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Comparison of Sunitinib and Sorafenib: Tumor Cell Response and Resistance Formation

Claus Bender

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Nature is all but intelligently designed

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I. Introduction

Under normal conditions, no mammalian cell lives in isolation and therefore has to respond to a wide variety of extracellular signals and coordinate them. Integration of these signals is necessary for multi-cellular organisms during embryonic development and adult life. This ability allows the organism to alter physiological processes in response to changes in its environment. Moreover, complex cell functions are adjusted by regulated information transfer along signaling pathways. It has become apparent, however, that these linear pathways are not free-standing but parts of large and highly complex networks. Communication pathways transduce and exchange informations between different cells but also from different compartments within a single cell. Neighboring cells often communicate by direct cell-cell contact, whereas distant cells use secreted signaling molecules, such as growth factors and hormones. These bind to cognate receptors at the target cells and ultimately induce distinct biological responses like cell proliferation, migration, differentiation or apoptosis. Deregulation of these signaling events was identified as a cause of many severe diseases e.g. cancer, diabetes, immune deficiencies and cardiovascular diseases [6-8].

Already in 1980, Hunter and co-workers defined the relative amounts of protein-derivedphosphoamino acids and found a distribution of 0.05 %, 10 % and 90 % for phospho-tyrosine (pTyr), phospho-threonine (pThr) and phospho-serine (pSer) under physiological cell conditions [9]. Recently, these observations could be verified in a global phosphoproteomic analysis by Olsen *et al.*, who identified more than 2000 phosphorylated proteins in HeLa cells containing 103 pTyr (1.8 %), 670 pThr (11.8 %) and 4901 pSer (86.4 %) sites [10]. Even though tyrosine phosphorylation accounts only for a small part of total protein phosphorylation, the reversible phosphorylation of proteins represents a major post-translational signaling mechanism and regulatory pathway that controls multiple cellular processes [11]. The phosphorylation of proteins is catalyzed by protein kinases, representing a large family of ATP-dependent phosphotransferases. As many as 518 putative kinase genes make up the human kinome [12]. In general, tyrosine phosphorylation is extensively utilized only in multicellular eukaryotes and it conducts crucial functions in the organization of higher ordered tissues. Protein phosphorylation by protein kinases and dephosphorylation catalyzed by protein phosphatases can reversibly modify protein function. This can be achieved by increasing or decreasing its biological activity, by stabilization or targeting proteins for

degradation, by alteration of the localization within the cell or by facilitating or disrupting protein-protein interactions.. According to their localization and their substrate specificity, both protein kinases and phosphatases can be subdivided into cellular and transmembrane proteins and into tyrosine or serine/threonine-specific kinases and phosphatases.

I.1. Cancer biology and genomic instability

The on-set of carcinogenesis is usually initiated by DNA alterations in the affected cell that mostly arises as a result of combination of several factors including genetics, environment, diet, immune-system as well as several others still to be discovered. The individual risk of cancer is influenced by the amount of exposure to environmental agents and one's intrinsic genetic predisposition [13]. This genomic diversity may include a single point nucleotide exchange, deletion, amplification, translocation, chromosomal rearrangement, methylation or other events that can subsequently lead to the activation of oncogenes and/or inactivation of tumor suppressor genes [14]. In addition the on-set of carcinogenesis, also the progression of the tumor to a malignant state is accompanied by genetic lability and this enhanced mutability even increases with tumor progression [15-16]. The increase in mutability is explained by the loss of function of genomic "care taker" systems like the protein p53 or other DNA damage repair genes [17]. Thus, genomic instability has the potential to generate extensively branching routes during tumor progression, generating corresponding intra-tumor genomic heterogeneity [18].
These and other observations of human cancers and animal models conclude that tumor development proceeds via a process formally analogous to Darwinian evolution. According to this model a succession of genetic changes, each conferring one or another type of growth advantage, leads to the progressive conversion of normal into cancer cells. Hence, genomic instability causes the basic principles of a malignant process, which proposes that human cancers should demonstrate a minimal set of capabilities that are necessary to progress to a malignant tumor state: (1) growing uncontrollably; (2) insensitivity to anti-growth signals; (3) evading apoptosis; (4) acquiring unlimited replicative potential; (5) ability to induce and sustain new blood vessels, e.g. angiogenesis; and (6) invasion and metastasis [6]. Moreover, tumor heterogeneity may result in the existence of cancer cell sub-populations within a tumor that exhibit genetic alterations and compensate the cytotoxic or cytostatic effects of an anti-cancer drug, leading to drug-resistance. Consequently, the identification of altered genes,

signaling pathways and their respective cellular targets could lead to substantial improvement in the prevention, diagnosis, prognosis and personalized therapy of cancers.

I.2. Receptor tyrosine kinases

The human genome contains 90 tyrosine kinase genes of which 58 encode transmembrane receptor tyrosine kinases (RTKs). RTKs are classified into 20 subfamilies depending on the structural motives of the extracellular ligand binding domain. The most important subfamilies comprise proteins with cysteine rich domains, EGF-like (epidermal-growth factor-like)

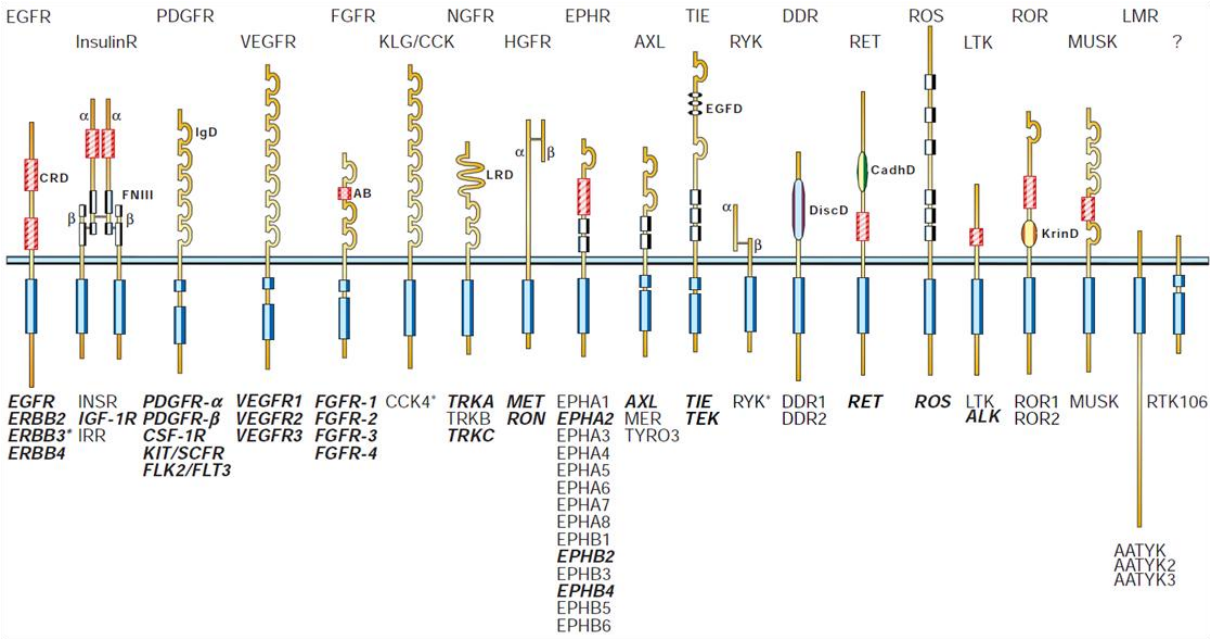


Figure 1: **Human receptor protein-tyrosine kinases**. The prototypic receptor for each family is indicated above and the known members are listed below. Abbreviations of the prototypic receptors: EGFR, epidermal growth factor receptor; InsR, insulin receptor; PDGFR, platelet-derived growth factor receptor; VEGFR, vascular endothelial growth factor receptor; FGFR, fibroblast growth factor receptor; KLG/CCK, colon carcinoma kinase; NGFR, nerve growth factor receptor; HGFR, hepatocyte growth factor receptor, EphR, ephrin receptor; Axl, a Tyro3 PTK; TIE, tyrosine kinase receptor in endothelial cells; RYK, receptor related to tyrosine kinases; DDR, discoidin domain receptor; Ret, rearranged during transfection; ROS, RTK expressed in some epithelial cell types; LTK, leukocyte tyrosine kinase; ROR, receptor orphan; MuSK, muscle-specific kinase; LMR, Lemur. Other abbreviations: AB, acidic box; CadhD, cadherin-like domain; CRD, cysteine-rich domain; DiscD, discoidin-like domain; EGFD, epidermal growth factor-like domain; FNIII, fibronectin type III-like domain; IgD, immunoglobulin-like domain; KrinD, kringle-like domain; LRD, leucine-rich domain. Distinct RTK subunits are denoted by a and b. RTK members in bold and italic type are implicated in human malignancies. An asterisk indicates that the member is devoid of intrinsic kinase activity. Adapted from Blume-Jensen and Hunter [2].

domains, immunoglobuline-like domains, cadherin-like domains and kringle-like domains among others (*Figure 1*) [2]. The extracellular domain selectively binds growth factors, such as epidermal growth factor (EGF), insulin-like growth factor (IGF), or vascular endothelial growth factor (VEGF). Upon binding of these ligands, the RTKs dimerize, which juxtaposes

and thereby activates their intracellular protein kinase domain. The intracellular domain catalyzes the transfer of the γ-phosphate of ATP to hydroxyl groups of tyrosines on various target proteins [19]. The generated phospho-tyrosines create docking sites for target proteins such as intracellular signal transducers that contain phospho-tyrosine interaction domains. These in turn cause alterations of the intracellular signal transduction. Thus, RTKs play a critical role in the regulation of various cellular processes including cell cycle, migration, metabolism, survival, proliferation and differentiation.

I.3. Cytoplasmic tyrosine kinases

There are ten known subfamilies of cytoplasmic, non-receptor tyrosine kinases (NRTKs, *Figure 2)*: Src, Abl, Jak, Ack, Csk, Fak, Fes, Frk, Tec and Syk [2]. NRTKs lack receptor-like features such as an extracellular ligand-binding domain and a transmembrane-spanning

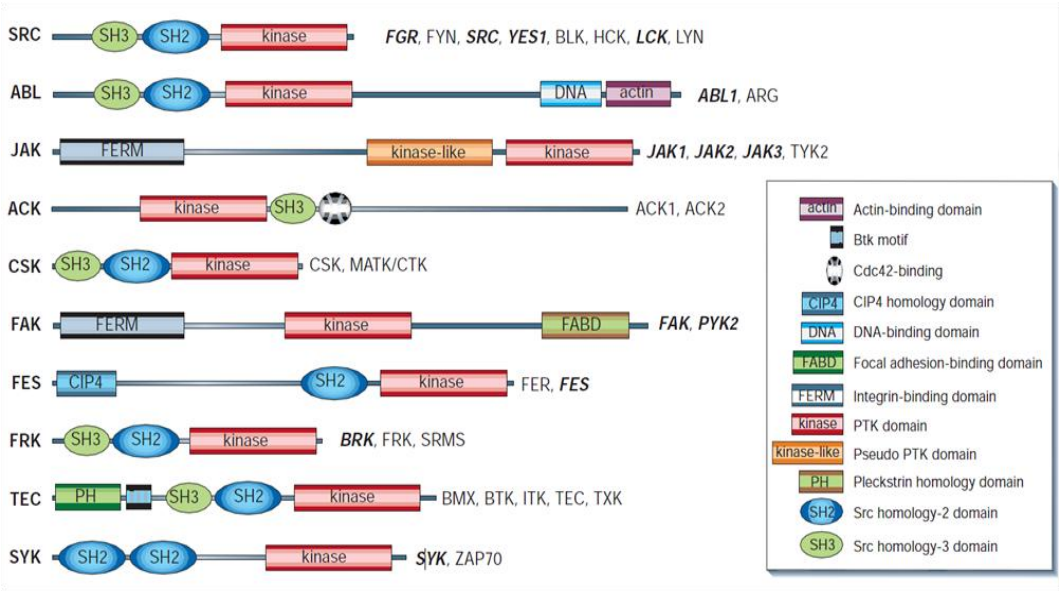


Figure 2: Human cytoplasmic protein-tyrosine kinases. The family members are indicated to the right and the family name to the left of each PTK. The PTK members in bold and italic type are implicated in human malignancies. Adapted from Blume-Jensen and Hunter [2].

region. Most NRTKs are localized in the cytoplasm, whereas some are anchored to the cell membrane through amino-terminal modifications, such as myristoylation or palmitoylation. In addition to a tyrosine kinase domain, NRTKs possess domains mediating protein-protein, protein-lipid, and protein-DNA interactions. The most common mechanism in NRTK regulation, as for RTK function, is tyrosine phosphorylation. In particular, phosphorylation of

tyrosines in the activation loop of NRTKs leads to an increase in phosphotransferase activity. Activation loop phosphorylation occurs via *trans*-autophosphorylation or phosphorylation by a different NRTK [20].

The largest subfamily of NRTKs, with nine members, is the Src family [2]. Src family members participate in a variety of signaling processes, including mitogenesis, T- and B-cell activation, and organization of the cytoskeleton. Various *in vivo* substrates have been described for Src and include the PDGFR and EGFR, the NRTK focal adhesion kinase Fak, the adapter protein p130Cas which is involved in integrin- and growth factor-mediated signaling and cortactin, an actin-binding protein important for the proper formation of cell matrix contact sites. Moreover, Src family members have been associated with various malignancies including renal cell carcinoma and melanoma [21]. Furthermore, the Syk family has been implicated as a tumor suppressor in breast and gastric cancers [22]. Another prominent NRTK, regarding its oncogenic potential, is the V-abl Abelson murine leukemia viral oncogene homolog 1 (ABL), which is the cause chronic myelogenous leukemia (CML). Here, as a consequence of translocation, ABL fuses with BCR, which in turn causes the CML on-set. Accordingly, to maintain a homeostatic state of the cell, NRTKs, like RTKs, must be tightly regulated. As an example for tight regulation Src should be named, as its catalytic regulation has been studied extensively. Src and its family members contain, next to others, a src-homology (SH) 3 domain, a SH2 domain, a tyrosine kinase domain, and a short carboxy-terminal tail. Src possesses two important regulatory tyrosine phosphorylation sites. Phosphorylation of Tyr-527 in the carboxy-terminal tail of Src by the NRTK Csk represses kinase activity. The importance of this phosphorylation site is underscored by v-Src, an oncogenic variant of Src that is a product of the Rous sarcoma virus and lacks the C-terminal tail. Hence, it is constitutively active, leading to uncontrolled growth of infected cells. A second regulatory phosphorylation site in Src is Tyr-416, an autophosphorylation site in the activation loop. Maximal stimulation of Src-kinase activity occurs when Tyr-416 is phosphorylated.

I.4. RTK downstream signaling and protein interaction domains

Ligand-induced RTK activation induces specific intracellular signal transduction pathways, depending on the stimulus and the cellular context. To regulate many different cellular processes, most proteins involved in intracellular signaling contain modular protein domains that specifically interact with other protein domains, lipids, and nucleic acids. These

interaction domains either target proteins to a specific sub-cellular localization, providing means of recognition for post-translational protein modification or chemical second messengers. Furthermore, they can control the conformation, activity and substrate specificity of enzymes [1]. The most important domains in RTK signaling are those recognizing the phosphorylated tyrosine itself [7]. Phospho-tyrosine residues are recognized by SH2 and phospho-tyrosine binding (PTB) domains with SH2 representing the most prevalent binding domain [23]. PTB domains are not restricted to bind phospho-tyrosine residues, because they can also bind to non-phosphorylated peptide sequences. WW and 14-3-3 domains bind to phospho-serine, while phospho-threonine residues are recognized by FHA and WD40 domains. The proline-rich sequence motif PXXP represents an additional binding moiety which binds specifically to SH3 domains. Pleckstrin homology (PH), phox homology (PHOX), FERM and FYVE domains bind to phosphoinositides. An overview of different interaction domains and their binding specificities is depicted in *Figure 3*.

A wide variety of proteins possesses both, an interaction domain and enzymatic activity. In addition to their SH2 domain, Src kinases have a protein kinase activity and PLC-γ a phospholipase C activity. In contrast, other signaling proteins exclusively consist of SH2 and SH3 domains, such as Grb2, Crk and SHC linking activated RTKs to downstream signaling events such as the mitogen-activated protein kinase (MAPKs). The ability of RTKs to recruit

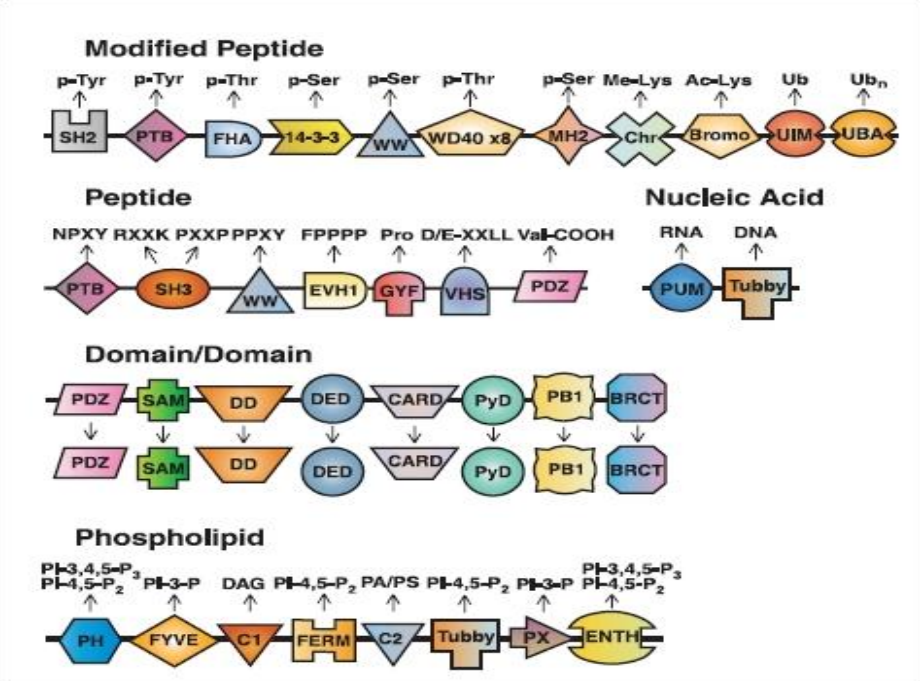


Figure 3: Modular interaction domains in signal transduction. Interaction domains bind proteins, phospholipids or nucleic acid. A subset of such domains is illustrated and their general binding functions are indicated [1].

and activate a wide variety of adaptor proteins provides a signaling platform for the cell to regulate miscellaneous biological responses.

I.5. Kinases as anti-cancer targets

In healthy cells, RTKs are strictly regulated to maintain the homeostasis of the cell. Deregulation and unspecific activation of RTK signaling can result in malignant transformation. Hence, unspecific RTK activation is often linked to hyper-proliferative diseases such as human cancer. In general, there are three mechanisms by which RTKs can become oncogenes with transforming potential. The first mechanism is based on RTK overexpression as a consequence of gene-amplification. This overexpression might increase

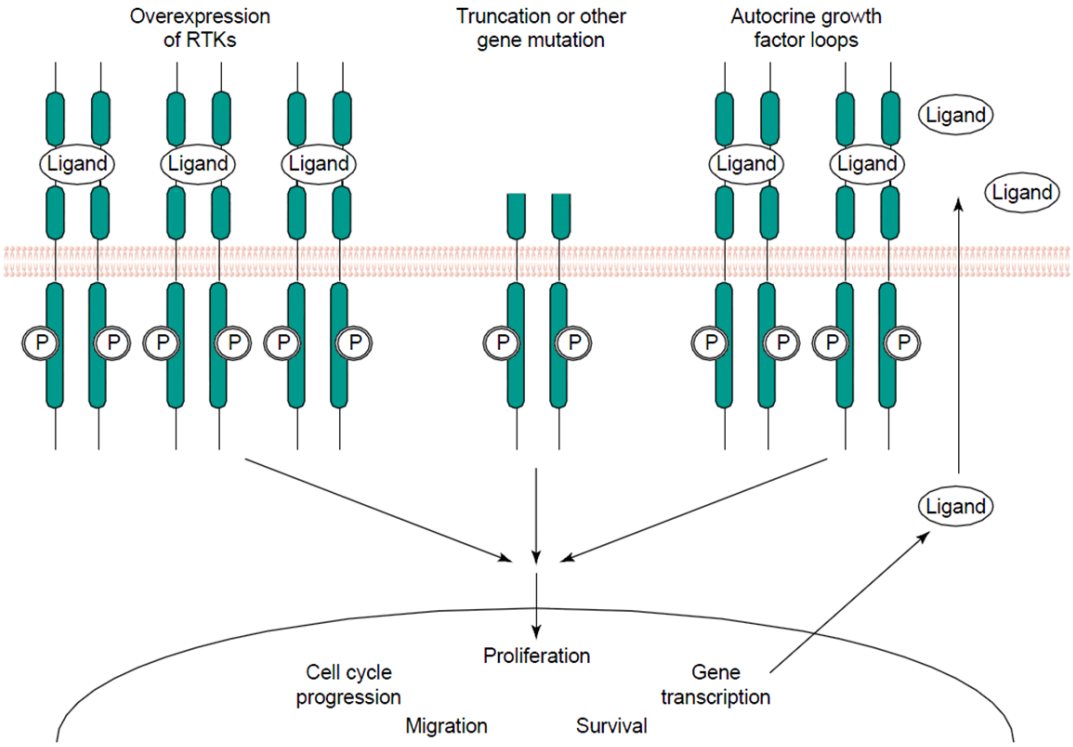


Figure 4: Dysregulated activation of RTKs. Overexpression and mutations of signal loops can trigger strongly enhanced and/or constitutive activation of RTKs. This can result in increased proliferation and gene-expression as well as migration and cell cycle progression causing a malignant transformation of the cell. Adapted from Zwick, Bange, Ullrich [3].

the incidence of activating RTK dimerization. Additionally, the abundance of receptors might enhance the cell response to normal ligand levels. The most prominent examples for this mechanism are the EGFR and HER2. The *EGFR* gene is amplified up to 60 times in certain

tumors and elevated EGFR expression levels are associated with increased tumor proliferation and a high rate of metastasis formation. Overexpression of the EGFR is frequently found in bladder, non-small-cell-lung, pancreatic and kidney cancer [24-25]. The *HER2* gene is, next to other indications, predominantly amplified in ovarian and breast carcinomas and its enhanced expression is correlated with a more aggressive progression and decreased survival time of patients [26-27].

In addition to elevated RTK expression, mutations or deletions can be responsible for an enhanced activity of RTKs, while these aberrations are mostly found within the extracellular domain or ATP-binding motif. One reason for enhanced RTK activation is increased receptor dimerization. For example, dimerization based activation can be mediated by a point mutation in the extracellular domain of the fibroblast growth factor receptor (FGFR) that causes an unpaired cysteine residue and consequently FGFR dimerization through disulfide binding [28]. Other mutations in the FGFR activation loop or somatic FGFR mutations have been shown in myeloma or cervical carcinoma, respectively [29-30]. A third mechanism is caused by para- or autocrine deregulated RTK activation. High plasma levels of Insulin like growth factor (IGF-1) are correlated with an elevated prostate cancer risk, which is exemplary for the paracrine mechanism [31]. An autocrine mechanism is carried out by transforming growth factor α (TGFα); its co-expression with the EGFR is frequently observed in glioblastomas and squamous-cell carcinomas [32].

However, not only RTKs but also cytoplasmic kinases, as mediators of RTK signaling, have emerged as strategic targets for therapeutic intervention. Like for the RTKs, aberrant expression or mutations can result in their oncogenic alteration. The Serine/Threonine kinase Akt plays an outstanding role in numerous processes which are known to be characteristic for cancer [33]. Those processes include the induction of different cell survival mechanisms by inactivation of pro-apoptotic BAD or activation of anti-apoptotic CREB and NFκB and regulation of the cell cycle by targeting cyclin-dependent kinase inhibitors such as p21 and p27 [2]. While no oncogenic mutations of Akt have been found in mammals, Akt-family member amplification is a frequent event in many different cancers [34]. As a consequence, Akt as well as Akt up- or downstream kinases like PI3K or mTor have emerged as drug targets. As Raf1 signaling also triggers survival mediating cascades, it represents another prominent cytosolic kinase drug-target (*Chapter I.12*). Another example is PLK1, which is a regulator of centrosome maturation and apoptosis [35-36]. Centrosomes play a critical role in generating

genetic instability in cancer cells, and hence PLK1 deregulation may be a driving force for tumor progression [37] (*Chapter I.*1).

With further insights into the multistep process of cancer formation a growing list of oncogenes with a potential therapeutic relevance emerged, resulting in the development of target-selective anti-cancer drugs. To date, there are basically two major strategies to interfere with the oncogenic potential of deregulated kinases. One strategy is blocking the ligand-receptor interaction using specific antibodies to inhibit ligand-induced RTK signaling and increase RTK downregulation and internalization. The strategy turned out to be successful as Herceptin®, an antibody directed against HER2, was approved by the FDA for the treatment of breast cancer. Today, there are various monoclonal antibodies for various cancer indications available, including Cetuximap®, which inhibits the EGFR, as well as the VEGF inhibiting Bevacizumab®. The second strategy is based on small molecule kinase inhibitors which interfere with RTKs and NRTKs and alter signal transduction by competing with ATP for the ATP-binding site [38]. Today, there are various different small kinase inhibitors targeting multiple cancer related targets. Those can be highly specific like Gefitinib or Tarceva®, which target the EGFR and thereby exhibit their cytotoxic effects [39]. Based on the known drug resistance mechanisms (*Chapter I.*6), there is a strong rationale for targeting multiple kinases simultaneously. Gleevec® was among the first tyrosine kinase inhibitors which target various kinases (Abl, Kit, PDGFR) [40]. Mono-therapy with Gleevec demonstrated a high clinical efficacy in chronic myeloid leukemia and GIST [41] but resistance formation remains as the major poor prognosis factor. As angiogenesis is an additional driver of tumorigenesis, a new strategy to increase the therapeutic potency and to decrease the drug resistance formation by targeting not only essential cancer cell pathways but also angiogenic components is required [42]. This strategy can either be achieved by combinatorial therapy with specific drugs or the use of rather unspecific drugs that affect sets of cancer relevant proteins [43]. Amongst the first multi target kinase inhibitors with clinical benefit were Sunitinib and Sorafenib (*Chapter I.*9 *and I.*10). As a consequence of targeting multiple pathways, Sunitinib or Sorafenib appear to be superior to single-targeted therapies, as they might restrain compensatory responses in alternative cellular signaling pathways and hence, avoid the formation of drug-resistance. However, the clinical application of Sunitinib for mRCC and GIST eventually results in the progression of the tumor [44-45], i.e. drug resistance formation.

