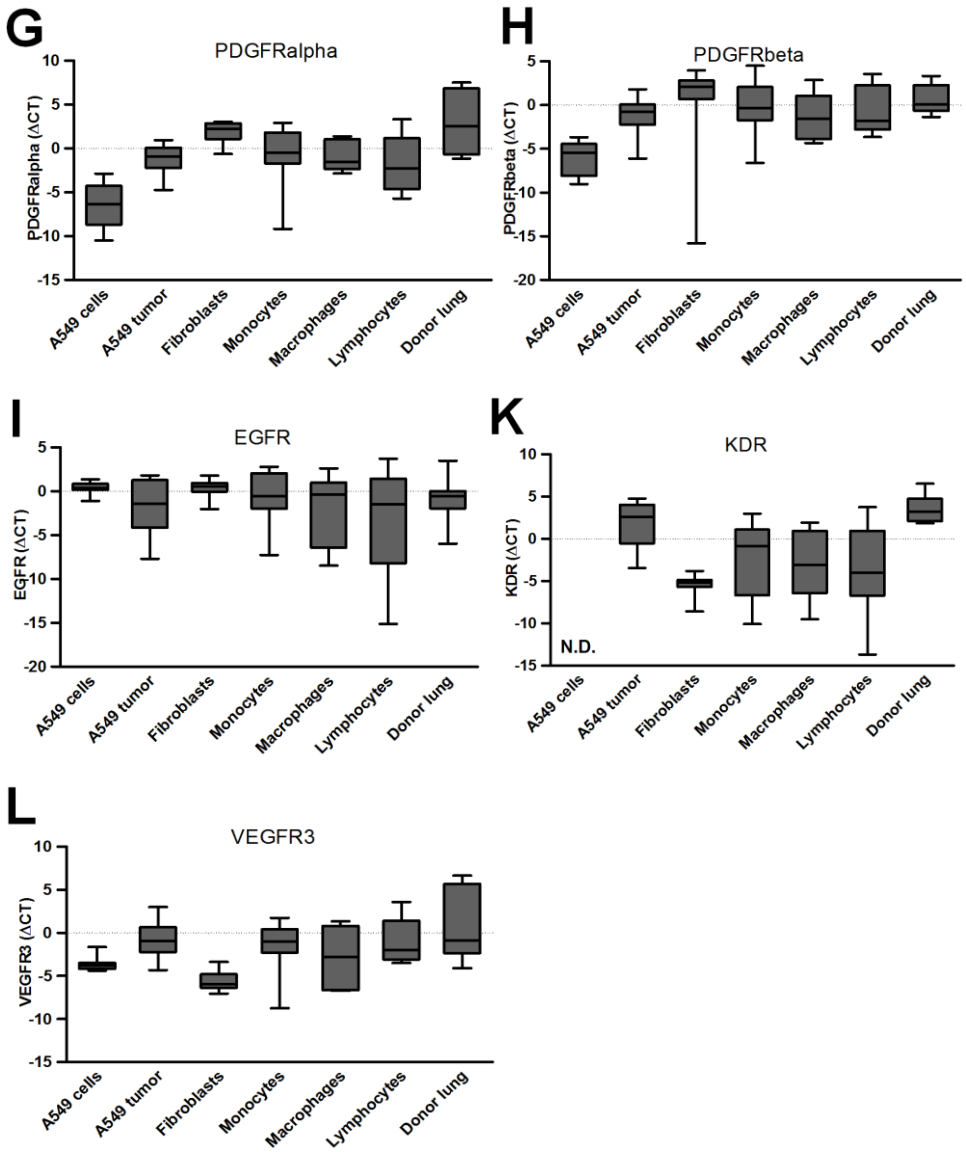


Figure 4: mRNA expression of tyrosine kinase receptors in A549 tumor cells, A549 tumor tissue, in human fibroblasts, monocytes, macrophages, lymphocytes and in human lung donor tissue. Expression of the cytokine receptor cKIT(A), the vascular endothelial growth factor Flt-1 (B), the cGMP-specific phosphodiesterase type-5A (C), the proto-oncogene RET (D) the colony stimulating factor 1 receptor CSF-1R (E), the receptor tyrosine kinase Flt3 (F), the platelet-derived growth factor receptor alpha (G) and beta (H), the epidermal growth factor receptor EGFR (I), the vascular endothelial growth factor KDR (K) as well as the vascular endothelial growth factor receptor 3 VEGFR3 (L) was analysed by real-time quantitative PCR using the ΔCt method, that is by comparing the Ct values of the samples with the control gene HRPT.

5.2 Tumor growth

No significant differences were observed comparing control and placebo groups to treated groups at day 4 and 8 after A549 cell injection. In the third measurement (day 12), both the control and the placebo group showed on average, an increased volume of tumor – twice the volume compared to the sunitinib + sildenafil group – while the other groups hardly showed any changes from the previous measurements. The fourth measurement (day 16) also demonstrated a significantly larger volume in the control and placebo groups than the other groups. There were also clear differences between the mono-therapy sildenafil compared to the mono-therapy groups sorafenib and sunitinib as well as the combined therapy groups sunitinib + sildenafil and sorafenib + sildenafil. The last four groups showed on average half the tumor volume of the first two mono-therapy group sildenafil; the control and placebo group showed, on average, triple the tumor volume.

Taking everything into consideration, the combined therapy of sunitinib + sildenafil showed, for the duration of the experiment and in comparison to the first measurement on day 4, no increase in volume of the tumor; that is, no increase in tumour growth.

On the days when the measurements were carried out, the groups sorafenib + sildenafil, sunitinib and sorafenib showed similar volumetric ratios with no significant differences.

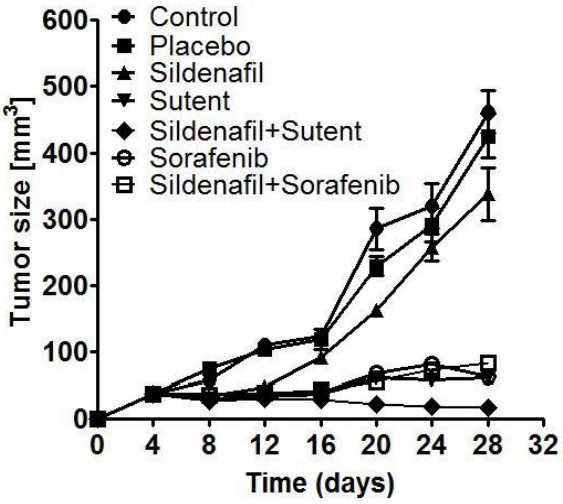


Figure 5: Subcutaneous tumor size over the course of time A549 cells were injected subcutaneously into balb/c nude male mice. There were seven groups: Control group (without treatment), placebo group (with methylcellulose), sildenafil (100 mg/kg bw), sutent (40 mg/kg bw) and sorafenib (50 mg/kg bw), sutent + sildenafil (combination of 40 mg/kg bw sutent and 100 mg/kg bw sildenafil, daily) and sorafenib + sildenafil (combination of 50 mg/kg bw sorafenib and 100 mg/kg bw sildenafil, daily) Tumor size was measured over 28 days by digital calipers in a time-dependent manner (every fourth day)

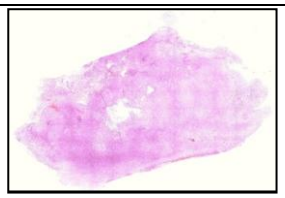
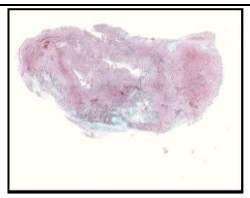
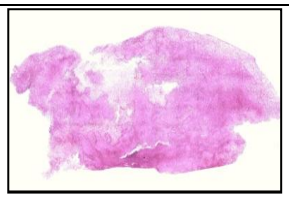
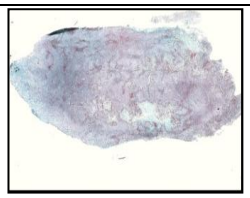
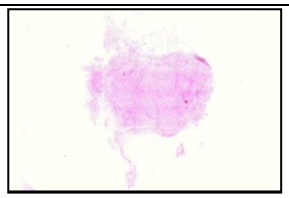
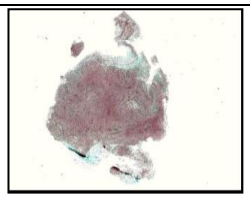
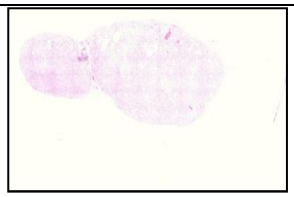
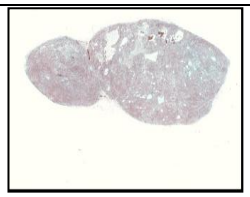
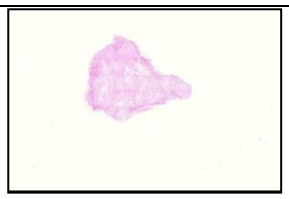
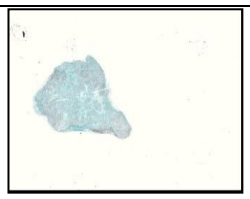
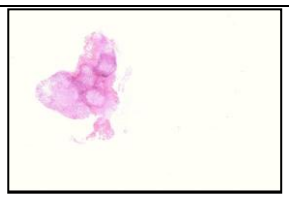
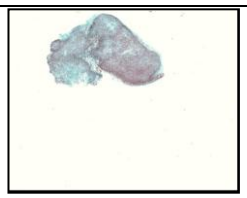
	A) H&E staining	B) Trichrome
Control		
Placebo		
Sunitinib		
Sildenafil		
Sorafenib		
Sunitinib+Sildenafil		
Sorafenib+Sildenafil		

Figure 6: HE (1) and trichrome (2) stainings of original scanned slides of A549 tumor tissue

Figure 6 shows the original proportions of the tumor volumes at day 28 after the injection of A549 tumor cells in relation to figure 5. Haematoxylin-eosin staining (HE) uses haematoxylin solution for staining of the nucleus (nucleus stains blue/ dark violet) and eosin solution for cytoplasmic staining (cytoplasm, collagen stain red). Masson trichrome staining is a classical staining method for fibrose tissue with the result that nuclei stain black, cytoplasm, muscle, erythrocytes stain red, and collagen stains blue. The intensity is not matter to compare the different sizes. Control and placebo group shows the biggest increase almost equal. The sildenafil group is bigger than the other monotherapie groups and combination therapie groups but taller than control and placebo. Sunitinib, sorafenib and sutent are almost equal in size, too. These three groups totally are smaller than placebo, control and sildenafil groups but still bigger than sildenafil + sutent group. In the trichrome staining it appears that both combination groups and sorafenib monogroup have a higher content of collagen compared to the other groups.

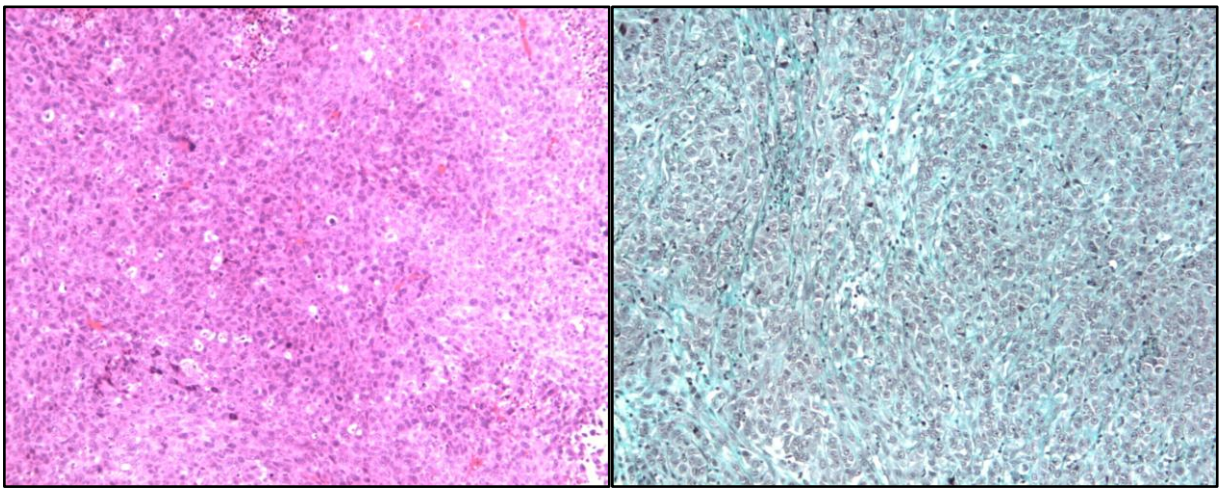


Figure 7: This picture shows an enlarged example of HE (left) and trichrome (right) staining