I.6. Drug resistance mechanims

As research has given major insights into cancer biology, the therapy of cancer has made significant advances in terms of the clinical benefit. However, as a consequence of genomic heterogeneity and an inevitable drug imposed selection process, drug resistance formation occurs, which causes a poor overall survival rate of cancer patients. Today, there are four major mechanisms causing drug resistance classified. (1) Reduced intracellular accumulation: an increased efflux of cytotoxic drugs is frequently observed in drug resistant cell lines. Two classes of energy-dependent efflux pumps, P-glycoprotein (Pgp) [46] and multi-drug-resistance-related protein (MRP) [47] that decrease the intracellular drug accumulation are known to cause resistance to many anticancer agents. (2) Compensation of targeted signaling pathways: Gleevec® resistance in CML patients is correlated with increased Lyn expression *in vitro* and patients, which can provide a bypass for the loss of Bcr/Abl signaling. Furthermore, an upregulation due to gene amplification of the Bcr/Abl fusion protein itself was observed in relapsed CML patients. (3) Diminishment of drug-target interaction: Several point mutations including the Bcr/Abl T315I mutation regarding Gleevec® treatment impede the molecular binding of anti-cancer drugs, which results in drug resistance [48]. (4) Modulation of the apoptotic threshold: Most chemotherapeutic drugs act through induction of apoptosis. In melanoma for example, constitutively low levels of spontaneous apoptotic cells compared to other malignant cell types occur, which likely relates to a defective apoptotic response compared to other malignant cell types [49-50]. This defective response can be caused by several dysregulated apoptosis related proteins including p53 or Ras. The assumed mechanism by which both are involved in drug resistance is an upregulation of Bcl-2 expression [51-52]. The high expression of Bcl-2 in human tumors has been correlated with resistance to chemotherapy and decreased survival [53], indicating it´s oncogenic potential. Moreover, antisense oligonucleotides against Bcl-2 or Bcl-X_L can increase the chemo-sensitivity against chemotherapeutics like cisplatin or dacarbacine [54-55].

While drug-resistance is a drawback of today´s cancer treatment, the growing list of available anti-cancer drugs and potential combinatorial therapy raises the hopes for an advanced treatment. Nevertheless, a better understanding of drug specific resistance mechanisms and a list-expansion of disease related proteins remain pivotal for the development of novel therapeutic strategies to improve patient treatment.

I.7. Renal cell carcinoma

Renal cell carcinoma is a kidney cancer that originates in the linen of the proximal convoluted tubule. Metastatic renal cell carcinoma (mRCC) accounts for more than 100 000 deaths annually worldwide, with a projected 54 390 new cases and 13 010 deaths in the United States in 2007 [56]. Metastatic lesions are developed during the course of the disease in one-third of the patients [57]. The research is mainly focused on clear-cell carcinoma as it presents the most common type of kidney cancer. As metastatic RCC is currently one of the most treatment resistant malignancies, treatment options have been limited to interleukin-2 and interferon-alpha (IFN-α) [56]. Although these immunotherapies produce an overall response of only ~12 % and high rates of toxicity, they have been considered effective. The complex molecular genetics of RCC are making it difficult to target specific pathways to interfere with tumor growth [58]. One of the most common findings are mutations in the Von Hippel Lindau (VHL) gene (~ 75 %). In conditions of hypoxia or conditions with defective VHL protein (pVHL) function, the hypoxia inducible factor (HIF) is constitutively activated [59]. By this process, several hypoxia inducible genes such as the VEGF, EGFR, PDGF, glucose transporters and transforming growth factor-a (TGF-α) [58]. These proteins are involved in the mechanism of angiogenesis, pH regulation, glucose metabolism and survival. As tumor-angiogenesis plays an important role in tumor growth and progression, two RTKs, VEGFR and PDGFR, emerged as initial targets for RCC. Hence, the scientific rationale for the treatment of RCC with two small molecule, multi-targeted receptor tyrosine kinase (RTK) inhibitors was given: Sunitinib and Sorafenib, which are introduced in detail in the *chapters I.*9 *and I.*10.

I.8. Melanoma

Melanoma disease occurs in 160 000 new cases worldwide and causes 48 000 deaths every year (1975 – 2000) [60], while it occurs more frequent in males, Caucasians and more prominent in sunny climates. Patients with stage 1 melanoma are generally treated with surgery, while adjuvant therapy is rather inefficient. In later stages of melanoma disease, chemotherapeutic agents like decarbazine (DTIC), cisplatin, paclitaxel, docetaxel and the DTIC analogue temozolomide are used for treatment, whereas DTIC displays the highest response rates. As single or combinatorial therapy with these chemotherapeutics did not result in a better

response rate or overall survival, immunotherapy with Interferon-α (IFN-α) and interleukin (IL)-2 became of interest, showing promising response rates for treatment of metastatic melanoma [61]. Combinatorial treatment with chemotherapeutics and immunotherapy, known as biochemotherapy, demonstrated also high response rates [62]. While initial response to single or combinatorial therapy rates 15-20 %, again no impact on overall survival was observed [61]. Hence, the prognosis with an overall survival time of 6 – 12 month for metastatic melanoma is poor [63]. The cause for this low survival rate is based on primary or secondary single- or multi-drug resistance, respectively [64]. The underlying mechanisms for drug resistance can be manifold, as further introduced in *Chapter I.*6. For the understanding of the on-set of the malignancy substantial advances have been made. These include: the up-regulation of the Ras/Raf/MEK/ERK pathway and activating mutations in c-Kit [65-67]. Down-stream of c-Kit and the Ras pathway, the microphthalmia-associated transcription factor (MITF) integrates multiple pathways and regulates survival, which is introduced in detail in *chapter I.*13. Next to others, these observations gave the scientific rationale for clinical trials of Sunitinib and Sorafenib for the treatment of metastatic melanoma.

I.9. Sunitinib

Sunitinib is an oral, small molecule, multi-targeted receptor tyrosine kinase (RTK) inhibitor. Sunitinib malate (SUl1248) is an indolin-2-one analogue with the chemical name N-(2diethylaminoethyl)-5-[(Z)-(5-fluoro-2–oxo-1H–indol–3-ylidene)methyl]-2,4–dimethyl-1H–pyrrole–3–carboxamide(2S)–2–hydroxybutanedioic acid. The structural formula of Sunitinib is depicted in *Figure* 5. It inhibits multiple RTKs, including the vascular endothelial growth factor 1/2 (VEGFR 1/2), the platelet derived growth factor receptor-β (PDGFR-β), the stem cell factor receptor (c-KIT), the colony stimulating factor-1 (CSF-1R), and the FMS-like tyrosine kinase-3 receptor (FLT-3) [68-71]. Upon activation, these RTKs initiate a signaling cascade that eventually blocks apoptosis and enhance proliferation as well as angiogenesis and metastasis [6]. Competitive inhibition of these RTKs might down-regulate transduced survival

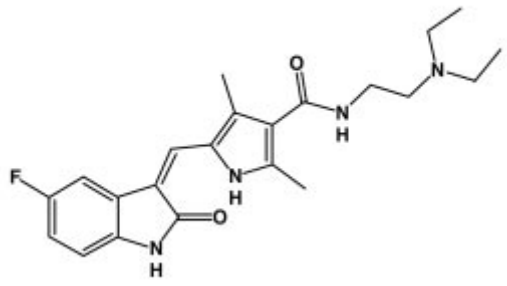


Figure 5: Chemical structure of Sunitinib.

effects resulting in the apoptosis of cancer cells. These cascades are also important within the growth of renal cell carcinoma and GIST [56]. Moreover, these pathways appear to be active in many tumors, suggesting that Sunitinib may have a broad spectrum of activity [72]. The Sunitinib targeted receptors are highly expressed in tumor cells, but also are also present in endothelial cells (VEGFR) and in tumor associated pericytes, fibroblasts, and smooth muscle cells (PDGFR-β) [73-75]. Hence, Sunitinib modulates tumor growth not only directly, but also by indirect action on tumor vasculature and cells of the tumor environment. Next to those RTKs, *in vitro* ATP site-dependent binding assays as well as proteomic analyses revealed panels of more than 70 kinases, which are targeted by Sunitinib [76-77].

The pharmacokinetic covariate analysis did not produce a reduction in systematic clearance and volume of distribution (Vd/F). Therefore, dose adjustment is not required based on age, body weight, race or sex [72]. Sunitinib is metabolized by the cytochrome P450 in two sequential N-de-ethylation steps with a calculated half-life of 40-60 hours. It is secreted 77 % in total, distributed to 61 % in the feces and 16 % in urine [78-79]. Altered taste, Hand-Foot-Syndrome, Mucositis, Astenia, and reversal hair and skin discoloration can be side effects of Sunitinib [78]. The clinical efficacy of Sunitinib was shown in several pre-clinical and clinical trials. Studies in xenografts revealed dose-dependent inhibition of tumors including colon, non-small lung, glioma, melanoma, AML and breast [69,71,80-81]. In phase 3 trials, Sunitinib demonstrated a significantly higher efficacy over IFN-α as first line treatment of good- or intermediate risk patients with mRCC. The response rates and progression-free survival were ~31 % (Sunitinib) compared to ~6 % (IFN-α) and 11 and 5 month, respectively [82-83]. In GIST, Sunitinib achieved 28.9 weeks of progression-free survival [84].

As a consequence, Sunitinib has been approved by the Food and Drug Administration (FDA) for the treatment of metastatic renal cell carcinoma (mRCC) and gastrointestinal stromal tumor (GIST) in January 2006 [85]. Today, multiple clinical trials including combinatorial therapy for melanoma, breast- and colon cancer are ongoing [86].

I.10. Sorafenib

Sorafenib (BAY 43-9006) is an oral, small molecule, multi-targeted inhibitor. The chemical name of the bi-aryl-urea Sorafenib is *N*-(3-trifluoromethyl-4-chlorophenyl)-*N*‘-(4-(2-methylcarbamoylpyridin-4-yl) oxyphenyl) urea. The molecular formula is shown in *Figure 6*. In an initial *in vitro* screening Sorafenib was identified as a potent inhibitor of Raf

serine/threonine kinase isoforms. This inhibition has been shown to be independent on the V600E *B-Raf* mutation, while inhibitory potencies can differ. As a consequence, the MEK/ERK pathway has been shown to be inhibited by Sorafenib. In addition to the inhibiton of Raf isoforms, Sorafenib inhibits VEGFR-1/2/3, PDGFR-β, c-Kit, FLT-3 and Ret [87-89]. Many of these RTKs mediate processes like tumor growth, progression, and metastasis by triggering multiple signaling pathways [3]. Like for Sunitinib, a wider kinase panel of targeted kinases was resented by Fabian *et al* [77]. Further an indirect action by inhibiting angiogenesis can be suggested. The volume of distribution of Sorafenib is not reported. However, since the drug is highly protein bound (99.5 %) and has a half life of 25 – 48 h, a large volume of distribution is expected. The oxidative metabolism of Sorafenib is mediated by CYP3A4, additionally the drug is glucuronidated by UDP glucuronosyltransferase (UGT) 1A9. Sorafenib is eliminated primarily through the liver. Of a 100 mg dose 77% is excreted with the feces and 19% is excreted as glucuronidated metabolites in the urine [90]. Sorafenib displays several side effects such as hand-foot skin reaction, rash and diarrhea [91-92]. Several pre-clinical and clinical trials have shown the clinical relevance of Sorafenib. In xenograft models, Sorafenib demonstrated a broad spectrum, dose-dependent anti-tumor activity against liver, colon, lung, breast, ovarian, pancreatic, and melanoma cancers [93-94]. Clinical phase 3 trials confirmed Sorafenib´s activity against RCC by significantly prolonging progression-free survival (PFS) compared with placebo in patients with advanced disease [95].

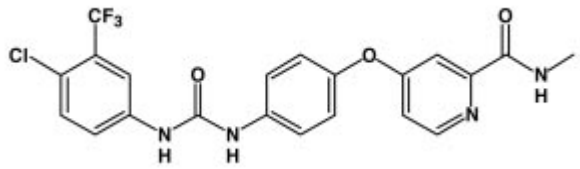


Figure 6: Chemical structure of Sorafenib.

Due to the clinical benefit, Sorafenib has been approved by the FDA for the treatment of RCC and HCC in November 2005 and November 2007, respectively. Today multiple clinical trials including mono- or combinatorial therapy for breast cancer and melanoma are ongoing [86].

I.11. Comparison of Sunitinib and Sorafenib

Sunitinib and Sorafenib have been the first small molecule, multi-targeted RTK inhibitors on the market. Both inhibit angiogenesis, display anti-tumor activity *in vivo* and share a pool of target kinases like the PDGFR-β and VEGFRs (*Figure 7*). Both drugs are used for the therapy of mRCC, whereas Sorafenib is reserved primarily for patients who are refractory to cytokine

therapy and Sunitinib for the treatment of cytokine naive patients [45,82]. Moreover, Sunitinib and Sorafenib are currently in clinical trials for a wide spectrum of tumor indications. The different chemical structures of both drugs, however, indicate different modes of action, which is supported by several observations: In a retrospective analysis of advanced renal cell carcinoma, patients who received sequential therapy, treatment with Sunitinib after Sorafenib failure provided more durable disease control than the reverse order [96]. In this context is it

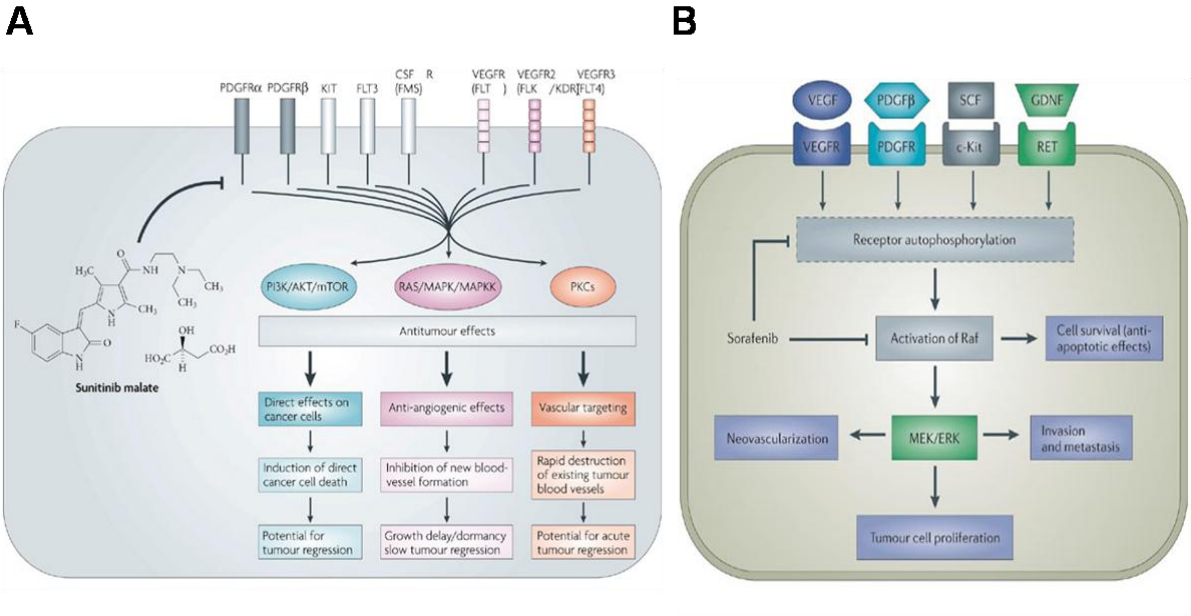


Figure 7: Schematic overview of Sunitinib´s and Sorafenib´s putative mode of action. (A) Sunitinib interacts with the intracellular domains of various RTKs and therby prevents their autophosphorylation. Thus, Sunitinib triggers multiple downstream signaling cascades, resulting in anti-tumor effects like induction of cell death, anti-angiogenesis and vascular targeting. (B) Next to these by Sunitinib inhibited RTKs, Sorafenib inhibits the activation of Raf-isoforms. Inhibition of Raf results in the inhibition of the MEK/ERK pathway and subsequent to inhibition of anti-tumor effects including anti-apoptosis, invasion and metastasis or cell proliferation. MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; GDNF, glial-derived neurotrophic factor; PDGFR, platelet-derived growth factor receptor; SCF, stem cell factor; VEGFR, vascular endothelial growth factor receptor. Drawings are adapted from Nature Reviews [4-5].

hypothesized that drug resistance after the use of Sorafenib would be overcome by Sunitinib, but not vice versa [96]. In GIST, only Sunitinib is approved by the FDA, while Sorafenib has shown no therapeutical benefit. Likewise, Sorafenib is used for therapy of HCC, while Sunitinib demonstrated no benefit for this tumor indication, which also suggests a different mode of action. A recent study demonstrated that Sorafenib but not Sunitinib induces the primary immune response and affects the function of dendritic cells [97]. The differences in the mode of action might be reasoned in the different kinase target-pools of both drugs. Moreover, even for the kinases that are inhibited by both drugs, different IC_{50}-values have been observed [77]. As Sorafenib was designed as a Raf-1 inhibitor, this kinase is the most prominent discrepancy between the drugs. While Sorafenib inhibits Raf-isoforms directly

independent of the V600E mutation, Sunitinib does not. Subsequently, Sorafenib directly inhibits the Ras/Raf/MEK/ERK pathway, which is involved in many processes like anti-apoptosis and drug resistance by multiple mechanisms [98]. A detailed view on the Ras/Raf/MEK/ERK pathway is introduced in *chapter I.*12. Summarized, the mode of action for both drugs is still poorly understood. Hence, except for drug related observed phenotypes and dissimilar inhibition coefficients (IC_{50}), no satisfactory comparision is available.

I.12. The Ras/Raf/MEK/ERK Pathway

The Ras/Raf/MEK/ERK is a signaling cascade that couples signals from the cell surface receptors to the transcription factors, which in turn regulate gene expression. The pathway has diverse effects that can regulate cell cycle progression, differentiation or apoptosis [99]. Upon ligand-induced activation of RTKs, the adaptor protein Grb2 is recruited. Grb2 associates with the RAS-GEF SOS complex thereby activating it, which in turn activates membrane-associated Ras, a small monomeric GTP-binding protein [7]. Next, the Raf-isoforms that are serine/threonine (S/T) kinases get activated by the recruitment of the Ras, dimerization, phosphorylation, and dissoziation from the Raf kinase inhibitory protein (RKip) [100-103]. Raf, in turn phosphorylates and thereby activates the Mitogen-activated protein kinase (MEK1), which is a tyrosine (Y-) and S/T dual specificity kinase [104]. The Extracellular-signal-regulated kinases 1,2 (ERK), which are positively regulated, when phosphorylated by MEK1 or MEK2, are the predominant downstream targets of MEK1. The functions of the Ras/Raf/MEK/ERK cascade are widely diversified. The cascade can induce a p15/p16 or p21 mediated premature G_1 arrest and subsequent senescence [105-106]. Over-expression of the Raf-isoforms is associated with

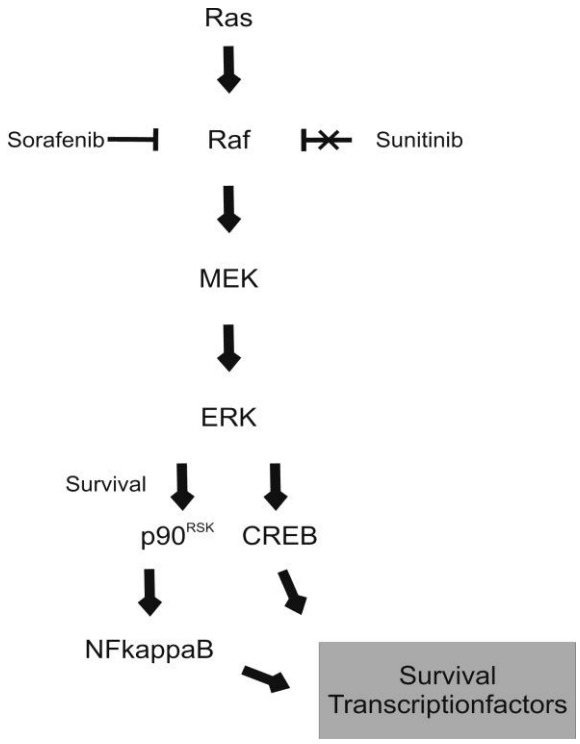


Figure 8: Schematic overview of the Ras/Raf/MEK/ERK pathway. The small GTP-binding protein Ras activates a signaling cascade of phosphorylation events, triggering and altering multiple cellular processes, including regulation of transcription factors for cell survival. These effects can be inhibited by the small molecule kinase inhibitor Sorafenib but not by Sunitinib. Abbreviations are described in the text.

divergent cell responses in dependence on the expression level and isoform. In hematopoetic cells, for example, over-expression of constitutively active A-Raf and Raf-1 mutant led to cell proliferation, while over-expression of B-Raf resulted in apoptosis [107-108]. The Raf/MEK/ERK cascade is moreover involved in processes preventing apoptosis: By BAD^{S112} phosphorylation a signaling cascade is started that generates an anti-apoptotic response via Bcl-2 homo-dimer formation. Recently, the inactivation of $Caspase\text{-}9^{T125}$ phosphorylation by the Raf/MEK/ERK cascade was demonstrated. As Bad and Caspase 9 phosphorylation is also triggered by the PI3K/Akt pathway a crosstalk between both pathways was suggested [109-110]. Next to the Raf-isoforms, the ERKs have multiple functions: They can activate multiple down-stream targets including the 90 kDa ribosomal S6 kinase ($p90^{RSK}$), which leads to the activation of the transcription factor CREB. Moreover, ERK can lead to the indirect activation of the nuclear factor immunoglobulin κ chain enhancer-B cell (NF-κB) by activating IKK-isoforms [99,111-112]. As those ERK targets are known to induce cell survival, the anti-apoptotic role of the Ras/Raf/MEK/ERK pathway is underlined. In AML, elevated expression of ERK is associated with poor prognosis [113]. Furthermore, ERK can enter the nucleus to phosphorylate multiple transcription factors and moreover phosphorylates several proteins that are involved in cell cycle regulation such as retino blastoma (RB) or p53.

The Ras/Raf/MEK/ERK pathway plays a critical role in human cancers, where ~30 % of constitutively active Ras was observed. While *Raf* oncogenes were thought not to be frequently mutated for years, recent studies have shown frequent mutations of *B-Raf* in certain tumors like melanoma or ovarian cancer [114]. Today, Raf is thought to be the most important kinase in the signaling cascade as it directly or - using Raf-1 as a bypass – indirectly activates MEK and ERK. While different *B-Raf* mutations have been mapped (D593V), the most common mutation is a change of valine to glutamic acid at position 600 (V600E), which in turn leads to constitutive activation of the Raf/MEK/ERK signaling cascade [115-116] with its diverse functions.

Due to its role in cancer, the Ras/Raf/MEK/ERK pathway has become a target for therapeutic intervention. Different strategies are pursued to inhibit the signaling cascade: (1) direct inhibition of Raf using competitive ATP-binding site inhibitors including Sorafenib (*Chapter I.*10); (2) indirect inhibiton of Raf by circumventing its dimerization using drugs like geldanamycin or coumermycin [117-118]; (3) inhibition of Raf by inhibition of targeting kinases such as Src, PKC, PKA and phosphatases (PP2A) involved in Raf activation; (4) direct inhibition of MEK, as it is aberrant expressed in many cancers, using specific inhibitors [119].

But as new therapeutics are being developed, classical chemotherapy such as doxorubicin remains the most used therapy for various cancer indications. The Ras/Raf/MEK/ERK pathway can protect cells from oxidative stress and thus counteract one of doxorubicins anti-tumor effects [120-121]. Other mechanisms of the pathway to induce drug-resistance include the modulation of drug pumps (Mdr-1) or down-stream transcription-factors like Bcl-2. Hence, inhibition of the pathway might help to overcome drug-resistance.

I.13. The Transcription Factor MITF

Transcriptions factors (TFs) are proteins that bind specific DNA sequences and thereby control the transcription of DNA to mRNA [122]. As they are essential for differential gene-regulation they can be found in all organisms. In human ~2600 genes contain a DNA-binding motif and thus a putative transcription regulating function [123]. By binding to the DNA, TFs regulate many important cellular processes including development and response to extra- or intracellular signaling cascades [124-126]. TFs themselves are regulated by their rate of synthesis and nuclear localization as well as accessibility of the DNA binding site and activation by

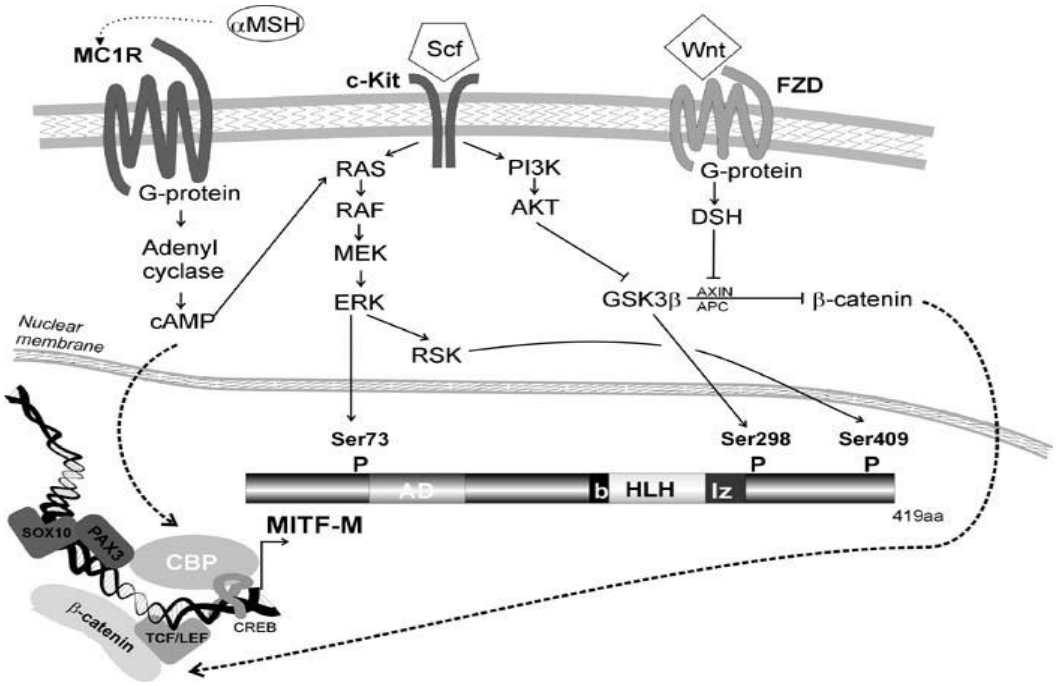


Figure 9: Regulation of MITF in melanoma. MITF is regulated on a transcriptional and post-translational level. In response to external stimuli like α-MSH, or WNT ligand binding, signaling cascades transduce multiple events that eventually activate MITF transcription. SCF ligand binding starts another cascade which results in altered stability or activity due to phosphorylation events. Schematic drawing adapted from Busca et al., 2000.

binding of co-factors or phosphorylation [127-128]. TFs are often tumor suppressors or oncogenes and hence, cancer and other diseases are associated with their mutations or deregulation and the clinical relevance for therapeutic intervention is given. TFs have not been the favorite targets in drug development but recent efforts have resulted in identification of several small molecule drugs that directly or indirectly affect transcription factor activity, e.g. tamoxifen® for breast- and bicaluteamid for prostate cancer [129].

The Microphtalamia-associated transcription factor (MITF) gene was discovered by Paula Hertwig in 1942 as a spotted mutant mouse. There are at least six MITF isoforms, which have been mapped to the 3p12.3-14.1 chromosome in humans. Together with TFE3, TFEB, and TFEC, MITF comprises a family of transcription factors that share a highly homologous basic-helix-loop-helixleucine zipper (bHLHzip) DNA binding and dimerization domain [130]. The MIT-family is critical for the normal development of several cell lineages [130]. In mice, homozygous MITF deficiency results in a loss of the melanoma cell lineage rather than albinism, suggesting an extended MITF function than pigmentation. Consistent with this vital role, MITF has been suggested to regulate genes important for cell proliferation and survival including BCL2, CDK2, p16/Ink4a, c-MET, and p21CIP [131-132]. In humans, germline heterozygous mutations of the MITF gene are associated with the congenital pigmentation/deafness condition Waardenburg syndrome type IIa [133]. Furthermore, approximately 20 % of melanomas harbor amplification of the MITF gene, in some cases exceeding 100 copies, and disruption of MITF activity is lethal to melanoma cells [134]. Mutations and/or aberrant expression of several *MITF* family member genes have also been reported in human cancer, including melanoma (*MITF*), papillary renal cell carcinoma (*TFE3,TFEB*), and alveolar soft part sarcoma (*TFE3*) which suggests a role for the MIT-family in oncogenesis [135-137].