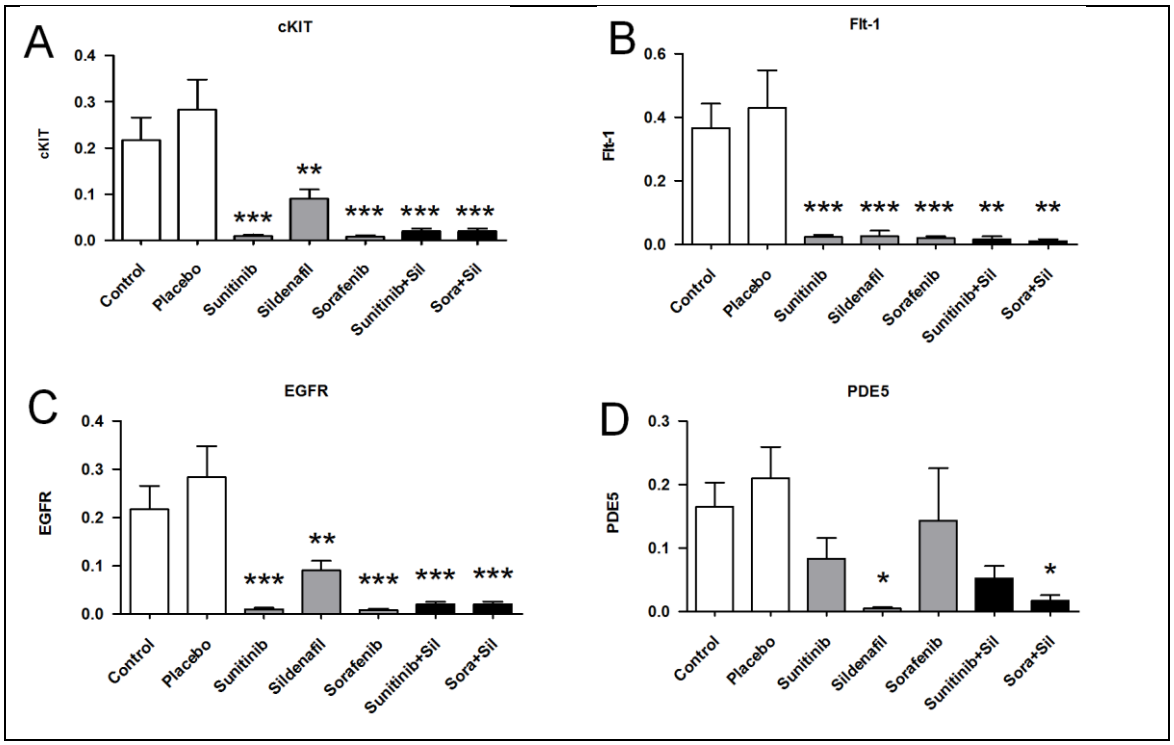
5.3 Tyrosine kinase receptor expression

Figure 8 shows the statistical analysis of the immunohistochemical quantification of the examined tyrosine kinase receptors (A - F).

In general, there was a high density of all tyrosine kinase receptors in both the placebo and the control group. Flt-1 shows significantly reduced values for the mono-therapy groups (sildenafil, sunitinib, sorafenib) and the combined therapy groups (sildenafil + sunitinib and sorafenib + sildenafil). For EGFR there was scarely any expression under

therapies with sutent and sorafenib, sorafenib + sildenafil and sutent + sildenafil appeared. As a mono-therapy, sildenafil showed a significantly higher density of receptors for EGFR, but compared to the two control groups (control and placebo), this was significantly lower. The expression of PDE5 subsided for the therapy with sildenafil and sorafenib + sildenafil; surprisingly, there was no significant reduction with the combination sildenafil + sunitinib. The two other mono-therapy groups also showed minimal influence on the PDE5 receptor expression.

The combined groups and sildenafil showed a stronger expression of PDGFR-alpha than the groups sunitinib and sildenafil. There was almost no expression for PDGFR-beta for the combined therapies and for the sorafenib therapy on its own. Nevertheless, sildenafil and sunitinib showed significantly fewer expressions compared to the control and placebo groups. There was no overall significant reduction in the tyrosine kinase receptor cKIT; however, the expression did decrease under the inhibition therapy. The smallest manifestation became apparent under the combined therapy of sunitinib + sildenafil. Overall the statistical evaluation confirms the optical impressions of the stainings seen in fig. 12



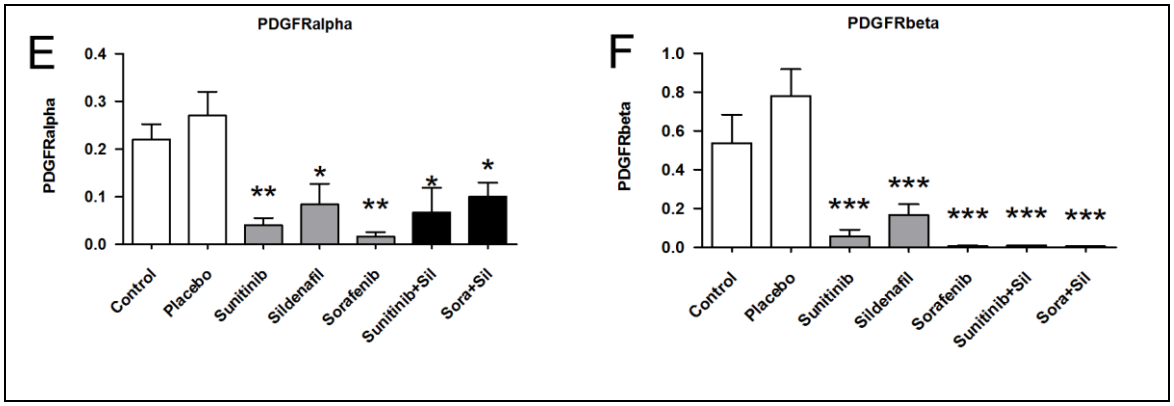


Figure 8: Immunohistochemical quantification of expression of tyrosine kinases receptors in A549 tumor tissue in control group and after 28 days of oral inhibitors administration with methylcellulose (placebo), sunitinib, sildenafil, sorafenib, sunitinib + sildenafil, sorafenib + sildenafil. *P<0.5, **P<0.001, ***P<0.001 vs placebo group. n = 7-10.

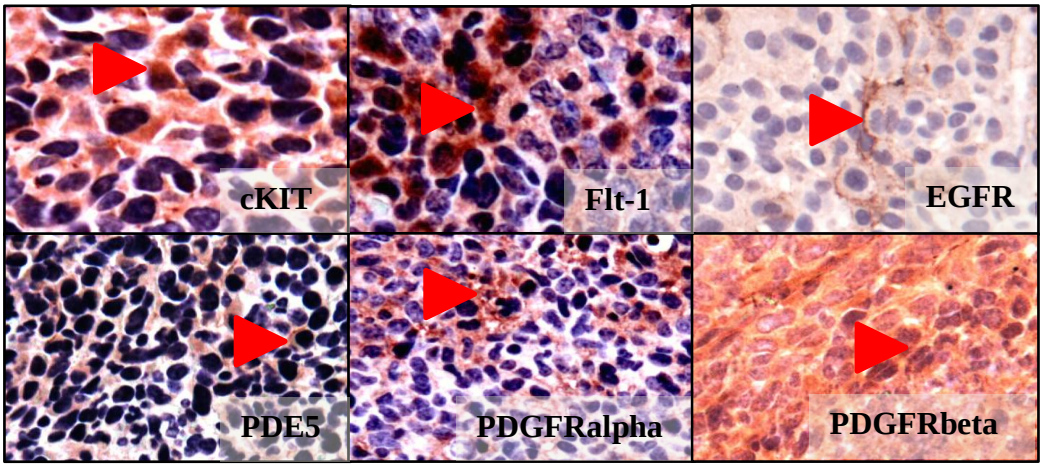


Figure 9: This picture shows a representative magnification of fig. 12. The arrows demonstrate the specific antibody staining.

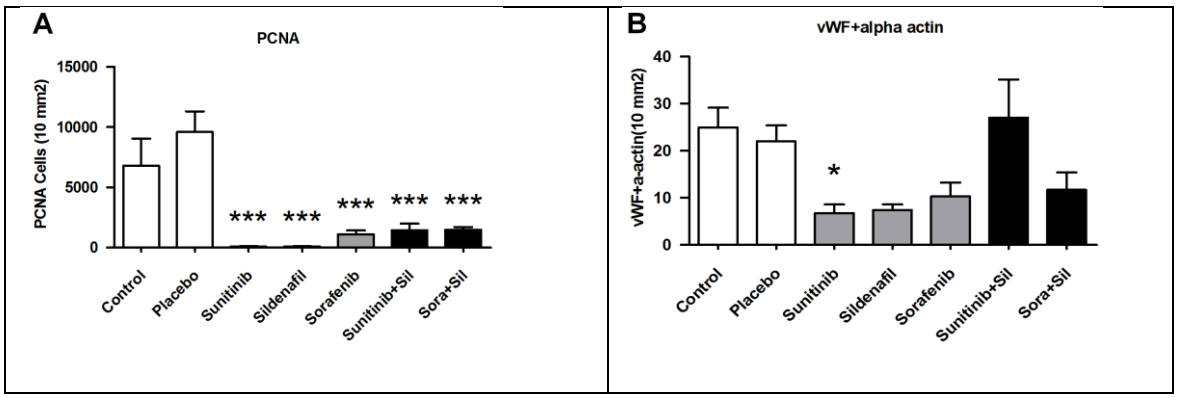


Figure 10: Immunohistochemical quantification of active cell divisions and expression of vWF + alpha actin receptor. 10 A shows a drastic decrease of active cell divisions in all therapeutic groups, compared to controls. In figure 10 B the amount of blood vessels is shown. The mono-therapy groups showed a decline of vascularization, the combination therapies rather showed an increase of vascularization. Of particular interest is the observation that for the therapy with sorafenib + sildenafil, there was just as high a density of blood vessels as was in the control groups in A549 tumor tissue in control group and after 28 days of oral inhibitors administration with methylcellulose (placebo), sunitinib, sildenafil, sorafenib, sunitinib + sildenafil, sorafenib + sildenafil. *P<0.5, **P<0.001, ***P<0.001 vs placebo group. PCNA (A), vWF+alpha actin (B). n = 3