The MITF gene is regulated on both, transcriptional and post-translational levels (*Figure 9*). In response to external stimuli such as the alpha-melanocyte stimulating hormone (α-MSH), the cyclic-adenosine monophosphate (cAMP) levels are elevated which in turn causes an upregulation of the MITF expression [138-140]. The WNT-signaling pathway, which is immensely studied in regards to cancer, is also associated to the transcriptional regulation of MITF. Secreted WNT proteins bind to seven-transmembrane receptors causing G-protein and subsequent disheveled (DSH) activation. The DSH protein inhibits an APC/Axin complex that in turn inhibits GSK3β induced β-catenin degradation, resulting in a nuclear accumulation of β-catenin, which functions a MITF gene-expression co-activator [141-142]. This

role of the WNT pathway and β-catenin is further underlined by observations in melanomas where significant fractions demonstrate mutational β-catenin or WNT pathway activation [143-144]. The post-translational modifications that regulate MITF activity have been mostly identified in mouse studies displaying overlapping phenotypes. Here, Kit-null mutant mice demonstrate a severe loss of melanocytes [145]. Furthermore, the tumor-suppressor neurofibrinogen-1, a negative regulator of the Ras/MAPK pathway, can partially rescue those Kit-mutations. The ERK protein itself can both cause the activation and degradation of MITF [146-147]. Downstream of ERK, the ribosomal S6 kinases RSK1 and RSK2 have been shown to phosphorylate and thereby activate several proteins of the Ras/MAPK signaling cascade including CREB and MITF [147-151]. In 2002, a genomic approach revealed the transcriptional regulation of target genes by MITF including the proto-oncogene BCL2, the interaction of which is a requirement for melanocyte lineages [152]. In summary, the transcription factor MITF is crucial for development and survival processes and hence is emerges a therapeutic target. But due to the difficulties to repress TFs with small molecules, additional insight of the pathways which converge in MITF might reveal more tractable targets.

II. Materials and Methods

II.1. Materials

II.1.1. Laboratory chemicals and biochemicals

Acrylamide	Serva, Heidelberg
Agar Difco	Detroit, USA
Agarose	BRL, Eggenstein
Ampicillin	Roche, Mannheim
Aprotinin	Sigma, Taufkirchen
APS (Ammonium peroxodisulfate)	Bio-Rad, München
ATP (Adenosine 3´-triphosphate)	Pharmacia, Freiburg
Batimastat	BritishBiotech, Oxford, UK
Bisacrylamide	Roth, Karlsruhe
Bromophenol blue	Sigma, Taufkirchen
BSA (Bovine serum albumin)	Sigma, Taufkirchen
Coomassie G250	Serva, Heidelberg
Crystal violet	Sigma, Taufkirchen
Deoxynucleotides (dG/A/T/CTP)	Roche, Mannheim
Dideoxynucleotides (ddG/A/T/CTP)	Pharmacia, Freiburg
DTT (Dithiothreitol)	Sigma, Taufkirchen
Ethidium bromide	Sigma, Taufkirchen
Heparin	Sigma, Taufkirchen
HEPES (N-(2-Hydroxyethyl)piperazine-N`-(2-ethanesulfonic acid))	Serva, Heidelberg
IPTG (Isopropyl b-D-1-thiogalactopyranoside)	Biomol, Hamburg
L-Glutamine	Gibco, Eggenstein
Leupeptin	Sigma, Taufkirchen
Lipofectamine® RNAiMAX	Invitrogen, Darmstadt
Lysozyme	Sigma, Taufkirchen
Marimastat Sugen	Inc., CA, USA
MBP (Myelin basic protein)	Sigma, Taufkirchen
PMSF (Phenylmethanesulfonyl fluoride)	Sigma, Taufkirchen
Poloxin	provided by Berg
Ponceau S	Sigma, Taufkirchen
RNAiMAX	Invitrogen, Karlsruhe
SDS (Sodium dodecyl sulfate)	Roth, Karlsruhe
Sodium azide	Serva, Heidelberg
Sodium fluoride	Sigma, Taufkirchen
Sodium orthovanadate	Aldrich, Steinheim
Sorafenib	ACCC, San Diego, USA

Sunitinib	ACCC, San Diego, USA
TEMED (N,N,N',N'-Tetramethylethylenediamine)	Serva, Heidelberg
TPA (Tetradecanoyl-phorbol-13-acetate)	Sigma, Taufkirchen
Triton X-100	Serva, Heidelberg
tRNA (from Baker Yeast)	Roche, Mannheim
Trypsin	Gibco, Eggenstein
Tween 20, 40	Sigma, Taufkirchen
Tyrphostin AG1478	Alexis, Grünberg

All other chemicals were purchased from Merck (Darmstadt).

II.1.2. Radiochemicals

[α^{33}-P] dATP 3000Ci(111TBq)/mmol	PerkinElmer, Köln

II.1.3. "Kits" and other Materials

Cell culture materials	Greiner, Solingen Nunclon, Dänemark Falcon, U.K.
Cellulose nitrate 0.45 µm	Schleicher & Schüll, Dassel
Dowex AG1-X8	Bio-Rad, München
ECL Kit	PerkinElmer, Köln
Glutathione-Sepharose	Pharmacia, Freiburg
Human Phospho-RTK Array	R&D, Minneapolis, USA
Hybond-N+ filter	Amersham, USA
Hyperfilm MP	Amersham, USA
Micro BCA Protein Assay Kit	Pierce, Sankt Augustin
Parafilm	Dynatech, Denkendorf
Protein A-Sepharose	Pharmacia, Freiburg
Protein G-Sepharose	Pharmacia, Freiburg
QIAquick Gel Extraction Kit (50)	Qiagen, Hilden
QIAquick PCR Purification Kit	Qiagen, Hilden
QIAGEN Plasmid Maxi Kit	Qiagen, Hilden
Random-Primed DNA Labeling Kit	Pharmacia, Freiburg
Sephadex G-50 (DNA Quality)	Pharmacia, Freiburg
Sterile filter 0.22 µm, cellulose acetate	Nalge Company, USA
Sterile filter 0.45 µm, cellulose acetate	Nalge Company, USA
Transwells	Corning, New York, USA
Whatman 3MM	Whatman, USA

II.1.4. Growth factors

EGF Sigma, Taufkirchen

II.2. Media

II.2.1. Bacterial media

LB was used for cultivation of all *Escherichia coli* strains. If required, 100 µg/ml ampicillin or 70 µg/ml kanamycin were added to the media after autoclaving. For the preparation of LB-plates 1.5 % Agar was added.

LB-Medium
1.0 % Tryptone
0.5 % Yeast Extract
1.0 % NaCl
pH 7.2

II.2.2. Cell culture media

Cell culture media and additives were obtained from Invitrogen (Eggenstein). Media were supplemented to the requirements of each cell line. Freeze medium contained 90% heatinactivated FCS and 10% DMSO.

Dulbecco's Modified Eagle Medium (DMEM) was supplemented with 4,5 mg/ml Glucose, 10 % FCS, 2 mM LGlutamine, 1 mM sodiumpyruvate, 1% Penicillin/Streptomycin

Minimum Essential Medium (MEM), 10 % FCS, 2mM L-Glutamine, 1% Penicillin/Streptomycin

RPMI 1640, 10 % FCS, 2 mM L-Glutamine, 1 % Penicillin/Streptomycin

II.2.3. Stock solutions and commonly used Buffers

Acrylamide solution (30/0,8%) 0.8% (w/v) Bisacrylamid	30% (w/v) Acrylamid
HNTG 50 mM NaCl 0.1 % TritonX-100 10 % Glycerol 10 mM $Na_4P_2O_7$	20 mM HEPES, pH 7.5
DNA loading buffer (6x) 0.05 % Xylencyanol 30 % Glycerol 100 mM EDTA pH 8.0	0.05 % Bromphenol blue
Laemmli buffer (2x)	65 mM Tris/HCl pH 6.8 2 % SDS 30 % Glycerol 0.01 % Bromphenol blue 5 % ß-Mercaptoethanol
Laemmli buffer (3x)	100 mM Tris/HCl pH 6.8 3 % SDS 45 % Glycerol 0.01 % Bromphenol blue 7.5 % ß-Mercaptoethanol
NET	50 mM Tris/HCl pH 7.4 5 mM EDTA 0.05% Triton X-100 150 mM NaCl
PBS	137 mM NaCl 27 mM KCl 80 mM Na_2HPO_4 1.5 mM KH_2PO_4 pH 7.4

SD-Transblot	50 mM Tris/HCl pH 7.5 40 mM Glycine 20% Methanol 0.004 % SDS
Western Blot “Strip” buffer	62.5 mM Tris/HCl pH 6.8 2 % SDS 100 mM β-Mercaptoethanol TAE 40 mM Tris/Acetate pH 8.0 1 mM EDTA
TE10/0.1	10 mM Tris/HCl pH 8.0 0.1 mM EDTA pH 8.0 DS 25 mM Tris/HCl pH 7.5 200 mM Glycine 0.1 % SDS
Triton X-100 lysis buffer	50 mM HEPES, pH 7.5 150 mM NaCl 1 mM EDTA 10 % Glycerin 1 % Triton X-100 10 mM $Na_4P_2O_7$ 2 mM VaO5 10 mM NaF 1 mM PMSF 100 µg/l Aprotinin

II.3. Eukarytotic cell lines

Cell line	Description origin	Reference
A498	Human kidney carcinoma	Sugen
A590	Human pancreas adenocarcinoma ascites	Schmiegel, W
A704	Human kidney adenocarcinoma	ATCC
C8161	Human skin melanoma	Gillies, B
CAKI1	Human kidney clear cell carcinoma	Sugen
CAKI2	Human kidney clear cell carcinoma	ATCC
Capan1	Human colon colorectal adenocarcinoma	ATCC
HT-29	Human colon colorectal adenocarcinoma	ATCC
KA II	Human skin melanoma	Hermeking, H
MDA-MB 231	Human mammary gland adenocarcinoma	U3
MDA-MB 435S	Human mammary gland ductal carcinoma	ATCC

MM-LO	Human skin melanoma	Hermeking, H
MM-SU	Human skin melanoma	Hermeking, H
U118	Human brain glioblastoma, astrocytoma	Sugen
U1242	Human brain glioblastoma	Sugen
WM115	Human skin melanoma	ATCC
WM266-4	Human skin melanoma	ATCC

All cell lines used in this study were grown as recommended by the supplier.

II.4. Antibodies

II.4.1. Primary antibodies

Antibody	**Immunogen origin**	**Reference**
Aurora kinase A	Goat, polyclonal, directed against N-terminus; sc-14318	Santa Cruz, USA
Aurora kinase B	Goat, polyclonal, directed against internal domain; sc-14326	Santa Cruz, USA
β-actin	Rabbit, polyclonal, directed against a C-terminal peptide	Sigma, Taufkirchen
β-catenin	Mouse, monoclonal; BD 610159	BD Transduction Labs
Erk	Rabbit, monoclonal	Cell signaling, MA
pErk	Rabbit, monoclonal, Thr202/Tyr204	Cell signaling, MA
JNK1	Mouse, polyclonal, directed against N-terminus	Santa Cruz, USA
pJNK1/2	Goat, polyclonal, detects pJNK1/2/3	Santa Cruz, USA
MEK1/2	Rabbit, monoclonal	Cell signaling, MA
pMEK	Rabbit, monoclonal, Ser217/S221	Cell signaling, MA
$p27^{Kip1}$	Mouse, monoclonal; recognizes the full length p27 protein	BD Transduction Labs
PLK-1	Rabbit, monoclonal	Cell signaling, MA
PRKX	Rabbit, polyclonal, directed Against C-terminus	Abcam, UK

TTBK2	Mouse, polyclonal	Abcam, UK
Tubulin	Mouse, monoclonal; ascites	Sigma, Taufkirchen

II.4.2. Secondary antibodies

For immunoblot analysis corresponding secondary antibodies conjugated with horseradish peroxidase (HRP) were used.

Antibody	**Dilution**	**Origin**
Goat anti-mouse-HRP	1:10.000	Sigma, Taufkirchen
Goat anti-rabbit-HRP	1:50.000	Bio-Rad, München
Goat anti-goat-HRP	1:25.000	Jackson ImmunoResearch Labs, USA

II.5. Oligonucleotides

II.5.1. RT-PCR Primers

AKT1 fwd	5´ AACCGCGTCCTGCAGAACTCCA 3´
AKT1 rev	5´ CGGGGGCCAGGTACTCAGGTGT 3´
AKT3 fwd	5´ AATGGGCAGTGAAGGCTAAGAACC 3´
AKT3 rev	5´ TAACCCCCGTCAGTCCCAGTG 3´
CHUK fwd	5´ TACAGAAGAGCCCCTATGGAAGACG 3´
CHUK rev	5´ CTACAAGGGACCGGGCAGAACTC 3´
CSK fwd	5´ AGGGCGGGCTCTACATCGTCACTG 3´
CSK rev	5´ CCGTCGGGGGCATCCATCTTGTAG 3´
EPHB4 fwd	5´ GTGGCCGGTAGCTGCGTGGTGGAT 3´
EPHB4 rev	5´ GGGGCCGGGGTCAAAAGTCAGGTC 3´
GAPDH fwd	5’ ACCACAGTCCATGCCATCAC 3’
GAPDH rev	5’ TCCACCACCCTGTTGCTGTA 3’
GADD45B fwd	5´

GADD45B rev	5´
ICK fwd	5´ GCAGCTCGGGGATGGAACCTACG 3´
ICK rev	5´ TTCTCGGGCCAAACCAAAGTCTG 3´
IKBKB fwd	5´ GCCCCAGCCCCGCCTTCC 3´
IKBKB rev	5´ CCTTCCCGCAGACCACAGCAGTTC 3´
IKBKE fwd	5´ GTACAAGGCCCGCAACAAGAAAT 3´
IKBKE rev	5´ GCACCGCCCGCTCATACAT 3´
IKBKG fwd	5´ GAGGAGGCCGAGCAGCACAAGAT 3´
IKBKG rev	3´ GCACTGGCCGGCCCTACTCAATG 3´
IRAK1 fwd	5´ GAGCCCGCCCCTTTCCGTTTTG 3´
IRAK1 rev	5´ GGCTGGGGCTGTCCTGATGTAGA 3´
MAP3K11 fwd	5´ CTCCACCCCCTGCGCAAAAAGACC 3´
MAP3K11 rev	5´ CACCCCCGCTGCCACTGCCATTC 3´
MAP3K14 fwd	5´ CCACCGCCGGCTCCACACTGC 3´
MAP3K14 rev	5´ GGCTCGCCCACCCCTTCTGACC 3´
PIM1 fwd	5´ GTCGCCGGGGCCCAGCAAATAG 3´
PIM1 rev	5´ CACAGCCGGAGTCCCCACAGAAGG 3´
PLAUR fwd	5´ AACTCTGGCCGGGCTGTCACCTAT 3´
PLAUR rev	5´ TTCATGGGGCCTCGGCAGTCAAT 3´
PRKX fwd	5´ GCACCACGGGGCTCTTCTACTCTG 3´
PRKX rev	5´ CACATCATTCGCCCCGTTCTTCAT 3´
RSK4 fwd	5´ GCGAGCAGCGGCGAGGTAA 3´
RSK4 rev	5´ CAAGGGCCAGTTCTGCGAGGTAG 3´
SPHK1 fwd	5´ CCGGGAACTGGGCCACTTGTCG 3´
SPHK1 rev	5´ GCCGCCCGCTGGATCCATAACCTC 3´
STK39 fwd	5´ GGCCCACCCAATGCTAATGAAGA 3´
STK39 rev	5´ GGGGGTGGGAGGAAATGGGCAGAA 3´
TTBK2 fwd	5´ GCGGAGCCGGCAGCAGCAGTAA 3´
TTBK2 rev	5´ AGCCCCCACCCCCAATCTTTCTCA 3´

TYRO3 fwd	5´ TGCCTGCAGCCCCCTTCAACA 3´
TYRO3 rev	5´ AGGGGCCTCGGGGATCACTTCTTC 3´
ULK1 fwd	5´ GGCCTCGGCGTCCCCCAGTCTC 3´
ULK1 rev	5´ CCTCACCCGCCCGCCACACG 3´
WNK2 fwd	5´ GGCCCCCTCCTCCCCTCCTGTGAC 3´
WNK2 rev	5´ GGCCCTTCCCCATCCGACTCC 3´
WNK4 fwd	5´ TTCGGCGCCCTGCTCTTCCTCA 3´
WNK4 rev	5´ CTTCCCGCTCCCGCCGCCCGCTCTA 3´

II.5.2. siRNAs

NAME	Catalog number	Company
CHUK/IKBKA	L-003473-00-0005	Thermo scientific
IKBKB	L-003503-00-0005	Thermo scientific
IKBKE	L-003723-00-0005	Thermo scientific
IKBKG	L-003767-00-0005	Thermo scientific
JNK1	L-003514-00-0005	Thermo scientific
JNK2	L-003515-00-0005	Thermo scientific
MAP2K7	s11182, s11183, s11184	Abcam
MAP3K11	J-003577-09/10/11	Thermo scientific
MAP3K14	J-003580-15/16/17	Thermo scientific
PRKX	s224410, s224411, s11195	Abcam
TTBK2	s44812, s44813, s44814	Abcam
RSK4	s223751, s223752, s223753	Abcam
SPHK1	J-004172-11/12/13	Thermo scientific

II.5.3. Enzymatic amplification of DNA by polymerase chain reaction (PCR)

Pfu Polymerase (MBI Fermentas) was used for long and accurate cDNA amplification:

1 µl template cDNA, 1-10ng
1 µl "forward" oligonucleotide, 10pmol/µl
1 µl "reverse" oligonucleotide, 10pmol/µl
5 µl 10x PCR buffer
5 µl 20mM $MgCl_2$
5 µl dNTP-Mix, 2.5mM each
1 µl Pfu DNA Polymerase (2.5U/µl)
ad 50µl H_2O

PCR reactions were carried out using an automated thermal cycler (Eppendorf). The following standard protocol was adjusted to each specific application:

3 minutes 95°C (initial denaturation)

<u>30 cycles:</u>

1 minute 95°C (denaturation)
1 minute Tm (hybridization at appropriate temperature)
5 minutes 72°C (extension)

PCR products were subsequently purified with QIAquick Gel Extraction Kit or directly purified using the PCR Purification Kit.

II.5.4. cDNA-array hybridization

Plasmids were expressed in *E. coli* DH5α bacterial cells and plasmid cDNA was extracted using QIAGEN Plasmid Maxi Kits (QIAGEN GMBH, Germany) following the manufacturers´ protocols. The cDNA-plasmids were denatured at 95 °C and incubated with 0.35M NaOH and 0.3M NH_4O acetate and stained with bromo-phenol (0.1 M). The denatured cDNA was printed on Hybond-N+ nylon transfer membranes (Amersham Pharamacia Biotech, GE Healthcare) using the MicroGrid II automated microarrayer with connected TAS

suite (Genomic Solutions®, version: 4.00.2). The printed nylon membranes were incubated under vacuum conditions at 80 °C for 90 minutes. The cDNA was synthesized by using 5 µg extracted total RNA, oligo-dT Primers (K1, SMART; Clontech Inc., USA) and Avian Myeloblastosis Virus reverse transcriptase (AMV; Roche Applied Science, Germany).
The cDNA labeling was assessed by using the Megaprime Labeling Kit (GE Healthcare) with deoxyadenosine 5'-triphosphate, [alpha-33P]- 10 mM Tricine (pH 7.6), 3000 Ci (111 TBq)/mmol (GE Healthcare/PerkinElmer) as the radioactive label and following the manufacturer´s protocol. Hybond-N+ filters were pre-hybridized in a 10 ml solution of 5 % sodium chloride and sodium citrate buffer (SSC) containing 100 µg/ml yeast tRNA human Cot-1 DNA (Invitrogen) during 16 h in a roller oven at 65 ° C. Filters were then washed under stringent conditions. A phospho-imager system (Fuji BAS 1000; Fuji) was used to quantify the hybridization signals. Average values for each slot were calculated using the formula: $A = (AB - B) \times 100/B$; [*A*, final volume; *AB*, intensity of each slot signal (pixel/mm2); *B*, background (pixel/mm2)].

II.5.5. Transfection of siRNAs using Lipofectamin RNAiMAX®

The cells were seeded with equal cell numbers in 6-Well plates and transfected with 40 pmol siRNA and 5 µl lipofectamine RNAiMAX (Invitrogen, SKU 13778-075) following the manufacturers´ protocols. After 6 h, normal medium lacking antibiotics and containing twice the amount of FCS was added. After 24 h, medium was changed to normal medium only lacking antibiotics. To determine the knock-down efficiency, semi-quantitative RT-PCR was performed. To determine the impact of reduced gene-expression on Sunitinib induced apoptosis, cells were cultured in the presence of Sunitinb (4 µM to 16 µM - depending on the LD_{50}-values of the respective cell line) for 72 h. The apoptotic rate was monitored according to Nicoletti's procedure [153]. To measure the influence of reduced gene-expression on Sunitinib anti-migratory effects, transfected cells were serum starved in medium containing 0.1% FCS for 24 h. Then, cells (25,000) were seeded onto a membrane with 8 µm pores of a modified Boyden chamber (Schubert and Weiss) containing 500 µL serum-free medium; 10 % FCS served as chemo-attractant. Next, cells were treated with 5 µM Sunitinib for 12h. Migration was evaluated visually and via crystal-violet staining with subsequent photometric analysis.

II.5.6. Stimulation of cells

Cells were seeded in cell culture dishes of appropriate size and grown overnight to about 80% confluence. After serum-starvation for 24 to 48 h cells were stimulated with appropriate growth factors, washed with cold PBS and then lysed for 10 minutes on ice. In some cases cells were transfected 24 h after seeding followed by serum-starvation for one day and subsequent stimulation as indicated above.

II.6. Methods of Biochemistry and Cell Biology

II.6.1. Lysis of cells with Triton X-100 lysis buffer

Cells were washed with cold PBS and then lysed for 10 minutes on ice in buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 1 % Triton X-100, 1 mM EDTA, 10 % glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 10mM sodium fluoride, 1mM phenyl-methylsulfonyl fluoride, and 10 µg/mL aprotinin. Lysates were precleared by centrifugation at 13000 rpm for 10 minutes at 4 °C.

II.6.2. Determination of total protein concentration in lysates

The overall protein concentration was determined using the Micro BCA Protein Assay Kit (Pierce, Sankt Augustin) according to the supplied standard protocol.

II.6.3. SDS-polyacrylamide-gelelectrophoresis (SDS-PAGE)

SDS-PAGE was conducted as described previously (Sambrook et al., 1990). The following proteins were used as molecular weight standards:

Protein	**MW (kD)**	**Protein**	**MW (kD)**
Myosin	205	Ovalbumin	42.7
ß-Galactosidase	116.25	Carboanhydrase	29
Phosphorylase b	97.4	Trypsin-Inhibitor	21.5
BSA	66.2	Lysozym	14.4

II.6.4. Transfer of proteins on nitrocellulose membranes

For immunoblot analysis proteins were transferred to nitrocellulose membranes (Gershoni and Palade, 1982) for 3 hours at 0.8 mA/cm2 using a "Semidry"-Blot device in the presence of Transblot-SD buffer. After transfer, the proteins were stained with Ponceau S (2 g/l in 2 % TCA) in order to visualize and mark standard protein bands. Subsequently, the membrane was destained in water.

II.6.5. Coomassie staining of polyacrylamide gels

Polyacrylamide gels were stained with Coomassie-brilliant-blue G-250 using a colloidal staining method. In brief, gels were fixed in 12 % TCA solution for one hour and stained thereafter over night in Coomassie-brilliant-blue solution (0.1 % Coomassie-brilliant-blue G-250, 2 % H_3PO_4, 10 % $(NH_4)_2SO_4$, 20 % methanol). Destaining was performed in 25 % methanol for 2 h. For drying, gels were fixed between two cellophane foils and dried in a fan heater.

II.6.6. Immunoblot detection

After electroblotting the transferred proteins are bound to the surface of the nitrocellulose membrane, providing access for reaction with immunodetection reagents. Remaining binding sites were blocked by immersing the membrane in 1x NET, 0.25 % gelatine or 5 % milk, TBST for at least 4 h. The membrane was then probed with primary antibody overnight at 4 °C. Antibodies were diluted 1:500 to 1:2000 in NET, 0.25 % gelatine or 1 % BSA, TBS-T. The membrane was washed 3x 20 min in 1x NET, 0.25 % gelatine or TBS-T, incubated for 1 hour with secondary antibody and washed again as described above. Antibody-antigen complexes were identified using horseradish peroxidase coupled to the secondary anti-IgG antibody. Luminescent substrates were used to visualize peroxidase activity. Signals were detected with X-ray films. Membranes were stripped of bound antibody by shaking in strip-buffer for 1 hour at 50 °C. Stripped membranes were blocked and reprobed.

II.6.7. RNA isolation and RT-PCR analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden) and reverse transcribed using AMV Reverse Transcriptase (Roche, Mannheim). 2-10 µg of RNA and 1µl of random primer in a volume of 10 µl were incubated for 2 min at 68 °C, followed by 10 minutes incubation at room temperature. After addition of 0.5 µl RNase inhibitor, 4 µl 5x AMV RT buffer, 4µl dNTPs (2.5 mM each) and 1µl AMV RT the volume was adjusted to 20 µl. The reaction mix was incubated at 42 °C for 1 h and thereafter cDNA was purified using the Qiagen PCR purification kit (Qiagen, Hilden). PuReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ) and 1 µl RT-PCR products were used for PCR amplification according to the manufacturers´ recommendations. PCR products were subjected to electrophoresis on 1.5-2 % agarose gels and DNA was visualized by ethidium bromide staining.

II.6.8. Proliferation assay (MTT)

2.000 or 4.000 cells were seeded in 96- or 48 well plates and allowed to attach to the plates over night. The cells were then treated with Sunitinb or Sorafenib concentrations between 1.25 µM and 15 µM for 72 h. At 72 h, the tetrazolium dye MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolim bromide; thizolyl blue, Sigma, Taufkirchen) was added to each well to a final concentration of 1 mg/ml MTT. Plates were incubated in the presence of MTT for 4 h. Mitochondrial dehydrogenase activity reduces the yellow MTT dye to a purple formazan, which was solubilized (DMSO, acidic acid, SDS) and absorbance was read at 570 nm on a micro-plate reader.

II.6.9. Apoptosis assay

To analyze Sunitinib or Sorafenib sensitivity of the cell lines, equal cell numbers were seeded into 12-well plates and allowed to attach to the plates over night. The cells were then treated with Sunitinb or Sorafenib concentrations between 2.5 µM and 20 µM for 72 h and apoptosis was determined according to Nicoletti's procedure [153]. BD CellQuest Pro software (Becton Dickinson) for subG_1 peak detection was used to determine the extent of the apoptotic effect.

Statistical evaluation of the half maximal lethal dose (LD_{50}) values was assessed using SigmaPlot (Systat Software, Inc., version 10).