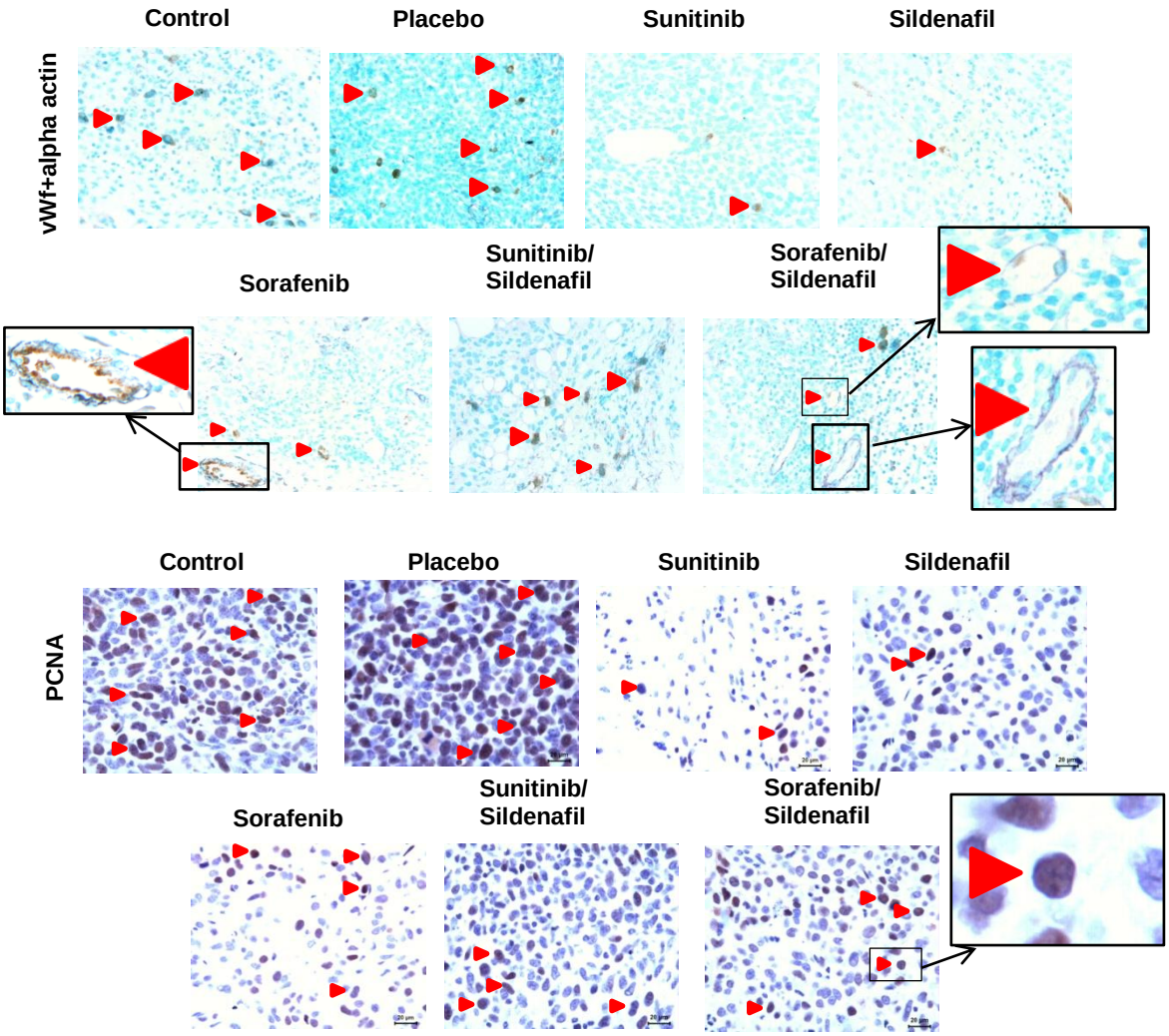


Figure 11: Representative pictures of immunohistochemical staining used for figure 10. vWF+alpha actin staining. The arrows exemplary denote the stained vessels. PCNA staining. The arrows exemplary denote the active cell devision. Vessel and active cell devision were counted under mikroskope.

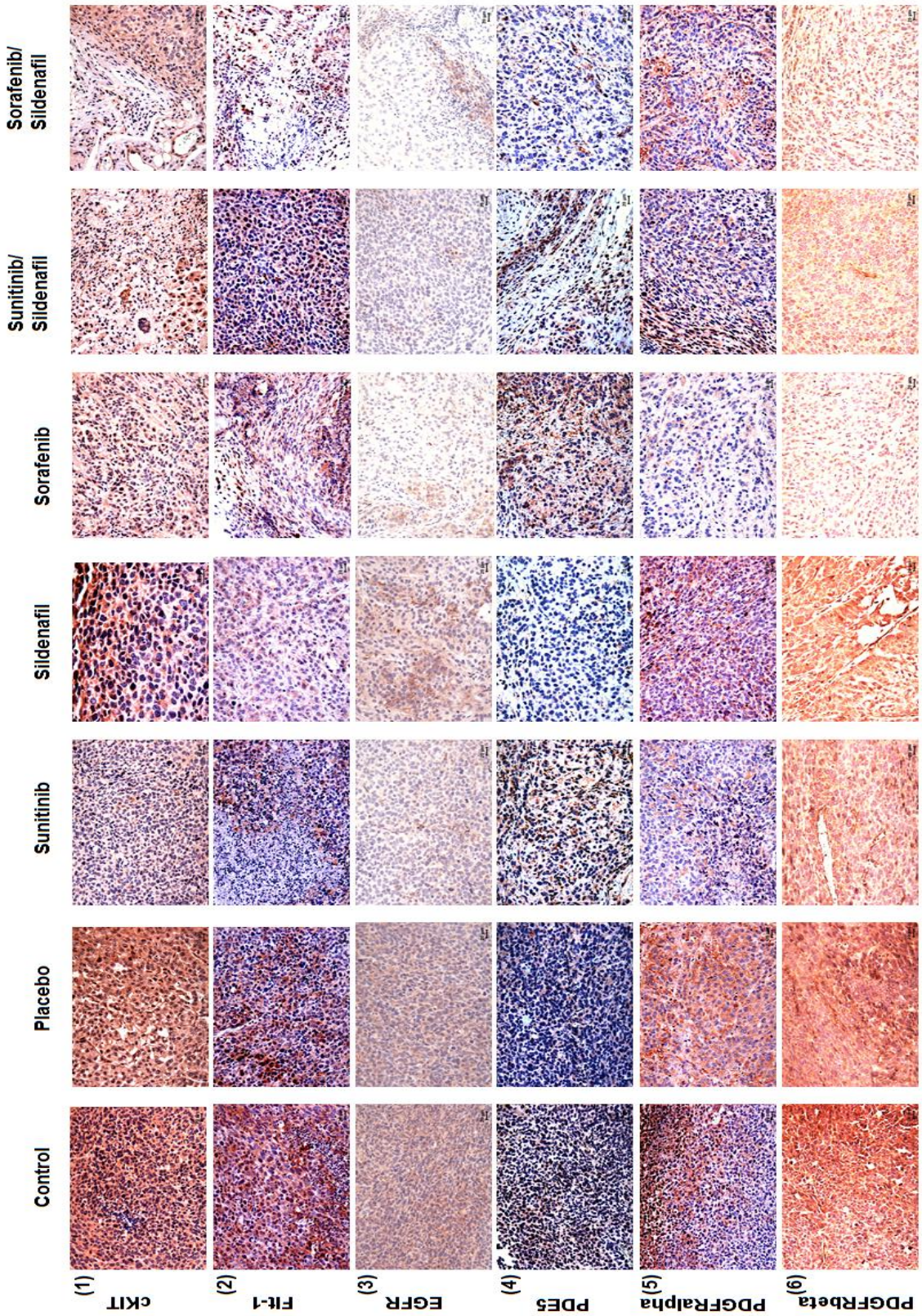


Figure 12: Representative pictures of immunohistochemical staining that were used for the quantitative analysis (figure 7). (Generic magnification see figure 2.) Expression of tyrosine kinase receptors in A549 tumor tissue in control group and after 28 days of oral gavage with methylcellulose (placebo), sunitinib, sildenafil, sorafenib, sunitinib + sildenafil, sorafenib + sildenafil, cKIT (1), Flt1 (2), EGFR (3), PDE5 (4), PDGFR-alpha (5) and PDGFR-beta (6).

6 Discussion

In this thesis, research was carried out on the influence of combined therapies, compared with treatments using mono-therapies, on the growth of tumors in immune-suppressed mice that have been injected with adenocarcinoma cells. The results show a reduction in tumor growth and an alleged increase in apoptosis, as a decrease of the active rate of cell division.

In addition to the study of the target parameters (tumor growth, active cell division, and blood vessels), the following receptors have been studied: cKIT, Flt-1, EGFR, PDE5, PDGFR-alpha, and PDGFR-beta. RET, CSF-1R, Flt3, KDR, and VEGFR3 were also studied at the mRNA level.

6.1 Expression profile of tyrosine kinase receptor in tumor, monocytes, macrophages, lymphocytes and fibroblasts

As mentioned above, Flt-1 belongs to the VEGF family. It has been documented numerous times in science that Flt-1 is highly expressed in tumor tissue (PMID: 21592963; Review Expert Onion). Also on the mRNA level an expression of Flt-3 and VEGFR3 appears, two additional representatives of the VEGF family. In the results we have obtained, it is apparent in all groups that those that have been treated with an inhibitor, or a combination of inhibitors, have had a minimal expression of Flt-1. It is known from other studies that PDE5 also has a deterministic influence on VEGF, and consequently on angiogenesis. A therapy with sildenafil has shown an increase in angiogenic activity. (PMID: 17585066 Sildenafil promotes ischemia-induced angiogenesis through a PKG-dependent pathway).