II.6.10. Establishment of Sunitinib and Sorafenib desensitized sub-populations

Drug resistant cell lines were obtained by applying a Sunitinib or Sorafenib based selective pressure. Cell lines were periodically treated with 20 µM Sunitinib or Sorafenib for 72 h to 96 h to induce cell death. At 72 h or 96 h, the drug selected cells were re-cultured in normal medium. This process was repeated two to seven times, depending on the desensitization progress. The drug sensitivity was monitored by performing aforementioned proliferation- and apoptosis assays.

II.6.11. Statistical analysis

For gene-expression analysis, densitometric data of at least three individual cDNA-gene array experiments were averaged. In order to identify Sunitinib or Sorafenib sensitivity related expression patterns regarding drug resistance, we performed a "Significance Analysis of Microarrays" (SAM) [154] using the Multi Experiment Viewer (SAEDD et al. 2003) with 3 different groups in dependence of the total cell population; Group 1: parental cell lines; Group 2: Sunitinib or Sorafenib desensitized sub-populations with a high degree of desensitization and; Group 3: Sunitinib or Sorafenib desensitized sub-populations with a low or no degree of desensitization. Subsequently, we used hierarchical clustering [155] for cluster analysis. P-values were obtained by comparing the gene-expression levels of each Sunitinib or Sorafenib desensitized- with the respective parental cell line. For primary drug response gene-expression, data were hierarchical clustered (Pearrson correlation) in correlation to the primary drug sensitivity of the cell lines (1 dimensional clustering).

Student's *t*-test was used to compare data between two groups. Values are expressed as mean ± standard deviation (s. d.) of at least triplicate samples. $P < 0.05$ was considered statistically significant.

III. Specific Aims

Advanced insights into cancer biology led to the development of the two small multi-target kinase inhibitors Sunitinib and Sorafenib. While both drugs have demonstrated clinical benefit for various tumor indications, the underlying mechanisms are still poorly understood. Moreover, drug resistance emerges as a critical drawback for Sunitinb or Sorafenib therapy, resulting in poor overall survival of patients. Our aim in this thesis was to advance the knowledge of the cytostatic and cytotoxic effects of both drugs in a comparative manner.

In order to systematically analyze and compare these effects, a drug defined correlation of transcriptome and drug dependent phenotype should be used.

A second comparison should be focused on drug specific resistance formation. To achieve this goal, a model system of Sunitinib and Sorafenib desensitized cell lines should be established. Subsequent gene-expression analyses in correlation to the degree of drug desensitization should be applied for the discovery of potential resistance markers or alternative targets for therapeutic interventions.

Since Sorafenib but not Sunitinib was developed as a Raf inhibitor, a third comparison should be focused on the Ras/Raf/MEK/ERK pathway. Next to the effects of both drugs on this pathway, differences to the respective drug insensitive cell lines would be of particular interest.

IV. Results

IV.1. Cell response upon Sunitinib and Sorafenib treatment

IV.1.1. Induction of apoptosis by Sunitinib and Sorafenib

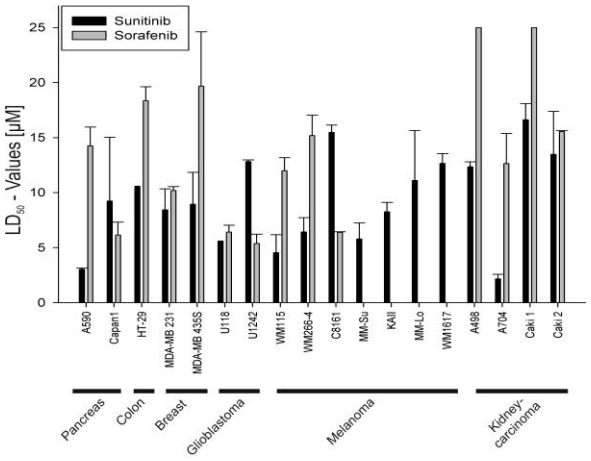
Today, Sunitinib and Sorafenib are in multiple clinical trials for various cancer indications, suggesting that their anti-cancer effects might be tissue independent. Consequently, we decided to establish a tissue in- but drug dependent cell line model system to study and compare the effects of both drugs. Thus, we used a model system displaying a broad variety of origin tissues and sensitivities for either Sunitinib or Sorafenib, which would allow us to correlate drug sensitivity and changes of the transcriptome. The drug sensitivity characterization was based on apoptosis induction and inhibition of proliferation. These effects were monitored by using Propidium Iodide- (PI) and MTT- assays, displaying the half maximal lethal dose- (LD_{50}-) or inhibition coefficient- (IC_{50}-) values after Sunitinib or Sorafenib treatment for 72h.

We tested 18 cell lines and observed a dose- and time-dependent apoptosis inducing effect of Sunitinib. However, the analyzed cell lines displayed a wide range of Sunitinib sensitivities regarding apoptosis induction while no correlation of sensitivity and the cell line tissue origin was observed. The cell lines A704 (kidney), A590 (pancreas), WM115 (melanoma) and U118 (glioblastoma) showed the highest sensitivity to Sunitinib treatment displaying LD_{50}-values of 2.2 ± 0.4 µM, 3.0 ± 0.1 µM, 3.6 ± 0.9 µM and 5.5 ± 0.2 µM. Mediocre Sunitinib sensitivity was demonstrated by the cell lines WM266-4 (melanoma) and A498 (kidney) with LD_{50}-values of 6.4 ± 1.3 µM and 12.4 ± 0.4 µM. Similar LD_{50}-values were observed for the breast cancer cell lines MDA-MB231 and MDA-MB435S (8.4 ± 1.9 µM and 8.9 ± 2.9 µM. The highest LD_{50}-values were monitored for the melanoma cell line C8161 (15.5 ± 0.7 µM) and, interestingly, for the kidney carcinoma cell lines Caki2 (13.5 ± 3.9 µM) and Caki1 (16.6 ± 1.5 µM).

Like Sunitinib, Sorafenib induced apoptosis in a dose-dependent manner with varying potencies in the respective cell lines. As for Sunitinib, the different potencies did not correlate with the cell line tissue origin. On one hand, the cell lines demonstrated similar LD_{50}-values for either Sorafenib or Sunitinib like observed for U118 or MDA-MB231. On the other hand however, different cell lines favored either Sorafenib or Sunitinib concerning apoptosis

induction. The differences in apoptosis induction were most pronounced in the melanoma cell lines WM115, WM266-4 and C8161. Sorafenib displayed the highest sensitivity for C8161 (6.4 ± 0.6 µM), followed by WM115 (12.0 ± 1.2 µM) and WM266-4 (15.5 ± 1.9 µM), while Sunitinib contrarily demonstrated the highest sensitivity for WM115 and 266-4 followed by C8161. Other examples are the cell lines U1242, which was more sensitive to Sorafenib-, and A704 that was more sensitive to Sunitinib treatment. Interestingly, the four kidney cell lines A498, A704, CAKI1 and CAKI2 demonstrated rather low sensitivities against Sorafenib, despite the already mentioned clinical benefit for RCC patients. The LD_{50}-values for Sunitinib and Sorafenib are depicted and listed in *Figure 10 A,B.*

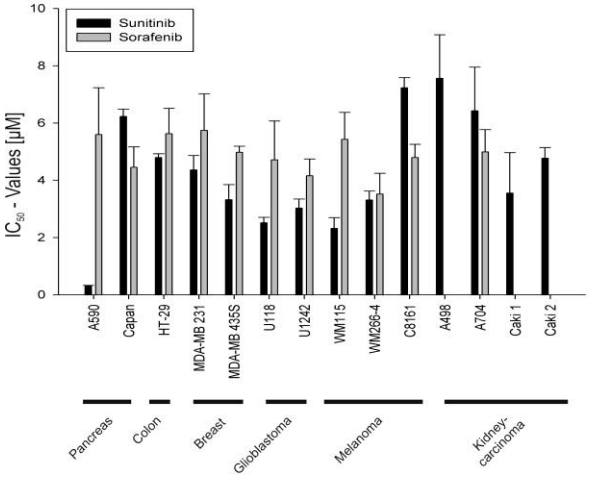
A



B

Cell line	Sunitinib LD_{50}-value [µM] Average	stdev	Sorafenib LD_{50}-value [µM] Average	stdev
A590	3.0	0.1	14.3	1.7
Capan1	9.2	5.8	6.1	1.2
HT-29	10.7	0.1	18.4	1.3
MDA-MB 231	8.4	1.9	10.2	0.4
MDA-MB 435S	8.9	2.9	19.7	4.9
U118	5.5	0.2	6.4	0.6
U1242	12.8	0.1	5.4	0.8
WM115	4.5	1.6	12.0	1.2
WM266-4	6.4	1.3	15.2	1.9
C8161	15.5	0.7	6.4	0.1
MM-Su	5.8	1.5	n. d.	n. d.
KAII	8.3	0.8	n. d.	n. d.
MM-Lo	11.1	4.5	n. d.	n. d.
WM1617	12.7	0.9	n. d.	n. d.
A498	12.4	0.4	25.0	0.0
A704	2.2	0.4	12.6	2.7
Caki 1	16.6	1.5	25.0	0.0
Caki 2	13.5	3.9	15.6	0.1
Avergage	9.3		13.7	

C



D

Cell line	Sunitinib IC_{50}-value [µM] Average	stdev	Sorafenib IC_{50}-value [µM] Average	stdev
A590	0,3	0,0	5,8	1,4
Capan1	6,2	0,3	4,5	0,7
HT-29	4,8	0,1	5,6	0,7
MDA-MB 231	4,4	0,5	5,7	1,3
MDA-MB 435S	3,3	0,5	5,0	0,2
U118	2,5	0,2	4,7	1,4
U1242	3,0	0,3	3,9	0,7
WM115	2,3	0,4	5,4	0,9
WM266-4	3,3	0,3	3,5	0,7
C8161	7,2	0,3	4,8	0,5
A498	7,6	1,5	n.d.	n.d.
A704	6,4	1,5	5,0	0,8
Caki 1	3,5	1,4	n.d.	n.d.
Caki 2	4,8	0,4	n.d.	n.d.
Avergage	4,2		4.9	

Figure 10: Sensitivity characterization of cancer cell lines upon Sunitinib or Sorafenib treatment. Sunitinib and Sorafenib demonstrated varying, cell tissue origin independent, potencies to induce apoptosis and inhibit proliferation. By trend, the cell lines displayed higher sensitivity against Sunitinib- than against Sorafenib treatment as depicted and listed in the bar chart (left panel) and table (right panel). Black bars: Sunitinib-; grey bars: Sorafenib treatment; Abbreviations: stdev, standard deviation.

IV.1.2. Inhibition of proliferation by Sunitinib and Sorafenib

In all tested cell lines, either Sunitinib or Sorafenib inhibited cell proliferation in a dose-dependent manner. In general, the inhibition of proliferation by both drugs occured at lower concentrations than the respective induction of apoptosis. The Sunitinib IC_{50}-values demonstrated a wide range of sensitivity, that displayed no correlation to the cell tissue origin. The cell lines A590, WM115 and U118 were strongest inhibited by Sunitinib (IC_{50}-values $\leq$ 2.5 µM), while WM266-4, MDA-MB 435S, CAKI1, CAKI2, MDA-MB231 and HT-29 displayed less inhibition (IC_{50}-values 3.3 – 4.8 µM). Lower sensitivity was observed for CAPAN1, C8161 and A498 (IC_{50}-values $\geq$ 6.2 µM). In most cases, the potency of Sunitinib to inhibit proliferation as a degree of sensitivity correlated with the potency to induce apoptosis.
For Sorafenib, no such vast discrepancy of the IC_{50}-values, which ranged in between 3.5 and 5.8 µM, was observed. Consequently, no correlation of cell tissue origin and Sorafenib sensitivity was determined. Additionally, the Sorafenib IC_{50}-values were by trend higher than the respective Sunitinib values ($IC_{50}^{Average}$: 4.9 µM or 4.2 µM), which was also observed for the LD_{50}-values ($LD_{50}^{Average}$: 13.7 µM or 9.3 µM). Nevertheless and again in line with LD_{50}-values, C8161 and Capan1 displayed higher Sorafenib sensitivity (4.8 ± 0.7 µM and 4.5 ± 0.7 µM, when compared with Sunitinib (7.2 ± 0.3 µM and 6.2 ± 0.3 µM). Interestingly, the kidney carcinoma cell line A704 demonstrated by trend a lower IC_{50}-value for Sorafenib than Sunitinib (5.0 ± 0.8 µM compared to 6.4 ± 1.8 µM), which is in contrast to the respective apoptosis inducing effect. The IC_{50}-values for Sunitinib and Sorafenib are depicted and listed in *Figure 10 C,D.*

IV.2. Cell response upon Sunitinib or Sorafenib treatment

IV.2.1. Workflow

Today, next to their major kinase targets, multiple other kinases are known to be directly inhibited upon Sunitinib or Sorafenib treatment [76-77]. As a consequence of these inhibition networks, both drugs exhibit their anti-cancer effects, i.e. apoptosis induction and anti-angiogenesis. However, with the increasing number of direct Sunitinib or Sorafenib kinase targets, the underlying mechanisms gain a new level of complexity. To advance the

knowledge about these cellular responses, we aimed to identify altered gene-expression patterns upon drug treatment, presuming that these indirect effects might be the best option for a comparative analysis of both drugs.

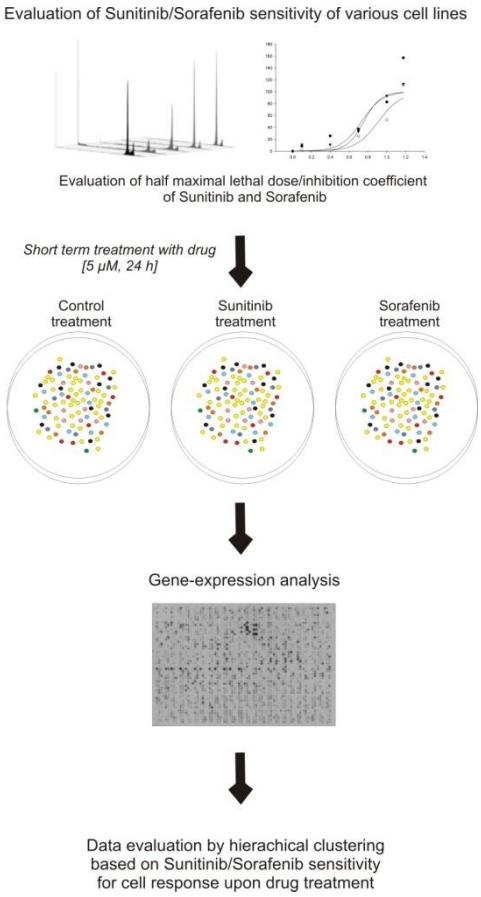


Figure 11: Cell response upon Sunitinib/Sorafenib treatment workflow. Cell lines, displaying a wide range of Sunitinib or Sorafenib sensitivities were treated with either Sunitinib or Sorafenib. Subsequently, gene-expression analysis was used to identify sensitivity based Sunitinib- or Sorafenib response patterns.

In order to identify drug dependent but tissue origin independent cell responses, we used a model system, which had to meet several requirements: All cell lines had to respond to both drugs in a dose-dependent manner. Moreover, the model system had to display a wide range of Sunitinib and Sorafenib sensitivity. Further it had to consist of various cell lines from various tissue origins. Finally, to compare the cell response of Sunitinib and Sorafenib, it had to consist of cell lines that display higher, lower or similar sensitivity for either of both drugs. All these requirements were met by the cell lines of the model system introduced in *chapter IV.1.1*. These cell lines were treated with 5 µM of either Sunitinib or Sorafenib. This drug

concentration was supposed to display discriminating gene-expression patterns in correlation with the sensitivity phenotype, while preserving sound mRNA levels for all cell lines of the model system, a requirement for comparative gene-expression analyses. The cell lines were treated for 24 h, which marks the time-point, at which the cytotoxic and cytostatic effects of both drugs became detectable (data not shown). Subsequently, we performed gene-expression analysis using in house cDNA arrays. The cDNA-arrays mainly consisted of kinases, phosphatases and genes functioning in cell-cycle regulation. Transcripts with altered expression in correlation to primary drug sensitivity expression and potential relevance in cytotoxic effects, such as those implicated in angiogenesis, metastasis, proliferation and apoptosis or survival, were of particular interest. To identify those genes, we correlated the mathematic ratios of the gene-expression of drug treated and untreated cell lines with the phenotypic drug sensitivity by performing one-dimensional hierarchical clustering.

IV.2.2. Cell response upon Sunitinib treatment

To identifiy the underlying mechanisms of Sunitinib´s ability to inhibit proliferation, induce apoptosis and prevent angiogenesis, we performed gene-expression analysis as described above. Based on one-dimensional hierarchical kmeans clustering, the expression analysis revealed a correlation of Sunitinib sensitivity and altered expression for 46 out of 1276 genes. The 46 genes could be subdivided into four gene-cluster representing two major groups (A and B, *Figure 12*): The first three clusters (Group A, 37 genes) consisted of genes, which were downregulated in primary Sunitinib high and moderate sensitive-, and upregulated (LD_{50}-values $\leq$ 12.5 µM) in primary insensitive (LD_{50}-values $\geq$ 12.5 µM) cell lines upon Sunitinib treatment. A fourth cluster displayed an antagonistic altered gene-expression pattern, demonstrating upregulated genes in the primary Sunitinib sensitive-, and downregulated genes in the insensitive cell lines. The genes of Group A could be further subdivided into several signaling cascades: Two genes, SFRS protein kinase (SRPK1) and cell division cycle 2-like 5 (CDC2L5) are involved in differential splicing of the VEGF and thus angiogenesis, in which integrin beta 3 (ITGB3) is also implicated. Since these genes are downregulated upon Sunitinib treatment, a decrease of angiogenesis can be presumed. Checkpoint kinase 1, ataxia telangiectasia mutated (CHK1, ATM) or tousled-like kinase 2 (TLK2) are moreover involved in chromosome stability and assembly. Other genes like LIM domain kinase 1 (LIMK1), pyruvate dehydrogenase kinase 3 (PDK3) and SRPK1 are

associated with drug resistance. Besides, the downregulation of I-kappa-B kinase-alpha (IKKA), ATM, Protein kinase C mu type (PKD1), receptor-interacting serine-threonine kinase 4 (RIPK4), mitogen activated kinase 8 (MAPK8) and caveolin (CAV1) indicated that NFκB-signaling plays a major role in the underlying mechanisms of Sunitinib´s cytotoxic effects. In this regard it is noteworthy, that expression of PLAUR, which is promoted by NFκB-signaling, was decreased in all Sunitinib sensitive cell lines but U118 (not visualized in heatmap). Other prominent pathways, in which genes of Group A are involved, are the WNT- and the AKT/PI3K pathway (CSNK1E, BMPR1A, MAPK8, FRAP1 and AKT).

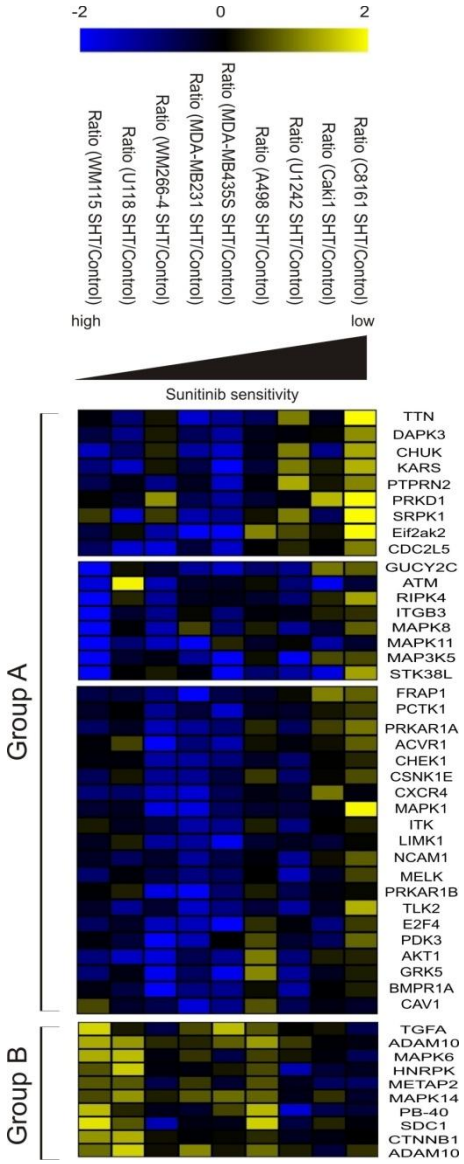


Figure 12: Sunitinib sensitivity based gene-expression analysis upon Sunitinib treatment. Sunitinib treatment resulted in a changed transcriptome. Correlative analysis of Sunitinib sensitivity and altered gene-expression revealed 46 genes, which are mainly associated with the fields of survival/apoptosis, angiogenesis and diabetes. Color code: Gene-expression scale from log2 (-2) to log2 (2), yellow to blue.

Interestingly, genes that are predominantly associated with diabetes (PTPRN2, GRK5, PKD1) were also members of Group A. The monitored expression-changes ranged between a 10-fold in- or decrease. However, most of the identified genes displayed gene-expression fold-changes of approximately two to three. In this regard, it is noteworthy, that family members of the aforementioned kinases are often regulated in a similar fashion. For example, all members of the AKT family were downregulated in WM115 and MDA-MB435S- and upregulated in A498 cells upon Sunitinib treatment. Another example for this observation is the PDK family. Here, PDK1/2/3 and 4 were downregulated in WM266-4, while in C8161 all four members were upregulated upon Sunitinib treatment. Next to those two examples, others gave further support for the assumption that a wide network of gene-expression alterations is required to discriminate between Sunitinib sensitivity and insensitivity.

In comparison to Group A, Group B with its antagonistic gene-expression pattern consisted of only one cluster comprising 9 genes. In Sunitinib sensitive cell lines, the upregulated expression of the metalloproteinase ADAM10, which is a prognostic marker for RCC, was observed for two different plasmids within the gene-array. Interestingly, the mitogen activated kinases MAPK6 and MAPK14, which are associated to NFκB signaling and survival, were also upregulated in primary Sunitinib sensitive cell lines. Furthermore, the heterogeneous nuclear ribonucleoprotein K (HNRPK1) together with the MAPK pathway has been associated to c-MYC regulation and the oncogenic potential of BCR/ABL [156]. Finally, it is noteworthy that β-Catenin (CTNNB1) expression was only upregulated in the most Sunitinib sensitive cell lines WM115 and U118, which provides a link to BMPR1A, CSNK1E, PRKD1 of Group A. Taken together, the genes of group B are associated to MAPK and NFκB signaling providing further support of the major role of these pathways to exhibit Sunitinib´s anti-cancer effects.

IV.2.3. Cell response upon Sorafenib treatment

To advance the knowledge regarding the mechanisms of how Sorafenib exhibits its anti-cancer effects, we performed gene-expression analysis as described above. For comparison of the cell response upon treatment with both drugs, we used the same algorithms as for Sunitinib-for the Sorafenib sensitivity based gene-expression analysis. The gene-expression analysis revealed a correlation of Sorafenib sensitivity and altered expression for 81 out of 1276 genes. Two gene-clusters responded to Sorafenib treatment with downregulation of

genes in the primary Sorafenib sensitive- and up-regulation of the respective genes in the insensitive cell lines (Group C, 39 genes). Like for Sunitinb, the switch for down- or upregulation was defined by a Sorafenib LD_{50}-value of 12-13 µM. In accordance to the comparably lesser sensitivity against Sorafenib, only U118, U1242, C8161 and MDA-MB231 displayed downregulated genes. Group D consisted of 42 genes that displayed the antagonistic pattern, i.e. enhanced expression of genes in Sorafenib sensitive and decreased expression in Sorafenib insensitive cell lines. The overexpression of tyrosine kinase-type cell surface receptor HER2 (ERBB2) and estrogen receptor alpha (ESR1) genes is a frequent characteristic of breast cancer. The matrix metalloproteinase-2 (MMP2), also characteristic for breast cancer, is involved in angiogenesis, migration and invasiveness.

These processes are moreover regulated by tyrosine kinase non-receptor protein 2 (TNK2), autocrine motility factor (GPI/AMF), which can be shedded by ADAM17, and p21 (CDKN1A)-activated kinase 4 (PAK4). Additionally, p21 protein (Cdc42/Rac)-activated kinase 4 (PAK4), PTEN induced putative kinase 1 (PINK1), cell division cycle 2 (CDC2) and the protein kinase, AMP-activated, alpha 2 catalytic subunit (PRKAA2) are known regulators of survival processes in cancer cells. PRKAA2 can modulate the activity of protein kinase C, zeta (PKCZ) and both are associated with positive regulation of NFκB-signaling. Next to NFκB-signaling, Sorafenib plays its apoptotic role also by regulating PI3K/AKT signaling. The average fold-change of downregulated genes of Group C in the Sorafenib sensitive- (0.8 x) was lower compared to the fold-change of upregulated genes in the insensitive cell lines (3.6 x), indicating that a negative modulation of the apoptotic threshold by increased genes expression in the Sorafenib insensitive cell lines might primarily trigger Sorafenib sensitivity. The transcriptional upregulation of genes was also favored over downregulated genes for the genes of Group D (average fold-change^{upregulated}: 2.1 x; average fold-change^{downregulated}: 0.7 x). The 42 genes of Group D can also be subdivided in respect to their functional cellular roles. In line with the Sorafenib induced phenotype, positive regulators of apoptosis or tumor supressors were represented by the STE20-related kinase (SLK), MAPK/ERK kinase kinase 1 (MEKK1) and LATS, large tumor suppressor, homolog 2 (LATS2). Next to these positive regulators, CAM kinase IV (CAMKIV), BCL2-like 2 protein (BCL2L2) and the fibroblast growth factor 2 (FGF2) account for negative regulators of apoptosis. Other functional subdivisions consisted of genes, which can be related to JNK - and NFκB signaling: Both, TAO kinase 3 (TAOK3) and dual specificity phosphatase 10 are negative regulators of JNK signaling.

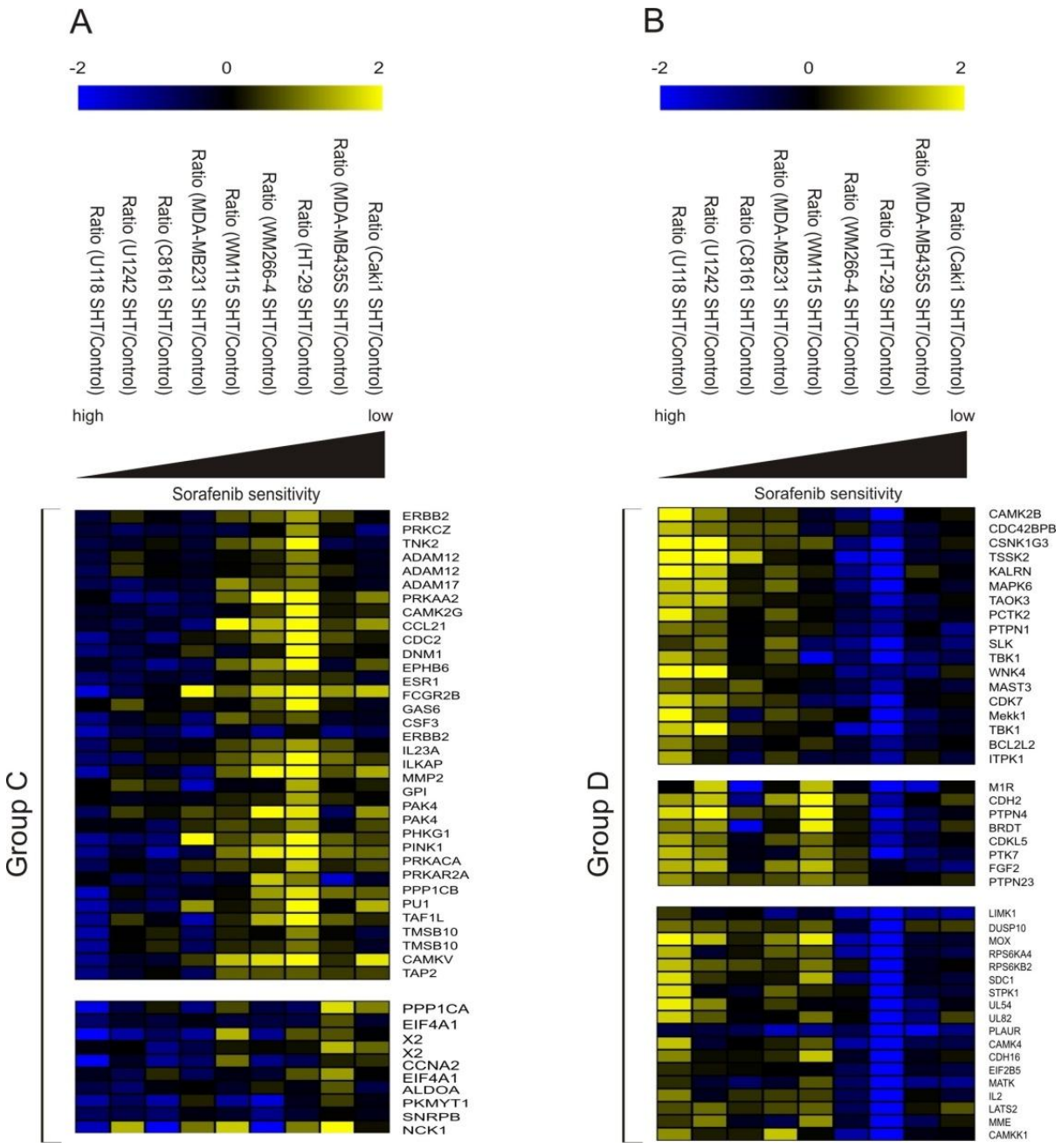


Figure 13: Sorafenib sensitivity based gene-expression analysis upon Sorafenib treatment. Sorafenib treatment resulted in a changed transcriptome. Correlative analysis of Sorafenib sensitivity and altered gene-expression revealed 81 genes, which are mainly associated to the fields of survival/apoptosis, angiogenesis and diabetes. (A) Group C consists of 39 genes, which were downregulated in primary Sorafenib sensitive- and upregulated in insensitive cell lines. (B) Group D demonstrated an antagonistic expression pattern. Color code: Gene-expression scale from log2 (-2) to log2 (2), yellow to blue.

While MAPK6 and TANK-binding kinase 1 (TBK1) are positive modulators-, megakaryocyte phosphatase (PTPN4/MEG) and MEKK1 are negative modulators of NFκB-signaling. The effects of Sorafenib on migration as well as angiogenesis are represented by FGF2, which is cleaved by membrane metallo-endopeptidase (MME), the tyrosine phosphatase HD-PTP

(PTPN23) as well as TBK1. LIMK1, which was downregulated in all analyzed cell lines but U118, positively regulates cell invasion. In contrast, N-Cadherin (CDH2) and CDC42-binding protein kinase beta (CDC42BPB), which were upregulated in Sorafenib sensitive cell lines, can trigger cell invasion. CDC42BPB, next to huntingtin-associated protein interacting protein (KALRN), PCTAIRE-motif protein kinase 2 (PCTK2), protein tyrosine phosphatase 1B (PTPN1), is moreover associated to cell skeletal reorganization. However, urokinase receptor (PLAUR/uPAR), which was downregulated in all cell lines upon Sorafenib treatment, is associated with all of the above mentioned processes, indicating its key role regarding the effects of Sorafenib. Moreover, plasminogen activator, urokinase (PLAU/uPA) gene-expression was down-regulated as well (not visualized in cluster analysis).

Altogether, Group D genes are involved in multiple cellular processes, whereas the Sorafenib sensitivity based expression alterations were mostly reflecting the Sorafenib induced phenotypes. Moreover, the genes of Group D were shown to exhibit antagonistic functions, indicating that Sorafenib sensitivity of cancer cells depends on a tightly controlled transcriptional network.

IV.2.4. RTK phosphorylation-levels upon Sunitinib and Sorafenib treatment

The treatment with either Sunitinib or Sorafenib resulted in gene-expression alterations correlating with the respective drug sensitivity of the cell lines. These changes, while indicating the modulation of the apoptotic threshold upon treatment with both drugs, were found to be highly different in a direct comparison. Both, Sunitinib and Sorafenib share a set of target kinases, while other kinases are specifically inhibited by either of both drugs.

However, for the congruently target kinases, different IC_{50}-values have been observed [77]. Thus, we postulated the distinct gene-expression patterns might be reasoned by a differential direct inhibition of kinases. Accordingly, by performing proteinarrays, we monitored the phosphorylation levels of 30 receptor tyrosine kinases (RTKs), as this protein family consists of prominent kinases like the PDGFRβ and VEGFRs that are targeted by both drugs. For this comparative analysis, we obtained the melanoma cell line WM115, as it displayed diverse sensitivities against Sunitinib (LD_{50}-value: 4.5 µM) and Sorafenib (LD_{50}-value: 12 µM). Moreover, WM115 displayed distinct changes of the gene-expression pattern upon treatment with either Sunitinib or Sorafenib. To correlate alterations of the RTK phosphorylation-levels,

WM115 cells were treated with 5 µM of Sunitinib or Sorafenib for 24 h, matching the experimental settings of the gene-expression analyses. Phosphorylation-level fold-changes were evaluated by performing phosphorylation-RTK arrays and subsequent scanning densitometry with DMSO treated WM115 cells, serving as a control. A scanning sensitivity value of 5000 [AU] was set as sensitivity minimum (*Figure 14*).

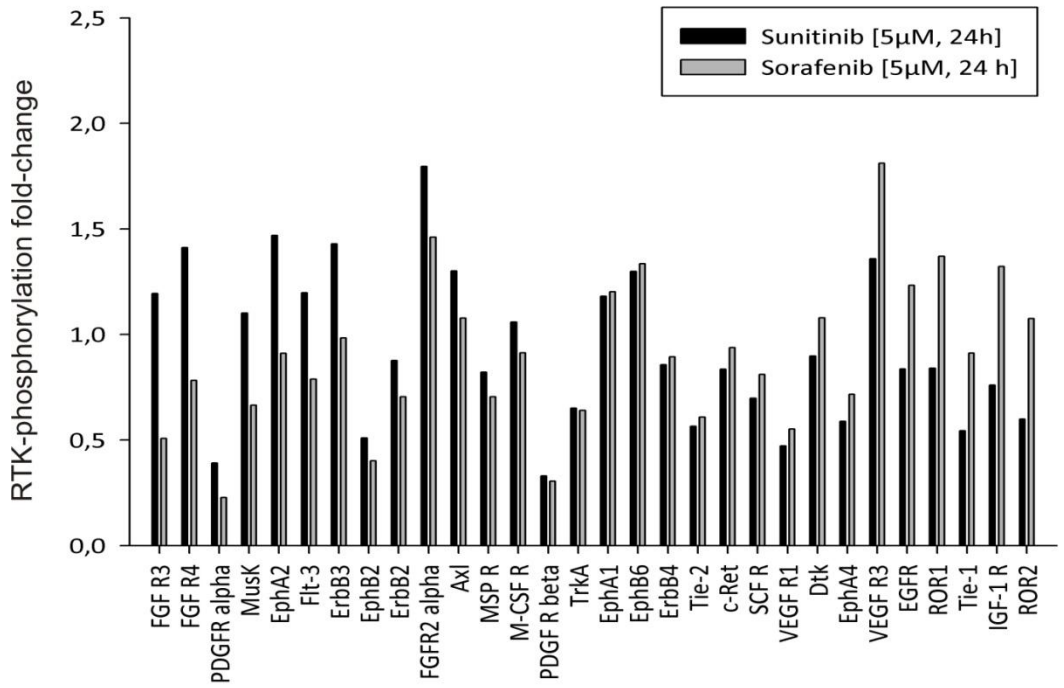


Figure 14: RTK-phosphorylation changed upon Sunitinib or Sorafenib treatment. The phosphorylation-levels of PDGFRα, PDGFRβ, ephrin receptor B2 (EphB2), neurotrophic tyrosine kinase receptor type 1 (TrkA), tyrosine-protein kinase receptor TEK (Tie-2) and VEGFR1 were reduced upon both Sunitinib and Sorafenib treatment (5 µM, 24 h). The phosphorylation-levels of receptor tyrosine kinase Tie-1 (Tie-1) and receptor tyrosine kinase-like orphan receptor 2 (ROR2) were reduced upon Sunitinib- but not Sorafenib treatment. Elevated RTK phosphorylation-levels were monitored for the fibroblast growth factor receptor 2 alpha (FGFR2α) upon Sunitinib treatment. Sorafenib treatment resulted in an increased phosphorylation of VEGFR3 and decreased phosphorylation of FGFR3. Black bars, Sunitinib treatment (5 µM, 24 h); grey bars, Sorafenib treatment (5 µM, 24h).

The treatment of WM115 with either Sunitinib or Sorafenib resulted in a decreased phosphorylation of the PDGFRα (2.6x or 4.4x), PDGFRβ (3.0x or 3.3x), ephrin receptor B2 (2.0x or 2.5x; EphB2), neurotrophic tyrosine kinase receptor type 1 (1.5x or 1.6x; TrkA), tyrosine-protein kinase receptor TEK (1.8x or 1.6x; Tie-2) and VEGFR1 (2.1x or 1.8x). Additionally, Sunitinib but not Sorafenib inhibited the phosphorylation of the receptor tyrosine kinase Tie-1 (1.9x or 1.0x; Tie-1) and receptor tyrosine kinase-like orphan receptor 2 (1.7x or 0.9x; ROR2). Stable phosphorylation-levels were displayed by FGFR3 upon Sunitinib treatment (0.8x), while Sorafenib treatment led to decreased FGFR3

phosphorylation (2.0x). Further, Sorafenib and Sunitinib treatment resulted in an increased phosphorylation of vascular endothelial growth factor receptor 3 (1.4x and 1.8x; VEGFR3). Moreover, Sorafenib treatment increased the phosphorylation-level of the fibroblast growth factor receptor 2 (1.8x; FGFR2). Interestingly, Sunitinib and Sorafenib treatment had no effect on stem cell tyrosine kinase 1 (1.2x and 0.8x; FLT-3) and only a slight effect on mast/stem cell growth factor receptor (1.4x and 1.2x SCFR/c-Kit) phosphorylation, although they both account for direct Sunitinib targets [78,157]. Other RTKs of the protein-array, like the EGFR, Axl or DTK did not display any change of their phosphorylation state upon treatment with either of both drugs (~ 1.0x).

IV.3. Sunitinib resistance

IV.3.1. Workflow

Sunitinib targets and thereby inhibits several RTKs and NRTKs [76] and competitive inhibition of these RTKs is thought interfere with crucial hallmarks of cancer, resulting in growth inhibition, prevention of invasivity and induction of apoptosis in cancer cells [3,6]. These functions are essential for the progression of RCC, GIST, pancreatic neuroendocrine- and other tumors. Because of Sunitinib´s broad spectrum of kinase inhibitory activity it appears to be relevant for the maintenance and progression of many other tumors [56,72]. Further, as a consequence of targeting multiple pathways, Sunitinib appears to be superior to single-targeted therapies, as it might restrain compensatory responses in alternative cellular signaling pathways and hence, avoid or delay the formation of drug-resistance. However, the clinical application of Sunitinib for mRCC and GIST eventually results in the progression of the tumor [44]. As a consequence, drug-resistance formation remains a key reason for bad prognosis in cancer and further understanding of resistance-mechanisms is pivotal for a significant and long lasting clinical success of these drugs. The aim of this study was to establish a molecular understanding of resistance mechanisms against Sunitinib in cancer therapy before the clinical reality of resistance formation emerges. Therefore, we reasoned that due to the instability of the cancer cell genome resistance is formed by selection of cancer cells that exhibit genetic alterations which compensate the cytotoxic or cytostatic effects of the drug. *In vitro* studies have indicated that Sunitinib sensitivity might likely be based on the genetic background of the tumor (Bairlein et al, unpublished data). Moreover, transcriptional changes in response to Sunitinib treatment correlated with Sunitinib sensitivity (Chapter IV.2.2), indicating that these changes might act as a modulator of the apoptotic threshold. Hence, we postulated that Sunitinib resistance might be based on transcriptional alterations as well.

To test this postulate, we established model-systems of parental and Sunitinib desensitized cancer cell lines from various tissues. Kidney carcinoma and melanoma cell lines, as for both indications the clinical and pre-clinical benefit of Sunitinib was shown, were of particular interest. Notably, we did not use resistant clones, as polyclonal desensitized cell lines represented the superior option to mimic Sunitinib resistance formation in patients. In order to identify drug specific- but tissue origin independent resistance mechanisms, the used model system had to meet several requirements: First, the cell lines should be derived from various

tissue origins. Second, the model system had to consist of cell lines with varying degrees of desensitization. Third, the degree of desensitization had to vary between Sunitinib and Sorafenib. Finally, the desensitized cell lines should not display morphologic changes due to the drug selection process. The degree of Sunitinib resistance was monitored by performing PI-assays to measure the cytotoxic effects of Sunitinib. Further, to determine the specificity of underlying mechanism causing Sunitinib resistance formation, we likewise established Sorafenib desensitized cell lines for a comparative analysis.

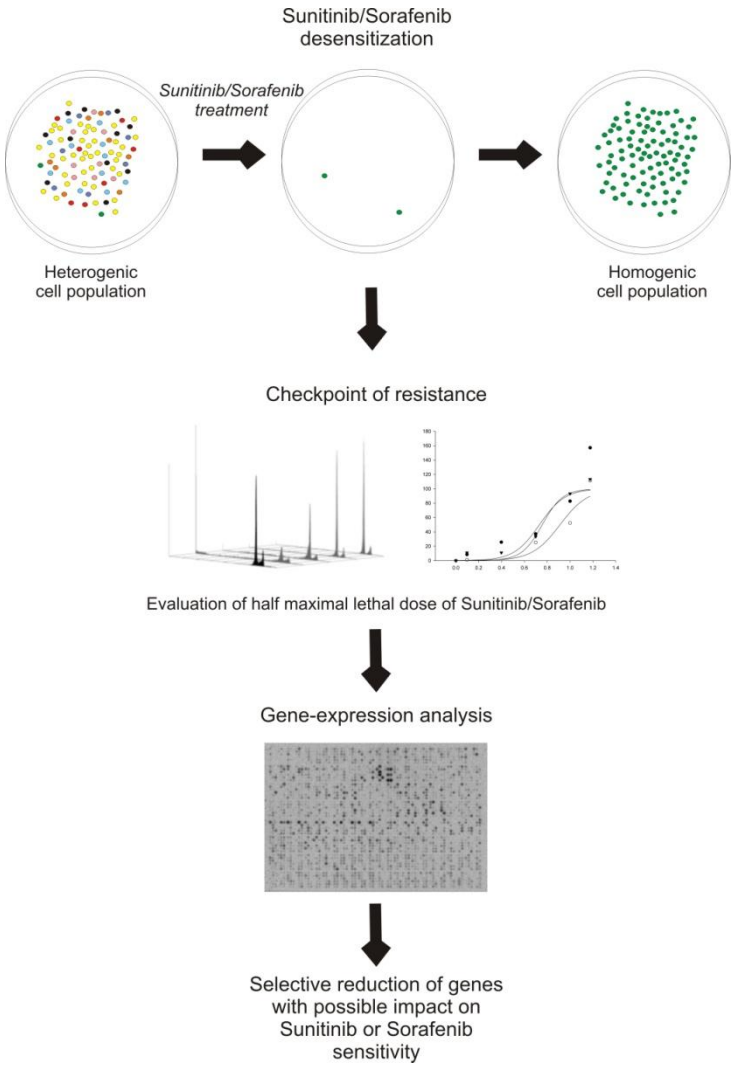


Figure 15: Mechanisms underlying Sunitinib or Sorafenib resistance workflow. Heterogenic parental cell populations were treated with either Sunitinib or Sorafenib to select for drug insensitive homogenic sub-populations. Subsequently, the selected sub-populations were tested in regard to their drug sensitivity (Checkpoint of resistance). Next, comparative gene-expression analysis of parental and drug-desensitized cell lines was performed to identify candidate genes for Sunitinib resistance formation. Finally, the expression of these candidate genes was individually reduced using siRNAs to validate their potential function in conferring drug resistance.

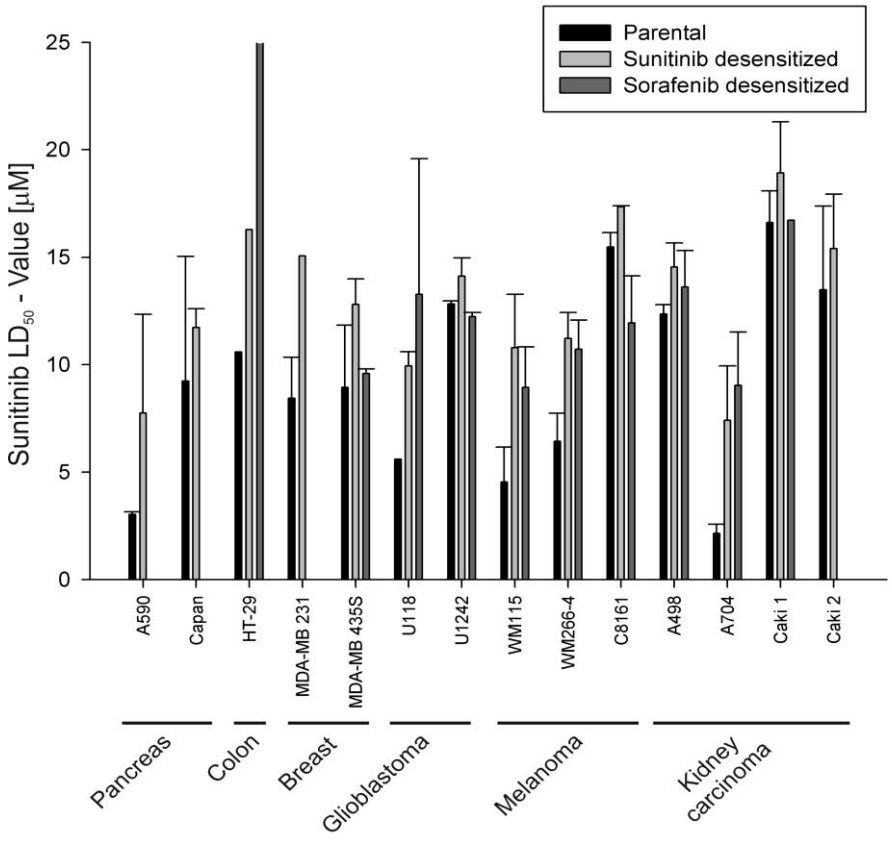
Subsequently, we determined changes in the kinome expression-profile of the desensitized versus the parental cell lines. Upregulated genes were then validated regarding their potential to confer drug resistance by individually downregulating them in the parental and resistant cancer cells. The workflow to identify drug-resistance associated genes is depicted in *Figure 15*.

IV.3.2. Generation of Sunitinib desensitized cell lines

To determine genetic alterations within a parental cancer cell population which compensate the cytotoxic effect of Sunitinib, we established Sunitinib selected homogenic sub-populations for gene-expression comparison. To select for those cell populations, parental cells were repeatedly treated with 20 µM of Sunitinib for two to seven times, mimicking the periodical treatment in the clinic. To determine the drug specificity of this selection process, we also established Sorafenib selected sub-populations using the same conditions. The progress of Sunitinib desensitization was monitored by determining the rate of apoptotic cell formation after 72 h of Sunitinib treatment. Compared to the parental-, the Sunitinib (^{SDes}) or Sorafenib (^{NDes}) desensitized cell lines displayed significant changes in the sensitivity against Sunitinib treatment, whereas the degree of the desensitization varied (*Figure 16*). Notably, this variation was in no correlation to the cell line tissue origin. Further, no change in cell-morphology due to the desensitization process with neither Sunitinib nor Sorafenib was observed. The exception of this observation was A704, for which size and shape of the Sunitinib desensitized cells were altered compared to its parental cell line.

The sub-population of the pancreas cell line A590 (A590^{SDes}) displayed an average increase of the Sunitinib LD_{50}-value from 3.0 to 7.7 µM (256 %), whereas the respective populations of Capan1 only demonstrated a increased LD_{50}-value. HT-29^{SDes} cells showed a LD_{50}-value of 16.4, compared to the parental value of 10.7 µM (153 %). Moreover, the Sorafenib desensitized cell line HT-29NDes did not reach an apoptotic value of 50 % within the used Sunitinib concentration range. For the two breast cancer cell lines MDA-MB231 and MDA-MB435S Sunitinib treatment also resulted in the selection of Sunitinib desensitized sub-populations. These selected sub-populations from MDA-MB231 cells showed a Sunitinib LD_{50}-value of 15.4 µM compared to the respective parental -value of 8.4 µM (183 %). For MDA-MB435S^{SDes} cells, this effect was less distinct (134 %). Sunitinib desensitized sub-populations could also be selected out of the glioblastoma cell lines U118 and U1242. Here,

U118^{SDes} displayed a Sunitinib LD_{50}-value of 9.9 µM, compared to 5.5 µM in the parental cell line (180 %). For U1242^{SDes}, the degree of desensitization was 110 % (14.4 µM compared to 12.8 µM). The Sunitinib desensitized melanoma cell lines WM115^{SDes}, WM266-4^{SDes}, C8161^{SDes} demonstrated increased LD_{50}-values of 217 %, 160 % and 112 %, respectively.



	Parental		Sunitinib desensitized		Sorafenib desensitized	
	Average	stdev	Average	stdev	Average	stdev
A590	3.0	0.1	7.7	4.6	n. d.	n. d.
Capan	9.2	5.8	11.7	0.9	n. d.	n. d.
HT-29	10.7	0.1	16.4	0.0	>20	0.1
MDA-MB 231	8.4	1.9	15.1	0.1	n. d.	n. d.
MDA-MB 435S	8.9	2.9	12.8	1.2	9.6	0.2
U118	5.5	0.2	9.9	0.7	13.3	6.3
U1242	12.8	0.1	14.1	0.9	12.2	0.2
WM115	4.5	1.6	10.8	2.5	8.9	1.9
WM266-4	6.4	1.3	11.2	1.2	10.7	1.4
C8161	15.5	0.7	17.4	0.0	11.9	2.2
A498	12.4	0.4	14.5	1.1	13.6	1.7
A704	2.2	0.4	7.4	2.5	9.0	2.5
Caki 1	16.6	1.5	18.9	2.4	16.7	n. d.
Caki 2	13.5	3.9	15.4	2.5	n. d.	n. d.

Figure 16: Sensitivity characterization of Sunitinib or Sorafenib desensitized cancer cell line sub-populations upon Sunitinib treatment. The Sunitinib and Sorafenib selected sub-populations demonstrated varying degrees of desensitization upon Sunitinib treatment for 72 h. The upper panel depicts the monitored LD_{50}-values, which are listed in the table (lower panel). The degree of desensitization varied in the Sunitinib and Sorafenib desensitized cell lines. Black bars: parental cell lines; light grey bars: Sunitinib desensitized-; dark grey bars: Sorafenib desensitized sub-populations. Abbreviations: stdev, standard deviation.

Interestingly, the Sorafenib desensitized cell lines WM115^{Ndes} and WM266-4Ndes also displayed an increase of the Sunitinib LD_{50}-values of 200 % and 137 %, respectively, while in contrast, C8161^{Ndes} demonstrated a decreased Sunitinib sensitivity of 85 %. The out of parental kidney carcinoma derived cell lines A498^{SDes} and A704^{SDes} demonstrated an increase of the LD_{50}-values of 112 % and 362 %, respectively. Furthermore, the respective Sorafenib desensitized sub-populations displayed a decreased Sunitinib sensitivity. For the kidney cell lines CAKI1 and CAKI2 no significantly less sensitive sub-populations could be selected. Summarized, these data prove the existence of comparably Sunitinib insensitive sub-populations with divergent genetic backgrounds within a parental cell population. Moreover, a converging mechanism in Sunitinib resistance formation due to Sunitinib or Sorafenib selection was indicated. However, the different degree of Sunitinib desensitization between the Sunitinib and Sorafenib selected sub-populations suggests the existence of multiple sub-populations with different genetic backgrounds.

IV.3.3. Gene-expression comparison of Sunitinib desensitized- and parental kidney carcinoma cell lines

The varying potencies of Sunitinib to induce apoptosis in the parental and desensitized kidney carcinoma cell lines suggested different mechanisms within the cells to evade the cytotoxic effects of Sunitinib. Assuming that the differences are reasoned in genetic alterations, we used cDNA-arrays to test the putative correlation of transcriptional profiles and Sunitinib insensitivity. The cDNA-array mainly consisted of kinases, phosphatases and genes functioning in cell-cycle regulation. Transcripts with elevated expression and potential relevance in resistance formation, such as those implicated in angiogenesis, metastasis, proliferation and apoptosis or survival were of particular interest. When comparing Sunitinib desensitized- with their respective parental kidney cell lines, ~29 % of all genes were at least two-fold changed (data not shown). In order to identify Sunitinib sensitivity related expression patterns we performed a “Significance Analysis of Microarrays” (SAM), employing three different groups, which compose a kidney population: Group 1 was represented by the four parental kidney carcinoma cell lines A498, A704, CAKI1 and CAKI2. Group 2 consisted of the Sunitinib desensitized cell lines A498^{SDes} and A704^{SDes}, which showed significantly changed Sunitinib sensitivity. The third group was represented by the Sunitinib selected sub-populations CAKI1^{SDes} and CAKI2^{SDes}, which did not display a

significant change of Sunitinib sensitivity when compared with the respective parental cell line and thus accounted as negative controls for the desensitization process.

For the kidney carcinoma population, SAM revealed 49 genes, the gene-expression of which was upregulated in the Sunitinib desensitized cell lines. In contrast, these genes did not display altered gene-expression in the CAKI1^{SDes} and CAKI2^{SDes} cell lines. As this pattern strongly correlated with the degree of the Sunitinib desensitization, a role of these genes in triggering Sunitinib insensitivity was indicated. Moreover, the identified genes are mostly associated with cellular pro-survival signaling, supporting the importance of these genes. The heatmap of the identified genes is depicted in *Figure 17*. Note that the gene-expression was compared to the average gene-expression of the kidney cell line population but not the direct fold-change of parental and respective Sunitinib desensitized cell lines.