It has been proven that the VEGF pathway plays a crucial role in the treatment of other types of tumor. Of particular importance is the treatment of renal cell carcinoma, NSCLC, mamma and colorectal cancer where the usage of medication, such as sunitinib or sevacicumab, results as neutralized antibodies for VEGF (Hurwitz 2004, PMID: 12636887 Miller 2003, PMID: 20085937 Sandler, PMID: 21318618, Development of second-generation VEGFR tyrosine kinase inhibitors: current status).

Another conclusion to be drawn from the present study is that the VEGF family, represented by Flt-1, plays a highly influential role in tumor growth. Surprisingly, only a scant amount of Flt-1 has been detected in both combined therapies as well as in the

mono-therapy with sorafenib. Sunitinib and sildenafil as mono-therapies reduce the density of receptors significantly; in combination, they are even more effective.

As already described, sildenafil has an influence on different growth factors which affect angiogenesis as well as the inhibitors of the above-mentioned proliferation processes, such as the VEGF pathway.

A further point of interest is the role of PDGFR-beta. As mentioned above, the growth factor receptor PDGFR-beta is a tyrosine kinase receptor. The downstream receptor signalling pathways influence angiogenesis, proliferation and the survival of the cell.

It is known from other studies that PDGF-beta and its receptor is highly expressed in various types of tumors, and that it plays a role in their development, for example chronic myelomonocytic leukaemia (CMML) and Ewing's sarcoma (PMID: 8962143 PDGFR beta Carroll 1996, PMID: 12700668 Uren PDGFR-beta Ewing). In the present study PDGFR-beta seems to play a crucial role concerning the size of the tumor. For the three groups that have kept the original size most constantly (sorafenib in the mono-therapy and the two combination groups), we found barely any expressions of PDGF-beta receptors. On a mRNA level it appears that PDGFR-beta is more prominent in cell forms of the tumor stroma, but also on isolated A549 cells, which would explain the strong occurrence even in tumor tissue.

The related PDGF-alpha receptors appear to play a less important role in tumorigenesis. Their expression is reduced through the inhibitors applied. Surprisingly, the combination groups show a similar presence as sildenafil in mono-therapy. Sorafenib has the strongest influence. For the receptor cKIT, the combination sunitinib + sildenafil resulted in the strongest decrease in receptor density; however, the rest of the inhibitor groups have also caused a decrease in receptor density. A study analysing the factors PDGFR-alpha, PDGFR-beta and cKIT focusing on chordoma showed that although PDGFR-beta was highly expressed, PDGFR-alpha and cKIT were less expressed, with the conclusion that through a with imatinib (also a tyrosine kinase inhibitor), all three receptors together achieved the desired effect (PMID: 17145809 Tamborin PDGFR-beta, alpha und cKIT). One can assume in this study that it is not just one factor that has an influence on tumor growth, but several that work together. The influence also varies from one inhibitor to another; different combinations of inhibitors produce different results.

EGFR is another important factor concerning tumor growth. This study shows that exclusive donation of sildenafil results in significantly distinct density of receptors for

EGF (when compared with the two control groups), but the remaining inhibitors show an even lower density of receptors. It is known that tumor cells produces EGF-family ligands to stimulate the proliferation of tumor cells and their expressions of EGFR by autocrine and paracrine stimulation (PMID: 16489018 Tumour endothelial cells express...).

All in all, for the sunitinib + sildenafil groups, Flt-1, PDGFR-beta, EGFR, and cKIT all showed little to no expression in the density of their receptors in tumor tissue of sunitinib + sildenafil groups acquired on the last day of measurement. Sorafenib, as a monotherapy, obtained good results in reducing the density of the receptors; however, there was also a significantly low density of PDGFR-alpha. This hypothesizes that PDGFR-alpha plays a less important role in the increase of the volume of the tumor.

6.2 In vivo effects of the PDE5 inhibitor sildenafil together with multikinase inhibitor sunitinib or sorafenib in the adenocarcinoma mouse model

The combination sorafenib + sildenafil as well as the combination sutent + sildenafil showed almost minimal growth increase of the injected tumor cells during the course of the study. It should be noted that sorafenib, when used in monotherapy until day 16 after the start of the experiment, shows almost the same average values as when used in combination with sildenafil. Likewise when compared with the other combination groups.

In contrast, sildenafil, when used in mono-therapy, shows an increase on the same level as those in the combined therapy groups and sorafenib, up to day 12. After day 12, there is an increase in the volume of the tumor, similar to the monotherapy with sunitinib.

Sildenafil is generally known as a specific inhibitor that affects an increase in the intra-cellular cGMP concentrations, and thereby induces vasodilation (Lugnier et al., 1986, Schoeffter et al., 1987). In Germany it is approved for the treatment of pulmonary hypertension due to its efficacy since 2006 (Journal med., 09.11.2005).

When combined with the multi-kinase inhibitors studied, it is obvious that the active agent only has an effect on the smooth muscle cells in the blood vessels of the tumor tissue and that the influence on the other growth factors and receptors is attributable to the combination compound. On the mRNA level, there is only a scant amount of PDE5a

in isolated A549 cells; however, it is evenly distributed in the remaining cells of the tumor stroma, human donor lung, and tumor tissue. It is interesting to note, that the immunohistochemical quantification shows that sildenafil, in addition to its well-known influence on PDE5 also shows a significant influence on Flt-1, PDGFR-beta and PCNA. Furthermore, sildenafil also shows an effect on EGFR and PDGFR-alpha, though this is noticeably weaker than those effects caused by the rest of the therapeutics. In other studies, PDE5 was described as a determinant in the proliferation of endothelial cells that created safe and stable conditions for angiogenesis by down regulation in the microvascular endothelium. (PMID: 19028977, Type 5 PDE expression is a critical determinant of the endothelial cell angiogenic phenotype).

In the immunohistochemical quantification of the vWF + alpha actin staining (which make the blood vessels visible), it becomes apparent that sildenafil and sunitinib show the least amount of blood vessels in mono-therapies; the most are shown when sunitinib and sildenafil are combined.

The smallest number of active cell divisions, however, occurs with sunitinib as well as with sildenafil in mono-therapy. Sorafenib shows a slightly higher rate of cell division, and both combination groups show the highest rate. This might lead to the hypothesis that tumor growth consists of a combination of active rates of cell divisions and vascularisation.