The 49 identified genes can be subdivided by their role in multiple cellular processes like cell survival, resistance, invasion (ABL2) and angiogenesis. The janus kinase 1 (JAK1), colony stimulating factor 1 (CSF1) and pim-3 oncogene (PIM3) represent a group of oncogenes, the overexpression of which is associated with multiple cancer types [158-161]. CSF expression is positively regulated by the transcriptionfactor NFκB [162]. Unsurprisingly, five identified genes, interleukin-1 receptor-associated kinase 1 (IRAK1), tank binding protein kinase (TBK1), mitogen activated kinase kinase 5 (MAP2K5), MAPK6 and TNF-related apoptosis-inducing ligand receptor 2 (TNFRSF10B/DR5), which positively regulate NFκB signaling, were upregulated in the Sunitinib desensitized cell lines A498^{SDes} and A704^{SDes}. Additionally, CSF1 induces the production of VEGF and promotes angiogenesis *in vivo* [163]. In line the gene-expression of VEGFA was also elevated in the Sunitinib desensitized kidney carcinoma cell lines. Moreover, src homology 2 domain containing adaptor protein B (SHB), ephrin receptor A2 (EPHA2), TBK, avian myelocytomatosis viral oncogene homolog (C-MYC) are known to promote angiogenesis. It is noteworthy that C-MYC expression often correlates with VEGFA expression in tumors [164]. Another prominent angiogenesis factor, neuropilin 1 (NRP1), which is a VEGF receptor, also displayed increased expression in A498^{SDes} and A704^{SDes}. NRP1 is moreover known to promote cell survival by modulation the p53/caspase axis [165]. Other genes revealed in this analysis, like tumor growth factor alpha (TGFα), apoptosis regulator Bcl-X(L) (BCL2L2), heat shock protein beta 8 (HSPB8), clusterin (CLU) and amphiregulin (AREG) are known to affect the apoptotic threshold of cancer cells in a survival manner. In contrast, STE20-like kinase 3 (STK24/MST3), death receptor 5 (DR5), protein kinase N1 (PKN1) were also increased in the Sunitinib desensitized cell lines. Those

genes are known negative modulators of cell survival, JNK- or NFκB signaling [166-168]. However, as those genes represent a minority compared to the aforementioned pro-survival genes their role in Sunitinib resistance formation should be not overrated.

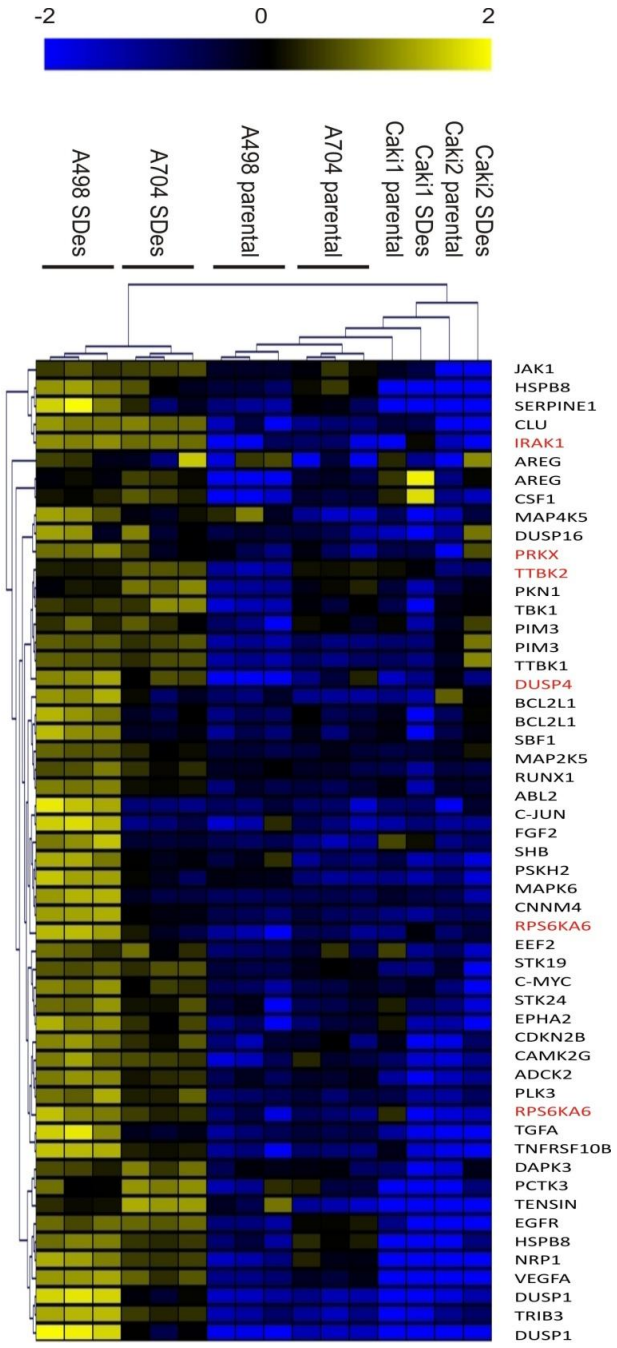


Figure 17: Comparative expression analysis of parental and Sunitinib desensitized (^{SDes}) kidney cell lines. Significance Analysis of Microarrays (SAM) of parental and Sunitinib desensitized kidney carcinoma cell lines revealed a subset of 49 genes that strongly correlated with the degree of Sunitinib desensitization. The identified genes are involved in many cellular processes that drive tumorigenesis including survival, angiogenesis and chemoresistance. Color code: Gene-expression scale from log2 (-2) to log2 (2), yellow to blue. Gene-names highlighted in red displayed a similar correlation of gene-expression and Sunitinib desensitization in melanoma cell lines.

Other genes revealed by the gene-expression analysis including protein kinase X linked (PRKX), tau tubulin kinase 1 and 2 (TTBK1 and TTBK2) and ribosomal protein S6 kinase 90kDa polypeptide 6 (RPS6KA6/RSK4) have yet not directly been associated to survival/apoptotic processes. However, since their gene-expression was found to correlate with the degree of Sunitinib desensitization and clustered with the aforementioned survival-genes, their role as a trigger for Sunitinib resistance emergence was indicated. The data for the depicted heatmap are listed in supplementary Table 2 (page 129).

IV.3.4. Gene-expression comparison of Sunitinib desensitized- and parental melanoma cell lines

The short term treatment with Sunitinib resulted in an altered gene-expression pattern. Moreover, this expression pattern was not correlated to the cell tissue origin but Sunitinib sensitivity (*Chapter IV.2.2*). Consequently, we postulated, that the underlying mechanism for Sunitinib resistance formation might be tissue independent as well. To test this postulate, we compared the altered gene-expression of parental and Sunitinib desensitized kidney- and melanoma cell lines. When comparing Sunitinib desensitized- with their respective parental melanoma cell lines, ~31 % of all genes were at least two-fold changed (data not shown). Therefore, like for the kidney cell lines, we employed SAM to identify Sunitinib sensitivity related expression patterns. Likewise, we subdivided the melanoma cell lines into 3 groups regarding their degree of Sunitinib desensitization, which compose a melanoma population: The parental melanoma cell lines constituted group 1. Group 2 was represented by the highly Sunitinib desensitized cell lines WM115^{SDes} and WM266-4^{SDes}. C8161^{SDes} was accounted as Group 3, as it was only minor desensitized compared to the respective parental cell line. Note that the heatmap (*Figure 18*) does not depict the direct fold-change of parental and respective Sunitinib desensitized cell line but the altered gene-expression compared to the average gene-expression of the melanoma cell line population.

For the melanoma population, SAM revealed the gene-expression of a subset of 26 up-regulated and three down-regulated genes, which strongly correlated with the degree of the Sunitinib desensitization, suggesting a role of these genes in triggering Sunitinib insensitivity (*Figure 17, left panel*). The three down-regulated genes were histone deacetylase 7 (HDAC7A), cyclinT2 (CCNT2) and doublecortin-like kinase 2 (DCAMKL2). The up-regulated subset consisted of 18 kinases, five phosphatases, one metalloprotease (MMP2), β-

catenin (CTNNB1) and Titin (TTN). The 18 kinases exhibit widely dispersed functions in different signaling pathways but can be mostly related to apoptotic processes, which supports the importance of the up-regulated genes within the cluster. Interestingly, not only anti-apoptotic kinases but also the pro-phagocytosis inducing kinase unc-51-like kinase 1 (ULK1) and the tumor-suppressor c-src tyrosine kinase (CSK) were up-regulated in the Sunitinib desensitized cell lines. While 11 kinases only showed an elevated gene-expression in WM115^{SDes} and WM266-4^{SDes} like RPS6KA6/RSK4, five serine/threonine kinases (PRKX, WNK4, STK39, TRIB1, TTBK2 and WNK2) and 2 tyrosine kinases (CSK, Tyro3) were of particular interest as they were up-regulated in all Sunitinib desensitized cells compared to their parental cell lines. A comparison of the identified genes of the kidney- and melanoma cell line population demonstrated that the majority of the genes is specific to either of the populations.

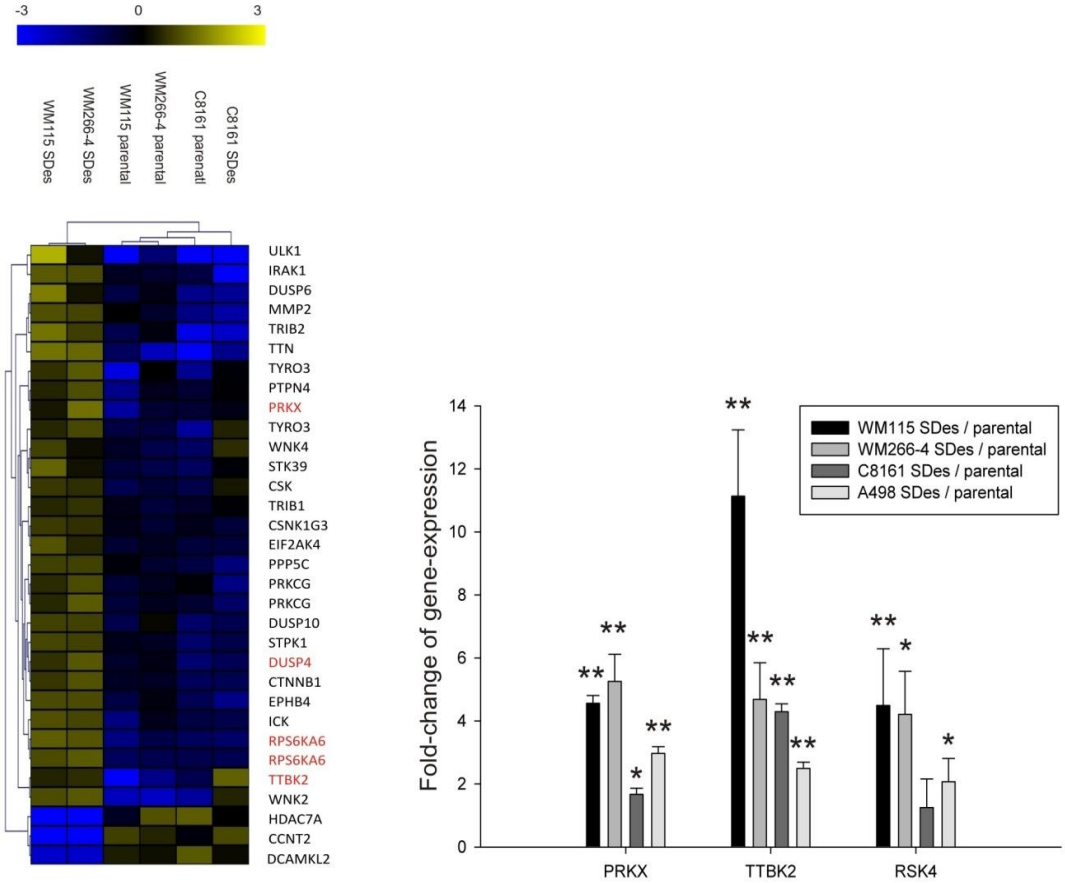


Figure 18: Comparative expression analysis of parental and Sunitinib desensitized (^{SDes}) melanoma cell lines. (Left panel) Significance Analysis of Microarrays (SAM) of parental and Sunitinib desensitized melanoma cell lines revealed a subset of 29 genes that strongly correlated with the degree of Sunitinib desensitization. The identified genes are mostly involved in apoptotic processes. (Right panel): Gene-expression fold-changes of Sunitinib desensitized and their respective parental cell line. The direct comparison demonstrated a strong correlation of increased gene-expression and the degree of Sunitinib desensitization. Data shown represent the average over 3 independent biological gene-expression analyses. Color code: Gene-expression scale from log2 (-3) to log2 (3), yellow to blue. Gene-names highlighted in red displayed a similar correlation of gene-expression and Sunitinib desensitization in kidney cell lines.

However, PRKX, TTBK2, RPS6KA6/RSK4, IRAK1 and DUSP4 demonstrated increased gene-expression independent of the cancer type. Direct comparison of the gene-expression fold-changes of PRKX, TTBK2 and RSK4 within the Sunitinib desensitized- and parental melanoma as well as kidney cell lines further demonstrated the strong correlation between the degree of Sunitinib desensitization and the increased gene-expression (*Figure 18, right panel*). PRKX displayed an approximately five-fold increased gene-expression in the highly Sunitinib desensitized cell lines WM115^{SDes} and WM266-4^{SDes}. The respective increase in C8161^{SDes} and A498^{SDes} ranged from 2 to 3 fold, which is in line with the less distinct degree of Sunitinib desensitization. Likewise, TTBK2 and RPS6KA6/RSK4 demonstrated the highest gene-expression elevation in WM115^{SDes} and WM266-4^{SDes} and a lesser increase in C8161^{SDes} as well as in A498^{SDes}. Moreover, it is noteworthy that the gene-expression of PRKX, TTBK2 and RSK4 also correlated with primary Sunitinib sensitivity within the parental melanoma cell line population. This mRNA-level alteration was less distinct but nevertheless further supported the crucial importance of these three kinases in triggering Sunitinib resistance formation. The data for the depicted heatmap are listed in supplementary Table 1 (page 128).

IV.3.5. Validation of candidate genes for Sunitinib resistance formation

To validate the results from the gene-expression analysis, we performed semiquantitative RT-PCR with subsequent scanning densitometry. The three serine/threonine kinases PRKX, TTBK2 and RPS6KA6/RSK4 were of particular interest as they we upregulated in both kidney- and melanoma Sunitinib desensitized cell lines (*Figure 19 A*). Moreover, eight kinases, the elevated gene-expression of which was specific to Sunitinib desensitization in melanoma, were validated by RT-PCR (*Figure 19 C*). Additionally, the expression of PRKX and TTBK2 was monitored on the protein-level by performing western-blotting (*Figure 19 B*). Here, the protein-expression was moreover compared to respective Sorafenib desensitized cell lines to address the question of specificity regarding multi-kinase inhibitor resistance formation. Note that the expressional alterations were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, RT-PCR) or actin-beta (ActB, Western-Blot).

The gene-expression of PRKX and TTBK2 was strongly increased in the Sunitinib desensitized cell lines WM115^{SDes} and WM266-4^{SDes}. In a less distinct degree, this increase was also observed for C8161^{SDes} and A498^{SDes}.

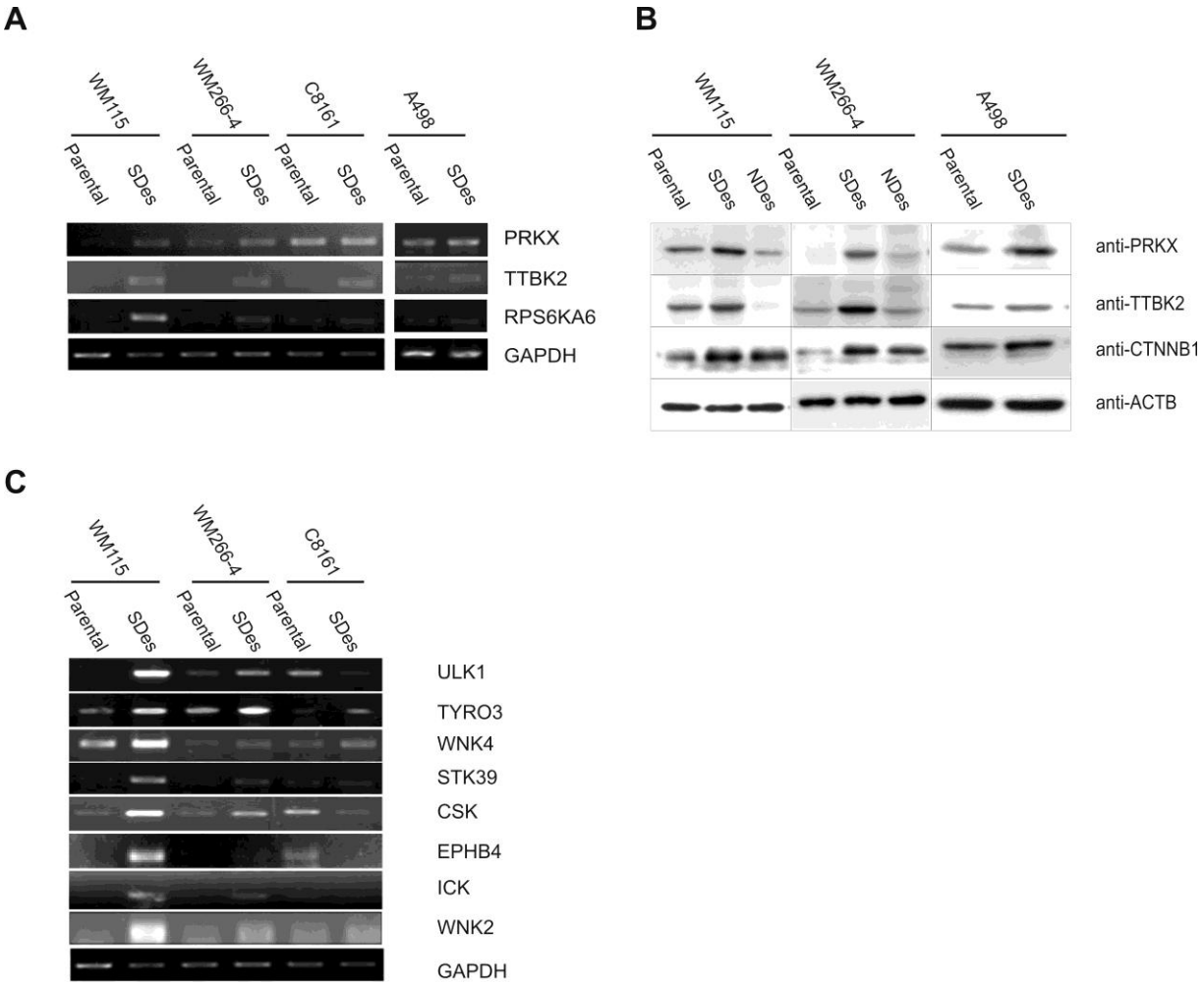


Figure 19: Validation of candidate genes that might trigger Sunitinib insensitivity. RT-PCR mimicked and thus validated the gene-array based monitored gene-expression alteration in Sunitinib desensitized cell lines. (A) PRKX, TTBK2 and RPS6KA6/RSK4 displayed an increased gene-expression in Sunitinib desensitized in WM115 and WM266-4 as well as kidney A498. This gene-expression alteration could be also translated on protein-level (B). No protein-expression change of PRKX or TTBK2 was observed in the Sorafenib desensitized melanoma cell lines. In contrast, CTNNB1 protein-expression was increased in both Sunitinib and Sorafenib desensitized melanoma cell lines. (C) Melanoma specific Sunitinib resitance candidate genes also mimicked the mined cDNA-array data.

On the protein-level, this increase was also observed in the Sunitinib desensitized cell lines WM115^{SDes}, WM266-4^{SDes} and A498^{SDes}. No change of protein-expression of PRKX and TTBK2 expression was monitored for the Sorafenib desensitized cell lines WM115^{NDes} and C8161^{NDes}, arguing for a Sunitinib specific mechanism of Sunitinib resistance formation. An increased PRKX and TTBK2 gene-expression was observed in the Sunitinib desensitized cell lines MDA-MB435^{SDes} (breast), U118^{SDes} (glioblastoma) and A590^{SDes} (pancreas) compared

to the respective parental cell lines (data not shown). The gene-expression of RPS6KA6/RSK4 was elevated in WM115^{SDes}, WM266-4^{SDes} and A498^{SDes}. In C8161^{SDes}, no significant change in RPS6KA6/RSK4 expression was monitored. TYRO3, STK39, WNK2 and WNK4, displayed an increased gene-expression in all Sunitinib desensitized melanoma cell lines. Furthermore, ULK1, CSK, EPHB4 and ICK demonstrated an elevated gene-expression in WM115^{SDes} and WM266-4^{SDes} but an expressional decrease in C8161^{SDes}. Finally, the protein-expression of catenin-beta (CTNNB1) was increased in WM115^{SDes}, WM266-4^{SDes} and, interestingly, in A498^{SDes}. Moreover, this increased protein-expression of catenin-beta was also observed in the Sorafenib desensitized cell lines WM115^{NDes} and WM266-4NDes, which argues for a drug-unspecific resistance mechanism.

In general, these results mimicked and consequently validated the results of the gene-array based gene-expression analyses. Hence, the monitored genes/proteins represent a group of potential candidates, which expression might trigger Sunitinib insensitivity.

IV.3.6. RTK phosphorylation-levels of Sunitinib desensitized- and parental WM115 cells

The Sunitinib selected sub-populations demonstrated correlations of expression pattern and decreased Sunitinib sensitivity. Altered gene-expression is often a result of altered signal transduction and Sunitinib primarily targets RTKs. Consequently, we next addressed the question, whether there is also an altered RTK-phosphorylation profile by comparing Sunitinib desensitized- and parental cell lines. Therefore, we monitored the phosphorylation levels of 30 receptor tyrosine kinases (RTKs) by performing a phospho-protein array with subsequent scanning densitometry to evaluate differences. A two-fold change in RTK phosphorylation was set as an arbitrary cut-off. As a model system we used the parental and Sunitinib desensitized melanoma cell line WM115 as it provided two key benefits: first, WM115^{SDes} demonstrated the highest degree of Sunitinib desensitization, while maintaining its cell size and shape. Secondly, since we employed the melanoma cell line WM115 for comparative analysis of short term treatment with either Sunitinib or Sorafenib, we would extend the already generated data for further comparative analyses.

The comparative analysis of Sunitinib desensitized- and parental WM115 revealed that only four of the 30 monitored RTKs displayed a more than two-fold change regarding their phosphorylation state (*Figure 20)*. The insulin-like growth factor 1 receptor (IGF-1R)

displayed an increased phosphorylation level with a 2.2 fold-change. A similar increase of the phosphorylation level (2.1 fold) was measured for v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (ErbB3/Her3), suggesting that deregulation of these RTKs might alter Sunitinb sensitivity.

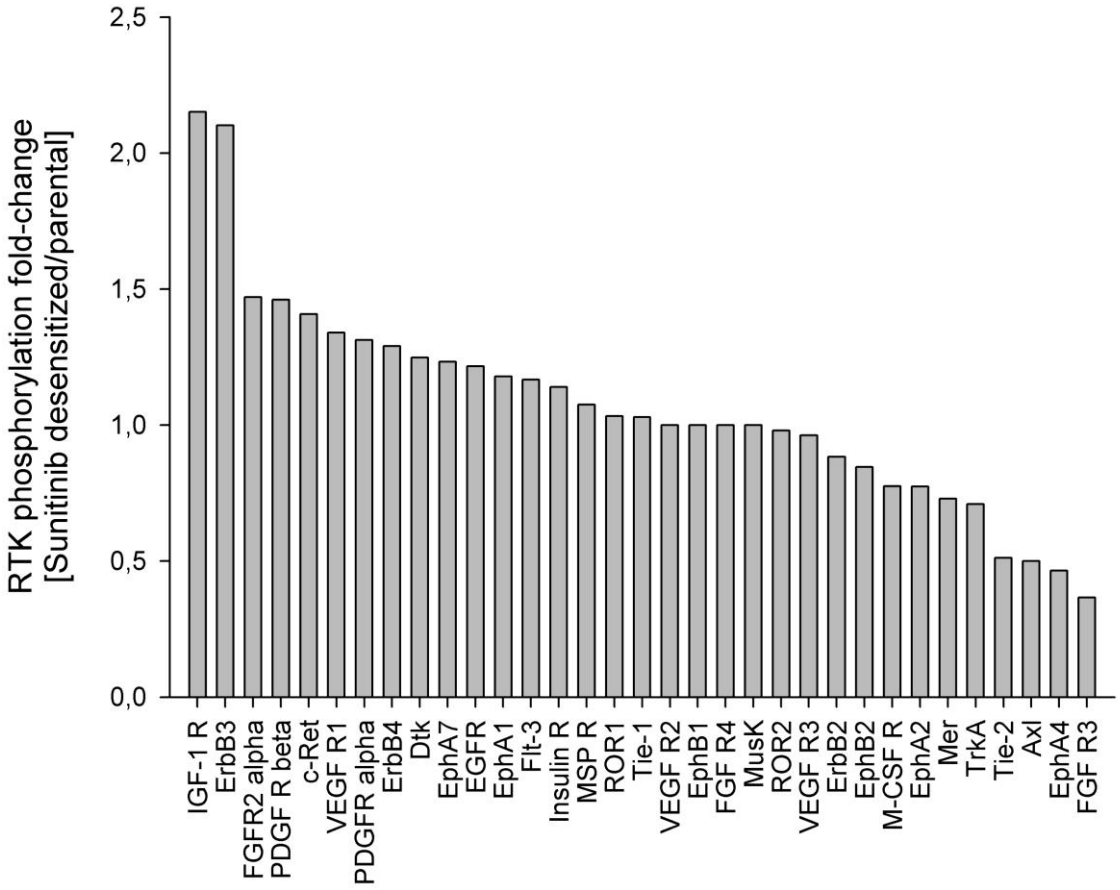


Figure 20: Comparative RTK phosphorylation of Sunitinib desensitized- and parental WM115 cells. By performing protein arrays with subsequent densitometric analysis, we determined the phospho-levels of 30 RTKs in a comparative analysis. The phospho-levels of IGF-1 R and ErbB3/Her3 were more than two-fold increased $WM115^{SDes}$ in comparison to its parental cell line. In contrast, a more than two-fold decreased phosphorylation-level was monitored for EphA4 and FGFR3 within the Sunitinib desensitized cell line. Prominent RTK targets of Sunitinib (PDGFRs, VEGFRs) displayed only minor altered phospho-levels comparing parental and Sunitinib desensitized WM115 cells.

This change might also be accomplished by the ephrin receptor A4 (EphA4), the phosphorylation level of which is decreased by a factor of 2.2. A correlative discussion of the gene-expression analyses and RTK phosphorylation regarding Sunitinib resistance formation is given in Chapter V.1.4.

IV.4. Specific reduction of candidate genes using short interference RNA technique

As a consequence of the correlation of the increased gene-expression and Sunitinib insensitivity in kidney and melanoma cell lines, we reasoned a potential function of PRKX, TTBK2 and RPS6KA6/RSK4 in an evasion-mechanism of Sunitinib induced apoptosis. To further investigate the role of PRKX, TTBK2 and RPS6KA6/RSK4, we determined whether selective reduction of their expression using siRNA is sufficient to trigger Sunitinib sensitization in parental and Sunitinib desensitized cell lines. To address the question of drug-resistance specificity, we likewise employed Sorafenib desensitized cell lines for comparative analyses. To analyze the additional Sunitinib induced apoptosis (AdA) upon candidate gene-reduction compared to the control siRNA, apoptosis was determined using PI-staining after 72 h, flow-cytometry and evaluation of the subG1-peak. Gene knock-downs ranged between 50 % and 90 %, as estimated by scanning densitometry while the knock-down efficacy was similar in parental and the respective Sunitinib or Sorafenib desensitized cell lines.