It is known from several studies that tumor growth proceeds slowly and moderately before vascularisation begins and continues rapidly and almost exponentially after vascularisation (PMID: 1688381 What is the evidence that tumours are angiogenesis dependent?).

The best outcome, defined as the smallest tumor growth, could be demonstrated for the combination sunitinib + sildenafil, even if, interestingly, the counted sections of vessels are the highest, almost as high as in the two untreated control groups.

The smaller the tumor, the better the vascularisation. This hypothesizes that there could be a coherence between the effect of the specific therapy at the designated destination and the predominant vascularisation. It is debatable whether the antiproliferative effects of the combination sunitinib + sildenafil is stronger than the proangiogenetic effect of sildenafil on its own.

Based on the measured growth of the tumor, it is apparent that combined therapy is the most efficacious. The question that arises, as considered previously in connection with

vascularisation, is what specific influence the various inhibitors and their combinations have on the studied receptors.

6.3 Analysis of morphology, angiogenesis and proliferation in tumor tissue

On the mRNA level the tyrosin kinase receptors Flt-1, EGFR, PDGF-alpha, PDGFR-beta, RET, CSF-1R, Flt3, cKIT, KDR, and VEGFR3 as well as the specific receptor PDE5a were examined concerning their appearance in different tumor tissues and cell kinds. The following were examined: A549 cells bred in an isolated culture medium; tumor tissues samples which had been injected with A549 cells in nude mice with no inhibitor treatment; human fibroblasts; monocytes; macrophages; lymphocytes; and human-donor lung tissue.

Further the study focused on the tyrosine kinase receptors Flt-1 and KDR. Interestingly all the examined factors were present in the tissues tested, with the exception of Flt-1 and KDR.

There have been no expressions of Flt-1 in cultivated A549 cells. For the rest of the tissues and cell types, it is possible to detect Flt-1 with the help of qPCR. This suggests that whilst the A549 cells themselves may not have receptors for Flt-1, it is quite likely that the tumor stroma do. It is understood that ‘tumor stroma’ refers to the tissue around the individual tumour cells. It has been proven that a tumor needs its stroma to interact (PMID: 17554513 The participation of mesenchymal stem cells in tumour stroma formation and their application as targeted-gene delivery vehicles). In this stroma, the following are located: monocytes, macrophages, mast cells, tumor-associated fibroblasts, and other immune cells (Review Expert Onion, Raj). It was not possible to continue with the immunohistochemical detection of KDR, as the markers used by us were not able to leave a visible staining of the tissue samples.

Although it was not possible to investigate additional factors, it should be possible, in further studies, to examine these factors (cKIT, RET, CSF-1R, Flt3 and VEGFR3), their occurrence, and their influence on the growth of the tumor and vascularisation in NSCLC.

6.4 Analysis of the role of angiogenesis in tumor tissue growth

Past research has shown that there is a connection between the size of the tumor and the vascularisation. Up to a certain point, the tumor is well supplied with blood by the surrounding tissue. Once the tumor has become too big, its own blood supply becomes necessary. (PMID: 8756718 Patterns and emerging mechanisms of the angiogenic switch during tumourigenesis). A good blood flow favors the growth of the tumor without ischemia. In the present study necrotic parts were found in the sections of the tumor of the control group as well as in the placebo group. The tumor appears to grow quickly, and from day 16 onwards, the growth appears to be almost exponential; however, from a certain tumor volume onwards, it appears that the development of the blood vessels is not able to keep up with the growth, and parts of the tumor decay. Thus, a small tumor does not need its own blood supply. Nevertheless, it is apparent in this study that the group that has, on average, the smallest and most constant tumor volume is also the one that has the highest number of sections of blood vessels.

This begs the question whether the receptors, which were the least adjusted in this group compared to the control groups, had the most influence on angiogenesis, in particular PDGFR-alpha. This could be investigated in detail in future studies.

It also seems dubious whether sildenafil, with its pro-angiogenic effects, is superior to sunitinib, with its anti-angiogenic effects. When used in monotherapy, both show a similar high number of sections with blood vessels.

It is a fact that a combined therapy of sunitinib + sildenafil results in a clearly reduced tumor volume. A smaller non-growing tumor in the body would increase the length of survival for a patient suffering from NSCLC. Both medications are, as already stated above, in use as therapeutic agents for humans. Further studies could probably determine to what extent this combination can be used as a therapy for NSCLC. It is still not clear why this combination is the most successful, even though, as had been expected, the density of amount of number of sections with blood vessels has decreased.

7 Summary

Lung cancer is still the leading cause of death amongst the various types of cancers.

This study researched the influence of a combined therapy, consisting of a multi-kinase inhibitor and a specific phosphodiesterase 5 inhibitor, compared with that of a single inhibitor used in mono-therapies and compared with that in a placebo.

On the day the experiment commenced, all the animals received a subcutaneous injection of isolated A549 cells just above the muscle of the right buttock.

Each of the seven animal test groups consisted of 6-10 laboratory animals (nude mice). For the duration of the experiment of 28 days, the animals were treated with different inhibitors in the form of oral medication: sildenafil, a specific PDE5 inhibitor, sorafenib and sunitinib as multi-kinase inhibitors, the combined therapy groups sorafinib + sildenafil and sunitinib + sildenafil, the placebo groups (these laboratory animals received an oral dose of methylcellulose), and finally the control group – there was no intervention with these animals.

The animals received the same dosage of medication. The size of the tumor was measured every four days.

The largest increase in volume occurred, as expected, in the control and the placebo groups. The smallest increase in volume occurred in the combined-therapy groups. The most constant was, however, for the combination of sunitinib + sildenafil.

The mRNA expression of individual growth factor receptors from tissues of the control group was examined with the help of real-time PCR. In order to capture the influence of tumor stroma, the following were also examined: human fibroblasts, monocytes, macrophages, lymphocytes, donor lung tissue, and, for comparison, isolated and cultivated A549 cells. The receptors cKIT, RET, CSF-1R, Flt3, PDGF-alpha, PDGF-beta, EGF, VEGF3 and also the receptor for PDE5a showed an expression in every sample examined. Flt.1 and KDR showed no expression in isolated A549 cells; however, they did show expressions in the rest of the tissues.

Furthermore, some of these factors were compared among themselves and examined for their expression in the tumors of the individual groups by means of immunohistochemical quantification. At the end of the experiment, the resulting tumor was dissected from the attached tissue and, after the usual preparation, cut for the immunohistochemical examination. We could demonstrate a decrease in the expression

of the various growth factor receptors that were inhibited. In particular, PDGFR-beta, Flt-1 and EGFR showed a significant decline in the density of receptors.

Additionally, the active rate of cell division (PCNA) and the existing section of the vessels were stained and compared with one another. It was interesting to note, that the one with the smallest increase in tumor volume had the largest number of vessel sections and a slightly higher active rate of cell division, in comparison to the mono-therapy group.

The combined therapy of a multi-kinase inhibitor and the specific PDE5 inhibitor appears to be a good therapy option for NSCLC, as the tumor growth is delayed and so the chance of survival increased. The influence of the factors studied provided no detailed information about the mode of action effective in this kind of therapy, in particular with regards to angiogenesis. This needs to be examined in further studies.

8 Zusammenfassung

Lungenkrebs ist noch immer die weltweit führende Krebserkrankung mit Todesfolge.

Diese Studie untersuchte den Einfluss einer Kombinationstherapie, bestehend aus einem Multikinaseinhibitor und einem spezifischen Phosphodiesterase-5-Inhibitor im Vergleich mit den einzelnen Inhibitoren als Monotherapiegabe und im Vergleich zu einer Placebogabe bzw. einer Versuchsgruppe ohne Intervention.

Am Tag des Versuchsbeginns wurde allen Tieren isolierte A549 Zellen subkutan oberhalb des Muskels am rechten Hinterlauf injiziert.

Die sieben Versuchstiergruppen bestanden aus den jeweils 6-10 Versuchstieren (Nacktmäuse) und wurden während der Versuchsdauer von 28 Tagen mit den unterschiedlichen Inhibitoren als orale Medikation behandelt. Die Behandlungen erfolgten mit Sildenafil, als spezifischer PDE5-Inhibitor, Sorafenib und Sunitinib als Multikinaseinhibitoren, die Kombinationentherapiegruppen bestehend aus Sorafenib + Sildenafil und Sunitinib + Sildenafil, des Weiteren die Placebogruppe. Diese Versuchstiere bekamen Methylcellulose oral verabreicht und zuletzt die Kontrollgruppe, an der keine Intervention stattgefunden hat.

Die Tiere erhielten jeden Tag die gleiche Medikamentendosis, jeden 4. Tag erfolgte eine Messung der Tumorgröße.

Die größte Volumenzunahme zeigte sich, wie zu erwarten war, bei der Kontroll- und bei der Placebogruppe, die kleinste Volumenzunahme zeigte sich bei den beiden Kombinationstherapiegruppen, am konstantesten jedoch bei der Kombination aus Sunitinib + Sildenafil.

An Tumorgewebe aus der Kontrollgruppe wurde die mRNA-Expression einzelner Wachstumsfaktorenrezeptoren untersucht. Um den Einfluss des Tumorstroma zusätzlich zu erfassen, wurden humane Fibroblasten, Monozyten, Makrophagen, Lymphozyten und humanes Donorlungengewebe und zum Vergleich isolierte und angezüchtete A549-Zellen untersucht. Die Rezeptoren cKIT, RET, CSF-1R, Flt3, PDGF-alpha, PDGF-beta, EGF, VEGF3 sowie der PDE5a-Rezeptor waren in allen untersuchten Proben exprimiert, Flt-1 und KDR zeigten keine Expression in isolierten A549-Zellen, jedoch in den übrigen Gewebearten.

Weiter wurden einige dieser Faktoren mit Hilfe immunhistochemischer Quantifikation auf ihre Expression in den Tumoren der einzelnen Gruppen untersucht und untereinander verglichen. Nach Versuchsende wurden dafür die entstandenen Tumore

von dem darunterliegenden Gewebe abpräpariert und nach üblicher Vorbereitung für die Immunhistochemie aufgeschnitten. Es zeigte sich eine Abnahme der Expression von diversen Wachstumfaktorrezeptoren unter Inhibition. Vor allem PDGFR-beta, Flt-1 und EGFR zeigten eine signifikante Abnahme der Rezeptorendichte.

Desweiteren wurden die aktive Zellteilungsrate (PCNA) und die vorhandenen Gefäßanschnitte (vWF+alpha actin) angefärbt und untereinander verglichen. Interessanterweise zeigte sich bei der geringsten Volumenvergrößerung des Tumors die größte Anzahl von Gefäßanschnitten und eine etwas höhere aktive Zellteilungsrate im Vergleich zu den Monotherapiegruppen.

Die Kombinationstherapie aus einem Multikinaseinhibitor und dem spezifischen PDE5-Inhibitor scheint eine gute Therapieoption für NSCLC zu sein, da das Tumorwachstum verzögert wird und damit die Überlebenswahrscheinlichkeit steigt. Der Einfluss auf die untersuchten Faktoren konnte jedoch keinen sicheren Aufschluss über den genauen Wirkmechanismus dieser Therapie geben, besonders in Hinblick auf die Angiogenese. Dies sollte in weiterführenden Studien näher untersucht werden.

9 Literature

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10 Abbreviations

A549 cells	adenocarcinoma 549 cells
AC	adenylyl cyclase
ANP	atrial natriuretic peptide
ATP	adenosine-5'-triphosphate
BNP	b-type natriuretic peptide
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CMML	chronic myelomonocytic leukaemia
COPD	chronic obstructive pulmonary disease
CT	computed tomography
DGAUM	Deutsche Gesellschaft für Arbeitsmedizin und Umweltmedizin e.V.
DMEM	Dulbeccos Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleosidetriphosphate
dsDNA	double stranded DNA
EGF	epidermal growth factor
EPAC	exchange protein activated by cAMP
EPAC	cAMP-activated exchange factors
ERK	extracellular signal regulated kinases
EtOH	ethanol
FCS	fetal calf serum
FLT	fms-like tyrosine kinase
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factors
GIST	gastrointestinal stroma tumor
GTP	guanosine triphosphate
HCC	hepatocelllular carcinoma
HE	haematoxilin-eosin staining
HRPT	hypoxynthinephophoribo-syltransferase
i.e.	for example
KCl	potassium chloride
KDR	kinase insert domain receptor

MAPK	mitogen-activated protein
mRNA	messenger RNA
NaCl	sodium chloride
NO	nitric oxide
NP	natriuretic peptides
NSCLC	non-small-cell lung carcinoma
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDE	phosphodiesterase
PDGF	platelet-derived growth factor
pGC	particulate guanylyl cyclase
PKA	protein kinase A
PPi	pyrophosphate
qPCR	quantitative polymerase chain reaction
RET	ret-protooncogen
RNA	ribonucleic acid
RT	reverse transcriptase
RTK	receptor tyrosine kinase
r.t.	room temparature
SCLC	small-cell-lung carcinoma
sGC	soluble guanylyl cyclase
SMBD	small molecule binding domains
TGF	transforming growth factor
TKR	tyrosine kinase receptor
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor
WHO	World Health Organisation

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