IV.4.1. Reduction of PRKX increased Sunitinib induced apoptosis

When PRKX was reduced, parental WM115 cells with only minor endogenous PRKX expression showed a slight increase in sensitivity for Sunitinib treatment compared to the control siRNA transfected cell line (AdA^{WM115}: 3.2 ± 0.4 %) (
Figure 21). A more distinct effect of PRKX reduction on Sunitinib sensitivity was observed in the parental cell lines WM266-4, C8161 and A498 ($AdA^{WM266\text{-}4}$: 22.4 ± 6.7 %; AdA^{C8161}: 19.4 ± 2.4 %; AdA^{A498}: 6.5 ± 3.2 %), which correlated with their higher endogenous gene-expression levels of PRKX. Furthermore, the Sorafenib desensitized cell lines displayed increased Sunitinib sensitivity subsequent to PRKX reduction, matching the monitored effect within the parental cell lines ($AdA^{WM115NDes}$: 3.9 ± 4.8 %; $AdA^{WM266\text{-}4NDes}$: 23.0 ± 5.4 %; $AdA^{C8161NDes}$: 18.1 ± 8.8 %). However, in the respective Sunitinib desensitized cell lines the increase of Sunitinib induced apoptosis upon PRKX reduction was strikingly higher. PRKX reduction in $WM115^{SDes}$ cells resulted in an additional apoptosis of 36.1 ± 3.2 % compared to the control siRNA. Similar observations were made in the Sunitinib desensitized cell lines $WM266\text{-}4^{SDes}$, $C8161^{SDes}$ and $A498^{SDes}$, where PRKX reduction triggered Sunitinib sensitivity ($AdA^{WM266\text{-}4SDes}$: 34.1 ± 4.9 %; $AdA^{C8161SDes}$: 45.8 ± 9.9 %; $AdA^{A498SDes}$: 14.7 ± 4.4 %). In contrast, PRKX reduction alone did not have an pro-apoptotic effect on the cell lines (data not

shown). As PRKX reduction sensitized the cell lines for Sunitinib treatment in strong correlation with their endogenous gene-expression, a crucial role in compensating the cytotoxic effects of Sunitinib can be suggested.

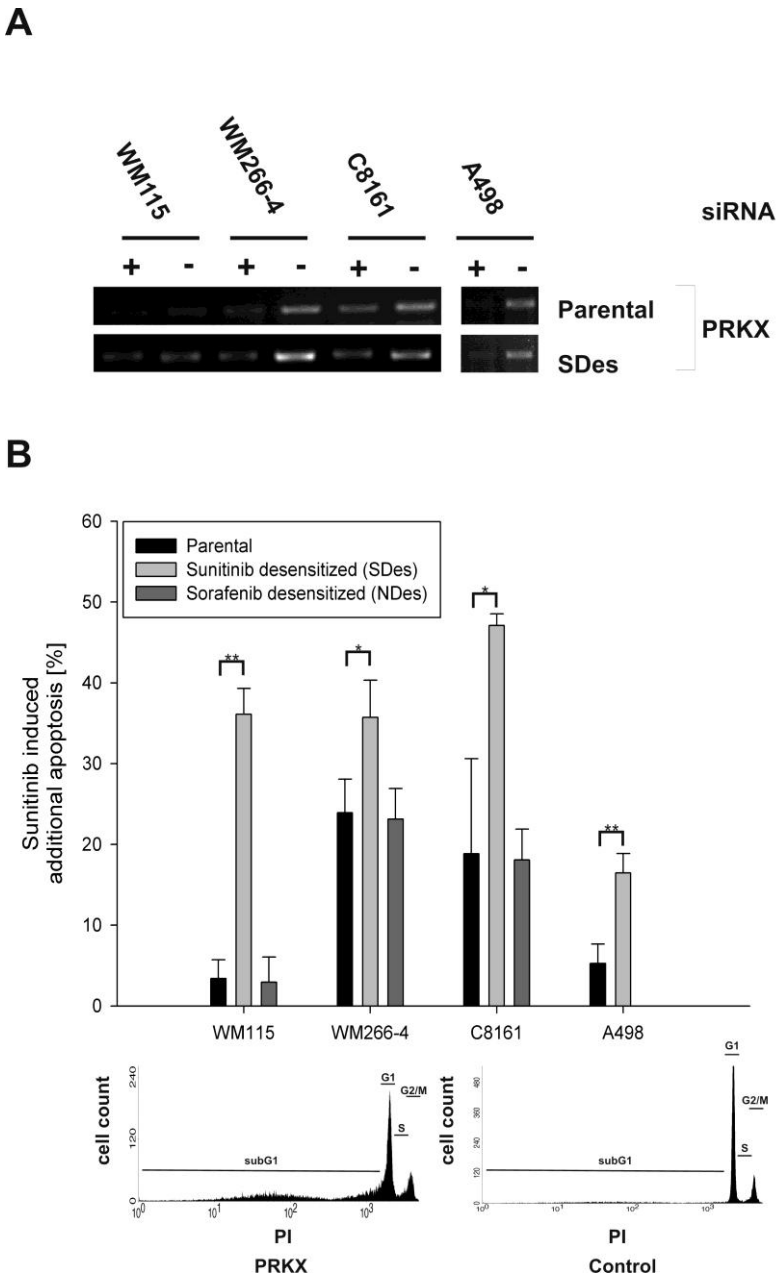


Figure 21: PRKX reduction increased Sunitinib induced apoptosis. (A) PRKX knock-downs using siRNA resulted in estimated 50 % to 90 % reduced PRKX gene-expression. This reduction was similar in parental and respective Sunitinib desensitized cell lines. Knock-down efficacy was monitored by performing RT-PCR after 96 h. (B) Bar chart, in parental and Sorafenib desensitized cell lines the effects of PRKX reduction on enhanced Sunitinib induced apoptosis ranged between ~ 3 % and ~ 22 %. In the respective Sunitinib desensitized cell lines this monitored effect upon PRKX reduction was strikingly higher (~ 15 to ~ 45 %). Lower panel, representation of the increased Sunitinib induced apoptosis (subG1-peak) upon PRKX and control siRNA transfection in $WM115^{SDes}$ cells. Color code: black bars, parental-; light grey bars, Sunitinib desensitized; dark grey, Sorafenib desensitized cell lines. **, p-value < 0.001; *, p-value < 0.05.

IV.4.2. Reduction of TTBK2 increased Sunitinib induced apoptosis

Like PRKX, TTBK2 revealed strong correlations between the increased gene-expression and Sunitinib insensitivity in kidney carcinoma and melanoma cell lines. Hence, we likewise used siRNA mediated TTBK2 reduction to evaluate a possible impact on Sunitinib induced apoptosis. The effects on Sunitinib induced apoptosis upon TTBK2 reduction are depicted in *Figure 22.*

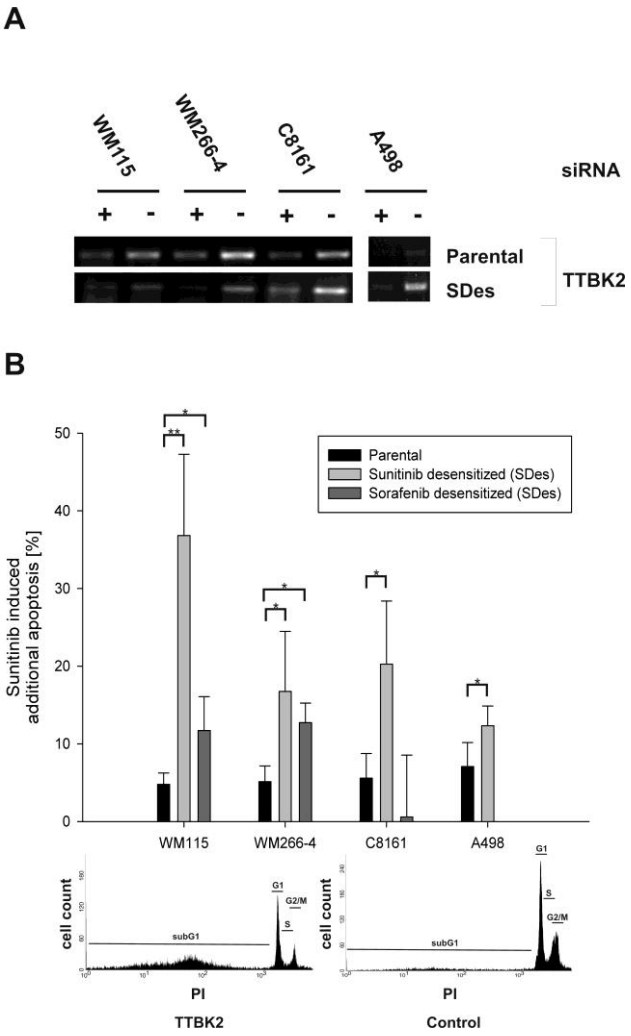


Figure 22: TTBK2 reduction increased Sunitinib induced apoptosis. (A) TTBK2 knock-downs using siRNA resulted in estimated 50 % to 90 % reduced TTBK2 gene-expression. This reduction was similar in parental and respective Sunitinib desensitized cell lines. Knock-down efficacy was monitored by performing RT-PCR after 96 h. (B) Bar chart, in parental melanoma and kidney cell lines the effects of TTBK2 reduction on enhanced Sunitinib induced apoptosis ranged between ~ 5 % and ~ 7 %. In the respective Sunitinib desensitized cell lines this monitored effect upon TTBK2 reduction was strikingly higher (~ 14 to ~ 36 %). In the Sorafenib desensitized cell lines WM115^{NDes} and WM266-4NDes the Sunitinib induced apoptosis ranged between ~12 % and ~13 %, while in C8161^{NDes} no enhanced apoptotic effect was observed upon TTBK2 knock-down. Lower panel, representation of the increased Sunitinib induced apoptosis (subG1-peak) upon TTBK2 and control siRNA transfection in WM266-4^{SDes} cells. Color code: black bars, parental-; light grey bars, Sunitinib desensitized; dark grey, Sorafenib desensitized cell lines. **, p-value < 0.001; *, p-value < 0.05.

The reduction of TTBK2 expression had a minor effect on Sunitinib sensitivity in parental WM115 and WM266-4 cells, correlating with a slightly higher endogenous TTBK2 gene-expression. In C8161 as well as in kidney A498 cells, the effect was slightly more distinct (AdA^{WM115}: 4.8 ± 1.5 %; $AdA^{WM266\text{-}4}$: 5.1 ± 2.0 %; AdA^{C8161}: 7.3 ± 4.8 %; AdA^{A498}: 7.1 ± 3.1 %). In the Sunitinib desensitized cell lines, however, the Sunitinib re-sensitization effect was strikingly higher compared to the respective parental cell lines: $WM115^{SDes}$ 36.8 ± 10.5 %, $WM266\text{-}4^{SDes}$: 16.8 ± 7.7 %, $C8161^{SDes}$: 20.3 ± 8.1 % and $A498^{SDes}$: 13.6 ± 0.4 %. Interestingly, increased Sunitinib sensitivity was also observed in the Sorafenib desensitized cell lines $WM115^{NDes}$ and $WM266\text{-}4^{NDes}$. The measured additional apoptosis rate after TTBK2 reduction ranged in between the parental and Sunitinib desensitized cell lines and did not correlate with the observed intrinsic protein-expression ($AdA^{WM115NDes}$: 11.7 ± 4.4 %; $AdA^{WM266\text{-}4NDes}$: 12.7 ± 2.5 %). Within the Sorafenib desensitized cell line C8161 no Sunitinib sensitizing effect upon TTBK2 reduction was observed ($AdA^{C8161NDes}$: -2.2 ± 6.8 %). Without subsequent Sunitinib treatment there was no change in apoptosis upon TTBK2 reduction monitored within the tested cell lines (data not shown). Like for PRKX, these data indicate a striking gene-expression dependent role of TTBK2 in compensating Sunitinib induced apoptosis.

IV.4.3. Reduction of RPS6KA6/RSK4 increased Sunitinib induced apoptosis

The relative gene-expression of RSK4 was increased in the Sunitinib desensitized- compared to the parental cell lines. Like for PRKX and TTBK2, the increased gene-expression strongly correlated with the degree of desensitization. Likewise, we specifically reduced the RSK4-expression, to examine its potential role as a cellular mechanism to evade Sunitinib induced apoptosis. The effects of the reduced expression of RPS6KA6/RSK4 on Sunitinib induced apoptosis are illustrated in *Figure 23*.

Reduction of RSK4 expression resulted in an increased Sunitinib induced apoptosis rate in the kidney carcinoma- and melanoma cell lines. Within the parental cell lines only a small increase in sensitivity against Sunitinib was observed (AdA^{WM115}: 4.8 ± 2.3 %; $AdA^{WM266\text{-}4}$: 7.1 ± 2.0 %; AdA^{C8161}: 12.5 ± 3.6 %; AdA^{A498}: 6.5 ± 3.2 %). $WM115^{NDes}$ resulted in an increased Sunitinib sensitivity comparable with the sensitization of the respective parental cell lines ($AdA^{WM115NDes}$: 5.6 ± 3.8 %). In $WM266\text{-}4^{NDes}$ the additional apoptosis was by trend-, in

C8161^{NDes} notably lower than for their respective parental cell line (AdA$^{WM266\text{-}4NDes}$: 5.3 ± 5.2 %, AdA^{C8161NDes}: 6.0 ± 2.1 %).

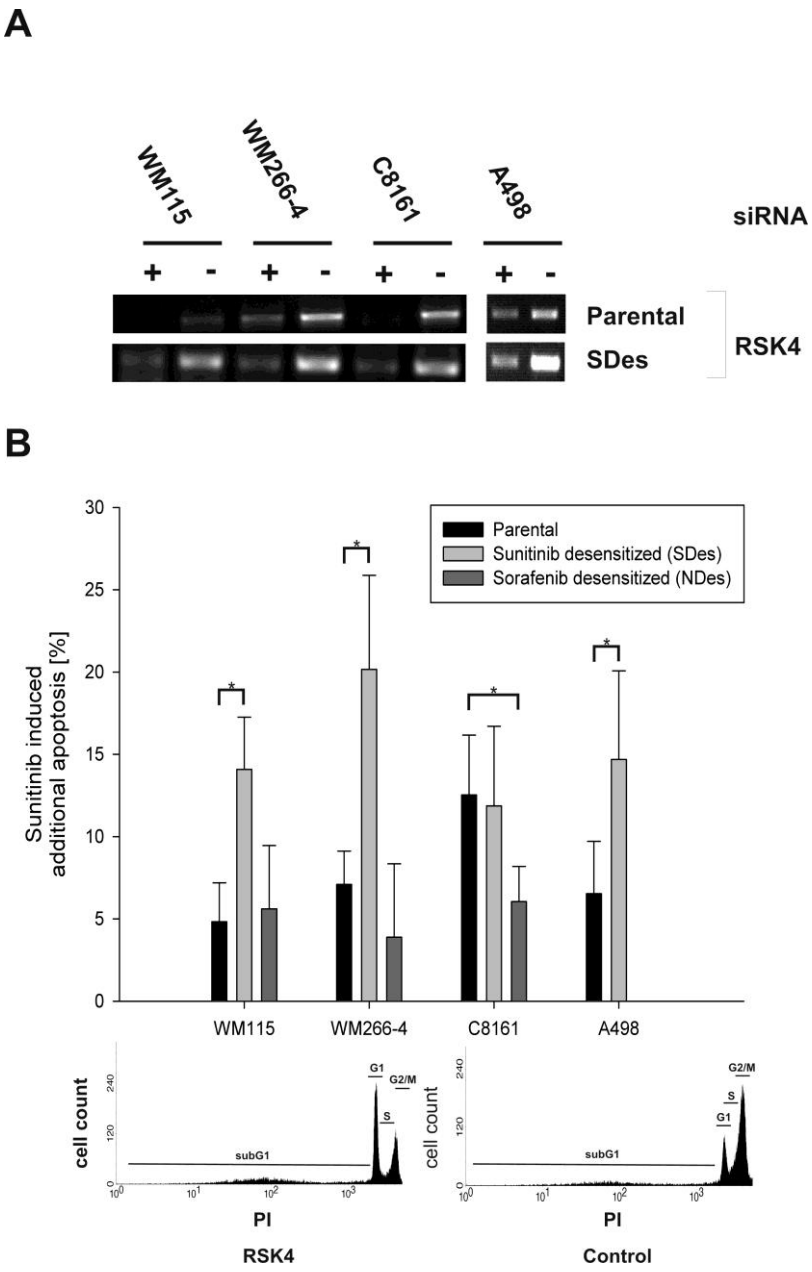


Figure 23: RSK4 reduction increased Sunitinib induced apoptosis. (A) RSK4 knock-downs using siRNA resulted in estimated 70 % to 90 % reduced RSK4 gene-expression. This reduction was similar in parental and respective Sunitinib desensitized cell lines. Knock-down efficacy was monitored by performing RT-PCR after 96 h. (B) Bar chart, in parental melanoma and kidney cell lines the effects of RSK4 reduction on enhanced Sunitinib induced apoptosis ranged between ~4 % and ~12 %. In the respective Sunitinib desensitized cell lines this monitored effect upon RSK4 reduction was strikingly higher (~12 % to ~20 %). In the Sorafenib desensitized cell lines WM115^{NDes} matched the respective parental one. In WM266-4NDes and C8161^{NDes} the Sunitinib induced apoptosis the effect was by trend or significantly decreased when compared to the parental cell lines. Lower panel, representation of the increased Sunitinib induced apoptosis (subG1-peak) upon RSK4 and control siRNA transfection in WM266-4^{SDes} cells. Color code: black bars, parental-; light grey bars, Sunitinib desensitized; dark grey, Sorafenib desensitized cell lines. **, p-value < 0.001; *, p-value < 0.05.

In line with the higher endogenous RSK4 gene-expression, the Sunitinib desensitized cell lines WM115^{SDes} (AdA: 14.1 ± 3.2 %), WM266-4^{SDes} (AdA: 20.2 ± 5.7 %) and A498^{SDes} (AdA: 14.7 ± 4.4 %) demonstrated notable higher Sunitinib induced apoptosis rates. However, within the Sunitinib desensitized cell line C8161^{SDes} - with no change in RSK4-expression - no change in Sunitinib sensitivity compared to the parental cell line was observed (AdA^{C8161SDes}: 11.9 ± 4.8 %). In the Sorafenib desensitized sub-populations WM115^{NDes} and WM266-4NDes the additional apoptosis rate was similar to monitored rate in the parental cell lines (5.6 ± 3.8 % and 5.3 ± 5.2 %). RSK4 reduction without a subsequent Sunitinib treatment did not result in a change in the level of apoptosis (data not shown). In summary, specific RSK4 reduction led to an increased Sunitinib sensitivity in terms of apoptosis induction. Like for PRKX and TTBK2, the strong correlation of endogenous RSK4 expression and Sunitinib sensitivity as well as the effects of RSK4 reduction on Sunitinib induced apoptosis suggest a role in triggering Sunitinib insensitivity.

IV.4.4. Reduction of PRKX, TTBK2 and RSK4 increased the anti-migratory effect of Sunitinib

Since Sunitinib is known to inhibit cell-migration and PRKX, TTBK2 or RSK4 reduction resulted in an increase of Sunitinib induced apoptosis, we consequently addressed the question whether these genes also had an impact on Sunitinib anti-migratory effects. For this purpose, we specifically reduced the PRKX, TTBK2 or RSK4 gene-expression. Subsequently, we treated the cells with Sunitinib and performed Boyden Chamber migration assays employing the parental and Sunitinib desensitized cell line A498 as a model system. The reduction of PRKX, TTBK2 and RSK4 resulted in an increased anti-migratory effect of Sunitinib within the tested cell lines (*Figure 24*). In parental A498 cells, PRKX reduction resulted in 64.4 ± 11.1 % less migration compared to the siRNA control. TTBK2 or RSK4 reduction, with 37.1 ± 1.0 % or 27.3 ± 2.7 %, led to a less distinct increase in Sunitinib inhibition of migration. The reduction of PRKX and TTBK2 in the Sunitinib desensitized cell line A498^{SDes} matched the results of the parental ones. Here, PRKX or TTBK2 reduction initiated an increased Sunitinib inhibitory effect of 76.4 ± 4.1 % and 45.5 ± 15.6 %. RSK4 reduction also resulted in an increased Sunitinib anti-migratory effect (8.6 ± 3.1 %). Interestingly, this effect was weaker compared to the respective effect in the parental cell line. Taken together, PRKX, TTBK2 and RSK4 reduction not only triggered induced apoptosis,

but also affected Sunitinib induced anti-migratory effects. Hence, an overall Sunitinib sensitivity triggering effect based on PRKX, TTBK2 and RSK4 expression can be assumed.

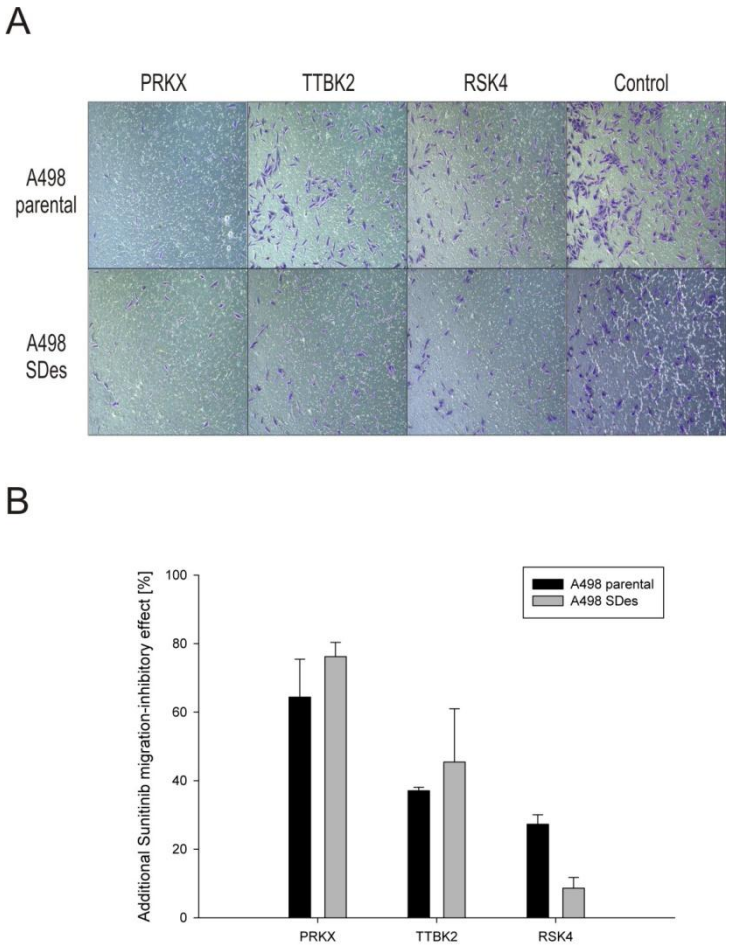


Figure 24: Reduction of PRKX, TTBK2 or RSK4 increased the Sunitinib inhibitory migration effect. (A) Subsequent to gene reduction, parental and Sunitinib desensitized A498 cells were seeded with equal cell numbers in Boyden Chambers. Microscopic visualization showed the effect of PRKX, TTBK2 or RSK4 reduction compared to the control-siRNA in the parental and Sunitinib desensitized cell line A498. PRKX, TTBK2 or RSK4 reduction resulted in an increased Sunitinib inhibitory migration effect. The effect was highest for PRKX, followed by TTBK2 and RSK4 reduction. Interestingly, RSK4 reduction affected the Sunitinib anti-migratory effect less in $A498^{SDes}$ than in the A498 parental. (B) Bars depict the quantification of increased Sunitinib induced anti-migratory effects due to gene-expression reduction. Data shown are the means ± standard deviation of two independent experiments (black bars: parental-; light grey bars: Sunitinib desensitized A498 cells).

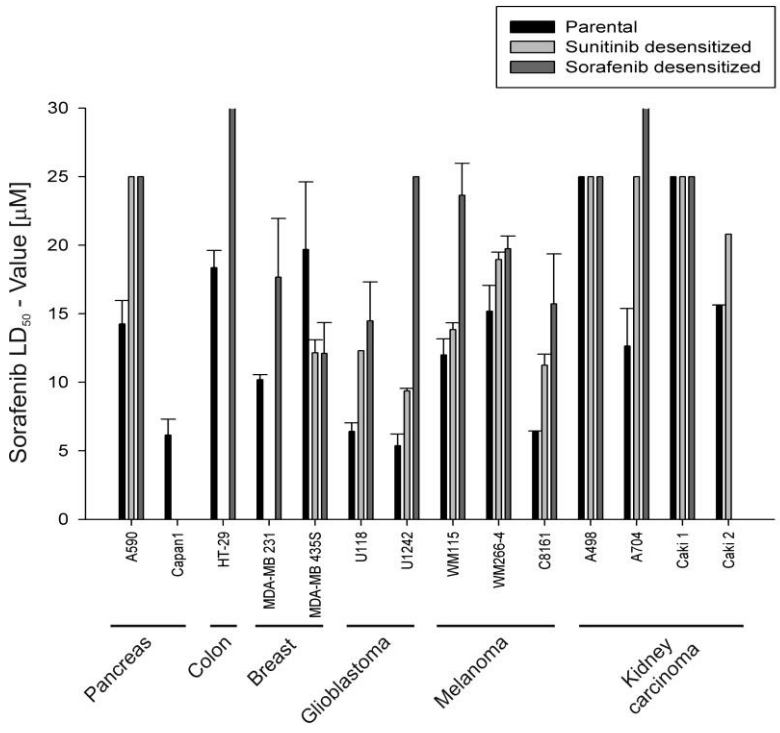
IV.5. Generation of Sorafenib desensitized cell lines

The generation of Sunitinib desensitized cell lines and subsequent gene-expression analysis resulted in the identification of potential candidate genes regarding Sunitinib sensitivity.

Postulating that Sorafenib sensitivity might be also based on a change of the apoptotic threshold as a consequence of altered gene-expression, we likewise selected homogenic sub-populations out of parental cell lines from various cell tissue origins. To select for those cell populations, parental cells were repeatedly treated with 20 µM of Sorafenib for two to five times, mimicking the periodical treatment in the clinic. The rate of apoptotic cell formation after Sorafenib treatment was monitored for 72 h to determine contrastable LD_{50}-values. Compared to the parental-, the Sorafenib (^{NDes}) desensitized cell lines displayed significant changes in the sensitivity against Sorafenib treatment, whereas the degree of the desensitization varied *Figure 25*. Note, that Sorafenib treatment did not induce the formation of more than 50 % apoptotic cells in some tested cell lines. Hence, for these cell lines, no LD_{50}-value could be evaluated and consequently, these LD_{50}-values were set to an arbitrary value of 25 µM. Like for Sunitinib desensitization, the degree of Sorafenib desensitization was in no correlation to the cell line tissue origin. Further notably, no change in cell-morphology due to the desensitization process with either Sunitinib or Sorafenib was observed. Again, the exception was A704, for which size and shape of the Sorafenib desensitized cells were altered compared to its parental cell line.

The Sorafenib selected sub-population of the pancreas cell line A590 (A590^{SDes}) displayed an average increase of the Sorafenib LD_{50}-value from 15 µM to 25 µM. HT-29NDes cells showed a LD_{50}-value of 25 µM, compared to the parental value of 18.4 µM. For the breast cancer cell lines MDA-MB231 Sorafenib also selected a desensitized sub-population. The selected sub-populations from MDA-MB231 cells showed a Sorafenib LD_{50}-value of 17.7 µM compared to the respective parental -value of 10.2 µM (173 %). Surprisingly, Sorafenib selected MDA-MB435S^{NDes} cells demonstrated a decreased LD_{50}-value when compared with the particular parental cell lines (12.1 µM compared to 19.7 µM). This decreased value was also monitored for the respective Sunitinib desensitized cell lines MDA-MB435S^{SDes}. Sorafenib desensitized sub-populations could also be selected out of the glioblastoma cell lines U118 and U1242. Here, U118^{NDes} displayed a Sorafenib LD_{50}-value of 14.5 µM, compared to 6.4 µM in the parental cell line (226 %). For U1242^{NDes} an even higher degree of Sorafenib desensitization (25 µM compared to 5.4 µM) was monitored. Moreover, the respective Sunitinib desensitized cell lines displayed increased Sorafenib LD_{50}-values (12.4 µM and 9.4 µM). In case of melanoma, WM115^{Ndes}, WM266-4Ndes and C8161^{Ndes} demonstrated an increase of the Sorafenib LD_{50}-values of 196 %, 129 % and 245 %, respectively. Interestingly, the Sunitinib desensitized cell lines C8161^{Sdes} and WM266-4^{Sdes} also displayed an increase of the Sorafenib

LD_{50}-values of 176 % and 129 %. In contrast, $WM115^{Sdes}$ demonstrated no change in Sorafenib sensitivity. The parental kidney cell lines A498 and CAKI1 already demonstrated Sorafenib insensitivity and only less than 50 % of apoptotic cells could be counted within the used drug-concentration range. Hence, no degree of Sorafenib desensitization could be monitored for those cells within the employed drug-concentration range.



Cell line	Parental		Sunitinib desensitized		Sorafenib desensitized	
	Average	stdev	Average	stdev	Average	stdev
A590	14.3	1.7	25.0	0.0	25.0	0.0
Capan	6.1	1.2	n. d.	n. d.	n. d.	n. d.
HT-29	18.4	1.3	n. d.	n. d.	49.4	17.1
MDA-MB 231	10.2	0.4	n. d.	n. d.	17.7	4.3
MDA-MB 435S	19.7	4.9	12.1	1.0	12.1	2.3
U118	6.4	0.6	12.4	0.1	14.5	2.8
U1242	5.4	0.8	9.4	0.2	25.0	0.0
WM115	12.0	1.2	13.8	0.5	23.6	2.3
WM266-4	15.2	1.9	18.9	0.5	19.7	0.9
C8161	6.4	0.1	11.3	0.8	15.7	3.6
A498	25.0	0.0	25.0	0.0	25.0	0.0
A704	12.6	2.7	25.0	0.0	45.3	65.2
Caki 1	25.0	0.0	25.0	0.0	25.0	0.0
Caki 2	15.6	0.1	20.1	1.0	n. d.	n. d.

Figure 25: Sensitivity characterization of Sorafenib or Sunitinib desensitized cancer cell line sub-populations upon Sorafenib treatment. The Sorafenib and Sunitinib selected sub-populations demonstrated varying degrees of desensitization upon Sorafenib treatment for 72 h. The upper panel depicts the monitored LD_{50}-values, which are listed in the table (lower panel). The degree of desensitization varied in the Sunitinib and Sorafenib desensitized cell lines. Black bars: parental cell lines; light grey bars: Sunitinib desensitized-; dark grey bars: Sorafenib desensitized sub-populations. Abbreviations: stdev, standard deviation.

However, both A704^{NDes} and CAKI2^{SDes} displayed increased Sorafenib LD_{50}-values of 25 and 20.1 µM. Furthermore, A704^{SDes} also showed an increased Sorafenib LD_{50}-value of 25 µM. Like for Sunitinib, these data prove the existence of comparably Sorafenib insensitive sub-populations with divergent genetic backgrounds within a parental cell population. Sorafenib selection not only resulted in decreased sensitivities against Sorafenib but also Sunitinib selection. Together with the phenotypic data of the Sunitinib desensitization, a converging mechanism could be suspected. Nevertheless, the by trend different degree of Sorafenib desensitization between the Sorafenib and Sunitinib selected sub-populations suggested the existence of multiple drug-specific sub-populations with specific genetic backgrounds.

IV.5.1. Gene-expression comparison of Sorafenib desensitized- and parental melanoma cell lines

Sorafenib displayed varying potencies to induce apoptosis in a cancer type independent manner, while this potency was in correlation to a Sorafenib induced altered gene-expression pattern (Chapter IV.2.3). Hence, we postulated that differential gene-expression might play a key-role in Sorafenib resistance formation. To identify candidate genes which might trigger Sorafenib resistance formation, we performed gene-expression analysis using cDNA-arrays with subsequent cluster analysis, mimicking the settings used for Sunitinib desensitized cell lines. To compare Sorafenib- with Sunitinib resistance formation, we used parental- and Sorafenib desensitized melanoma cell lines, as we had already generated expression data regarding Sunitinib resistance that could be used for comparative analyses. To identify Sorafenib sensitivity related gene-expression patterns, we employed the SAM algorithm based on the gene-expression data of parental- and Sorafenib desensitized cell lines. Hence, we subdivided the melanoma cell lines into 3 groups regarding their degree of Sorafenib desensitization: Group 1 was represented by the parental cell lines; C8161^{NDes} and WM115^{NDes}, displaying a high degree of Sorafenib desensitization, were members of group 2; Group 3 consisted of WM266-4NDes, which showed only a slight degree of Sorafenib desensitization. Note that in case of Sunitinib desensitization, C8161^{SDes} displayed an only minor degree-, while WM266-4^{SDes} demonstrated a high degree of Sunitinib desensitization.
SAM revealed 77 genes (*Figure 26*), which expression correlated with the degree of Sorafenib desensitization within the melanoma cell line population. It is noteworthy that SAM only revealed genes, which were upregulated in Sorafenib desensitized-, when compared to

the respective parental cell lines. Furthermore and in line with the only minor degree of desensitization, only 33 genes were upregulated in WM266-4NDes cells. As the identified genes displayed a stark correlation between expression and degree of Sorafenib desensitization, their role in triggering Sorafenib resistance formation was suggested. Further support for this suggestion was given by the functionality of the identified genes. AKT1, CAV1, cyclin-dependent kinase inhibitor 1B (CDKN1B/p27), E2F transcription factor 6 (E2F6), mitogen-activated protein kinase 1 (MAPK1), regenerating islet-derived 3 alpha (REG3A/PAP) and PLAUR are known promoters of cell survival [169-175]. Furthermore, an enhanced metastatic state of the Sorafenib desensitized cell lines was apparent as ADAM17, annexin2 (ANXA2), the hyaluronate- (CD44) as well as the macrophage stimulating 1 receptor (MST1R) and CTNNB1 were upregulated in the Sorafenib desensitized cell lines [176-179]. Other upregulated genes play a functional role in the centrosome- and spindle assembly like aurora kinases A and B (AURKA and AURKB), budding uninhibited by benzimidazoles 1 homolog (BUB1), polo-like kinase 1 (PLK1) and nima related kinase 2 (NEK2). Additionally, twelve genes within the cluster namely E2F6, eukaryotic translation initiation factor 2-alpha kinase 2 (PKR), mechanistic target of rapamycin (mTor/FRAP1), GRB2-associated binding protein 1 (GAB1), IRAK1, TBK1, mitogen-activated protein kinase 3 (MAPK3), mitogen-activated protein kinase kinase kinase 7 and 11 (MAP3K7/MAP3K11), microtubule associated serine/threonine kinase 2 (MAST2), NFkappaB essential modulator (IKBKG) and IKK-a kinase (CHUK/IKKA) are activators of the pro-survival gene NFκB [180-185], indicating a crucial role of NFκB signaling in triggering Sorafenib insensitivity. Moreover, WNT signaling was represented by CTNNB1 and its activators cyclin dependent kinase 8 (CDK8), casein kinase 1 alpha1, -epsilon, -gamma2 (CSNK1A1, CSNK1E, CSNK1G2, NEK2 and the receptor related to tyrosine kinases (RYK) [186-189]. Besides, CAV1, histone deacetylase 1 (HDAC1) and sphingosine kinase 1 (SPHK1) are known mediators of drug resistance [190-192]. Finally, five markers of melanoma progression namely CD44, CDKN1B, Fas ligand (TNF superfamily, member 6, FASL/CD95L), transforming growth factor beta receptor II (TGFBR2) and vimentin (VIM) were upregulated in the Sorafenib desensitized cell lines [171,178,193-195]. It is noteworthy that v-akt murine thymoma viral oncogene homolog 3 (AKT3) and mitogen-activated protein kinase kinase kinase 14 (MAP3K14), which also function as activators of NFκB signaling , were also upregulated in the Sorafenib desensitized cell lines, although they were not identified by the SAM analysis. The data for the depicted heatmap are listed in supplementary Table 3 (page 130).

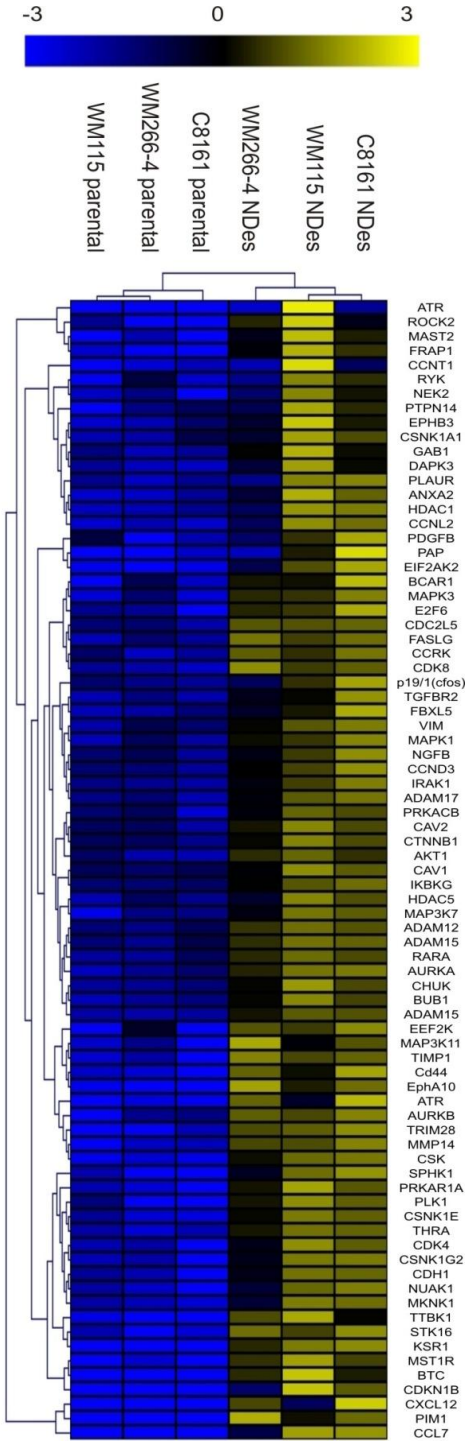


Figure 26: Comparative expression analysis of parental and Sorafenib desensitized (^{NDes}) melanoma cell lines. Significance Analysis of Microarrays (SAM) of parental and Sorafenib desensitized melanoma cell lines revealed a subset of 77 genes that strongly correlated with the degree of Sorafenib desensitization. The identified genes are mostly involved in pro-survival, pro-metastasis processes or centrosome- and spindle assembly. Data shown represent the average over 3 independent biological gene-expression analyses. Color code: Gene-expression scale from log2 (-3) to log2 (3), yellow to blue.

IV.5.2. Validation of candidate genes for Sorafenib resistance formation in melanoma cells

To validate the results from the array based gene-expression analysis, we performed semiquantitative RT-PCRs with subsequent scanning densitometry or western blotting (*Figure 27*), when anti-bodies were available. Of particular interest was the expression of genes with potential relevance in NFκB signaling and centrosome- and spindle assembly as cDNA-array based gene-expression analysis suggested their key role in triggering Sorafenib insensitivity. The protein-expression was moreover compared to respective Sunitinib desensitized cell lines to address the question of specificity regarding multi-kinase inhibitor resistance formation. The expression level alterations were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH, RT-PCR) or actin-beta (ActB, Western-Blot).

The first gene validated was SPHK1, which is associated with melanoma cell survival. SPHK1 was upregulated in all Sorafenib desensitized-, compared to the respective parental melanoma cell lines. The gene-expression of the NFκB activators CHUK/IKKA and MAP3K11 was elevated in WM115^{NDes} and C8161^{NDes}, while in WM266-4NDes no increased gene-expression was monitored. In all Sorafenib desensitized cell lines the increased gene-expression was also observed for AKT3. In contrast, the increased AKT1 expression observed in the cDNA-array was only validated for C8161^{NDes} cells, while WM115^{NDes} and WM266-4NDes demonstrated no change of the gene-expression. An elevated gene-expression was moreover observed for MAP3K14 and IKBKG in C8161^{NDes}. In both, parental- and Sorafenib desensitized WM115 cells, expression of these genes was not detectable. The increased gene-expressions of PLAUR, PIM1 and IRAK1 monitored in the cDNA-array could not be validated by RT-PCR. On protein level, the increased expression of CDKN1B, AURKA, AURKB and PLK1 in the Sorafenib desensitized cell lines was validated. In line with the cDNA-array based gene-expression data, CDKN1B expression was also elevated in C8161^{SDes}. Surprisingly, a slightly increased CDKN1B expression was also monitored in WM266-4^{SDes} cells, which is in contrast to the mined gene-expression data (data not shown). Furthermore, the monitored protein-expression of AURKA, AURKB and PLK1 correlated with the observed gene-expression in the Sunitinib desensitized melanoma cell lines.

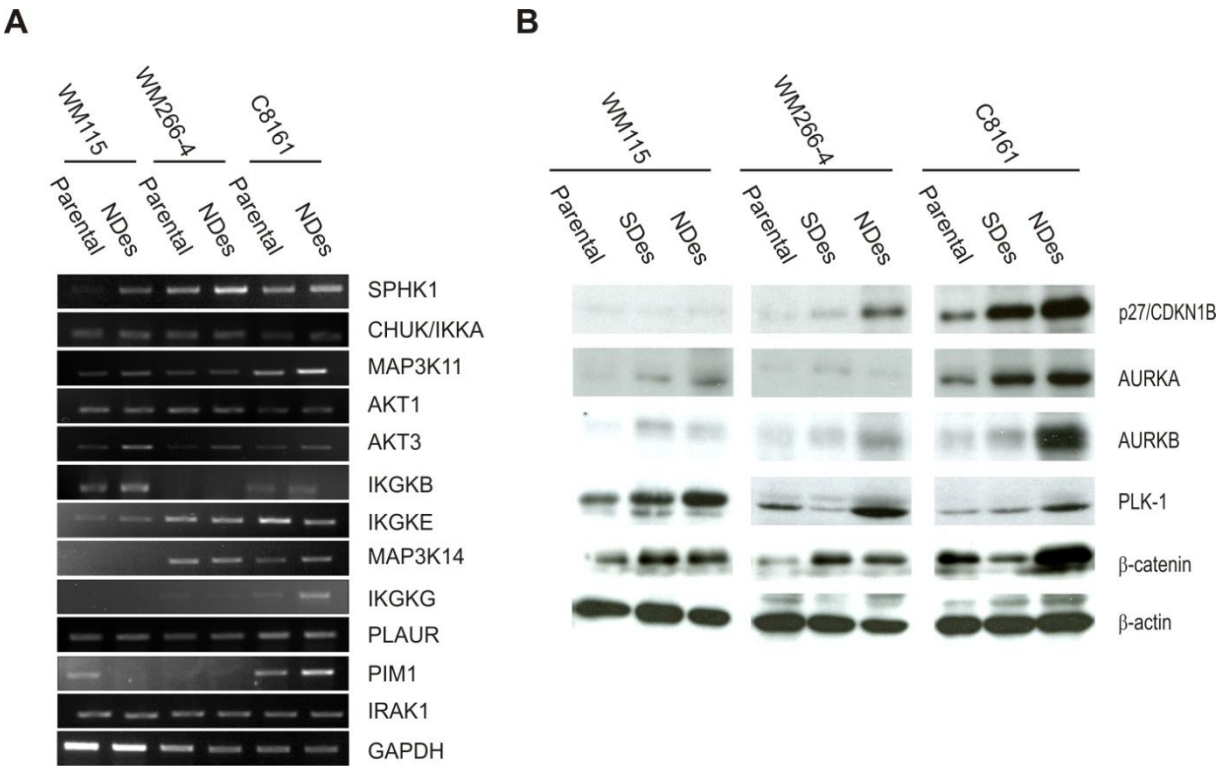


Figure 27: Validation of candidate genes that might trigger Sorafenib insensitivity. RT-PCR was performed to validate the gene-array based monitored gene-expression alteration in Sorafenib desensitized cell lines. (A) SPHK1, CHUK/IKKA, MAP3K11 and AKT3 displayed an increased gene-expression in Sorafenib desensitized WM115, WM266-4 and C8161. RT-PCR further validated the increased gene-expression of MAP3K14 and IKGKG in $C8161^{NDes}$ cells. No bands of these genes were observed for the parental- and Sorafenib desensitized WM115 cell lines. (B). Protein-expression of CDKN1B, AURKA, AURKB, PLK1 and β-catenin was increased in the Sorafenib desensitized melanoma cell lines. CDKN1B expression was also increased in $C8161^{SDes}$. Moreover, slightly increased expression levels of AURKA and AURKB were monitored in the Sunitinib desensitized melanoma cell lines. Additionally, PLK1 expression was elevated in $WM115^{SDes}$ cells.

IV.5.3. Reduction of CHUK/IKKA increased Sorafenib induced apoptosis

The gene-expression analysis revealed a correlation of CHUK/IKKA expression and Sorafenib desensitization suggesting its role in Sorafenib resistance formation. To further determine this role, we specifically downregulated CHUK/IKKA using siRNA. To analyze the additional Sorafenib induced apoptosis (AdA) upon gene-reduction compared to the control siRNA, apoptosis was determined using PI-staining after 72 h, flow-cytometry and evaluation of the subG1-peak. Gene knock-downs ranged between 70 % and 90 %, as estimated by scanning densitometry while the knock-down efficacy was similar in parental and the respective Sorafenib desensitized cell lines. The effects on Sorafenib induced apoptosis upon CHUK/IKKA reduction as well as the knock-down efficacy are depicted in *Figure 28*.

When CHUK/IKKA was reduced, parental C8161 and WM266-4 cells demonstrated only a minor increase in sensitivity for Sorafenib treatment compared to the control siRNA transfected cell line (AdA^{C8161}: ~ 2.5 %, $AdA^{WM266-4}$: ~ 3.5 %). In WM115 cells the reduction of CHUK/IKKA resulted in an increased apoptosis rate of ~ 5.5 %.

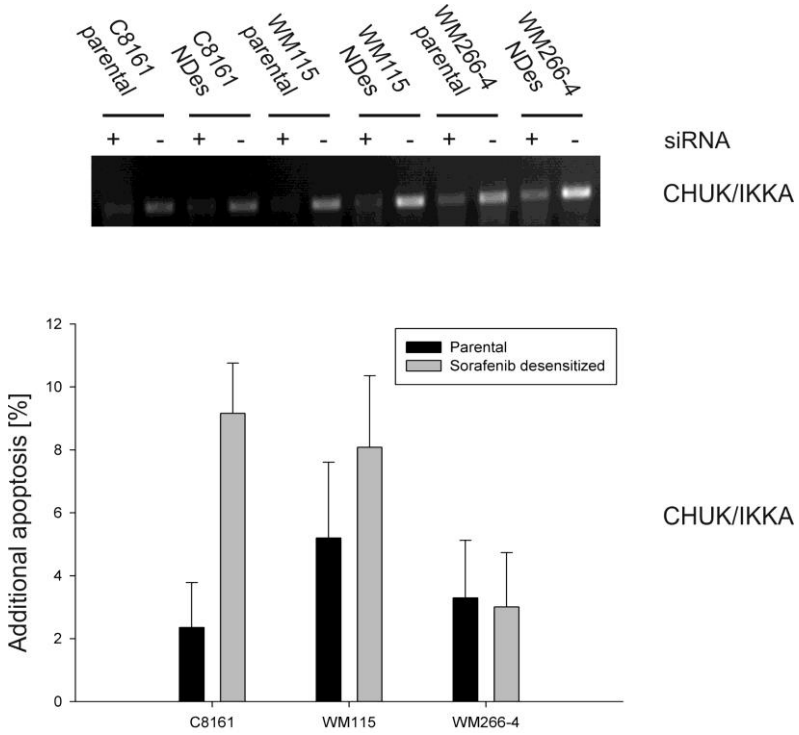


Figure 28: CHUK/IKKA reduction increased Sorafenib induced apoptosis. Upper panel: CHUK/IKKA knock-downs using siRNA resulted in estimated 70 % to 90 % reduced CHUK/IKKA gene-expression. This reduction was similar in parental and respective Sorafenib desensitized cell lines. Knock-down efficacy was monitored by performing RT-PCR after 96 h. Lower panel, bar chart: in parental melanoma cell lines the effects of CHUK/IKKA reduction on enhanced Sorafenib induced apoptosis ranged between ~ 2.5 % and ~ 5.5 %. In the respective Sorafenib desensitized cell lines $C8161^{NDes}$ and $WM115^{NDes}$ the monitored effect upon CHUK/IKKA reduction was higher (~ 9.5 % and ~ 8.5 %). In $WM266\text{-}4^{NDes}$ CHUK/IKKA reduction resulted in increased Sorafenib apoptosis matching the effect of the parental cell line (~ 3.5 %).

In $C8161^{NDes}$ CHUK/IKKA reduction resulted in a significantly higher increased apoptotic rate upon Sorafenib treatment (AdA: ~ 9.5 %). Likewise, in $WM115^{NDes}$ cells the reduction of CHUK/IKKA led to an increased Sorafenib apoptosis inducing effect (AdA: ~ 8.5 %) compared to the respective parental cell line. In contrast, within the Sorafenib desensitized cell line $WM266\text{-}4^{NDes}$ no change in Sorafenib sensitivity compared to the parental cell line was observed ($AdA^{C8161SDes}$: ~ 3.5 %). Notably, CHUK/IKKA reduction without a subsequent Sorafenib treatment did not cause a change of the appearance of apoptotic cells (data not shown).

IV.5.4. Reduction of NFκB signaling activators increased Sorafenib induced apoptosis in C8161 cells

The number of upregulated genes that can activate NFκB signaling within the Sorafenib desensitized melanoma cell lines indicated their key-role in regulating Sorafenib sensitivity. Furthermore, knock-down studies of CHUK/IKKA gave further support for this postulate. Hence, we addressed the question, whether other IKK-family members might exhibit a similar effect on Sorafenib induction of apoptosis. Consequently, we specifically reduced IKBKB, IKBKE and IKBKG using the same conditions as used for reduction of CHUK/IKKA to determine the additional apoptotic cell formation (chapter IV.5.3) in parental- and Sorafenib desensitized C8161 cells. Note that the impact of another NFκB activator, i.e. MAP3K11, is described in chapter IV.6.3, as it is also an activator of JNK1/2.

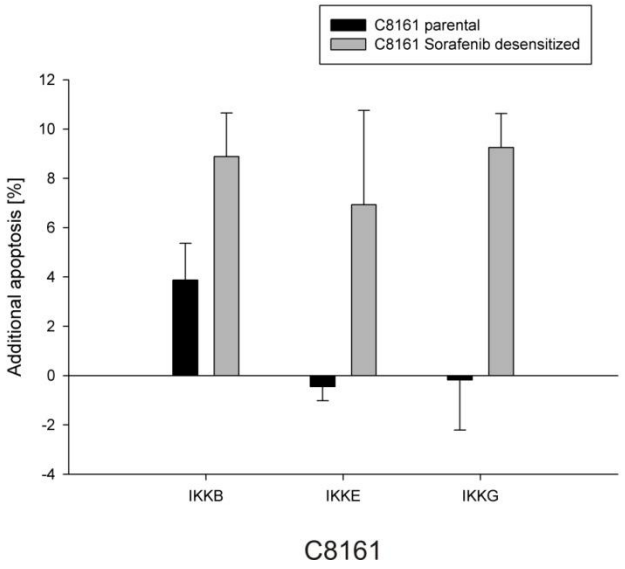


Figure 29: IKK-family member reduction increased Sorafenib induced apoptosis in C8161 cells. In parental C8161 cells, IKK-family member reduction demonstrated only minor or no effects on apoptosis induction upon Sorafenib treatment. In $C8161^{NDes}$ cells, the knock-down of the respective genes led to an increased susceptibility to Sorafenib induced apoptosis.

In parental C8161, the reduction of IKBKB resulted only in a minor increase of Sorafenib induced apoptosis (AdA^{C8161}: 3.9 % ± 1.5 %), while no effect was observed for IKBKE and IKBKG. In $C8161^{NDes}$ cells, reduction of IKBKB, IKBKE or IKBKG increased the susceptibility to Sorafenib induced apoptosis (AdA^{IKBKB}: 8.9 % ± 1.8 %: AdA^{IKBKE}: 7.0 % ± 3.8 %: AdA^{IKBKE}: 9.3 % ± 1.4 %).

IV.6. Sorafenib and Sunitinib play different roles in Ras/Raf/Mek /Erk- and JNK signaling

The Ras/Raf/MEK/ERK signaling cascade couples signals from the cell surface receptors to the transcription factors and has diverse functions like regulation of cell cycle progression, differentiation or apoptosis. Furthermore, Sorafenib but not Sunitinib was designed as a Raf-1 inhibitor. Hence, Raf-1 most prominent discrepancy between the drugs and the Ras/Raf pathway might explain differences in the mode of action between Sorafenib and Sunitinib. Consequently, we set our focus on the Raf-1 downstream kinases Mek and Erk (*Figure 30 A)*. Furthermore, since JNK phosphorylation was associated to Sorafenib treatment [196], we adressed the question if there is an impact of Sorafenib – compared to Sunitinib - on mitogen-activated protein kinase 8 (JNK1) phosphorylation in melanoma and kidney cell lines. Additionally, this implied the study of Mitogen-activated protein kinase kinase 7 (MAP2K7), an activator of JNK1 [197] and growth arrest and DNA-damage-inducible, beta (GADD45B) which is a repressor of MAP2K7 [198].

IV.6.1. Sorafenib but not Sunitinib inhibited the phosphorylation of Mek and Erk

To determine the inhibitory effect of Sorafenib and Sunitinib on the downstream targets of Raf-1 we treated the melanoma cell lines C8161, which was highly sensitive upon Sorafenib and comparably insensitive upon Sunitinib treatment, and WM115, which demonstrated an opposed drug-sensitivity pattern. Cells were treated under starving conditions (without FCS) with various drug-concentrations for 2 h and subsequently stimulated with 20 pmol epidermal growth factor (EGF). As a negative control we used cells incubated under starving conditions lacking FCS. Phosphorylation-levels were monitored by western-blotting using specific pMek or pErk antibodies (*Figure 30 B).*

In C8161 cells, Sorafenib inhibited the phosphorylation of MAPK/ERK kinase kinase (Mek) in a dose-dependent manner, while Sunitinib treatment demonstrated no effect on the phopshorylation of Mek. Similar inihibitory effects were monitored in the cell line WM115. Likewise, Sunitinib did not inhibit the phosphorylation of Mek, while Sorafenib displayed its inhibitory effect in a dose-dependent manner. A contrastable effect of Sorafenib and Sunitinib was monitored for the phosphorylation-levels of Erk. Here, Sorafenib inhibited the

phosphorylation of Erk in both cell lines in a dose-dependent manner, while Sunitinib treatment had no effect on the phosphorylation state. Note that the inhibitory effect of Sorafenib did not correlate with the primary Sorafenib sensitivity.

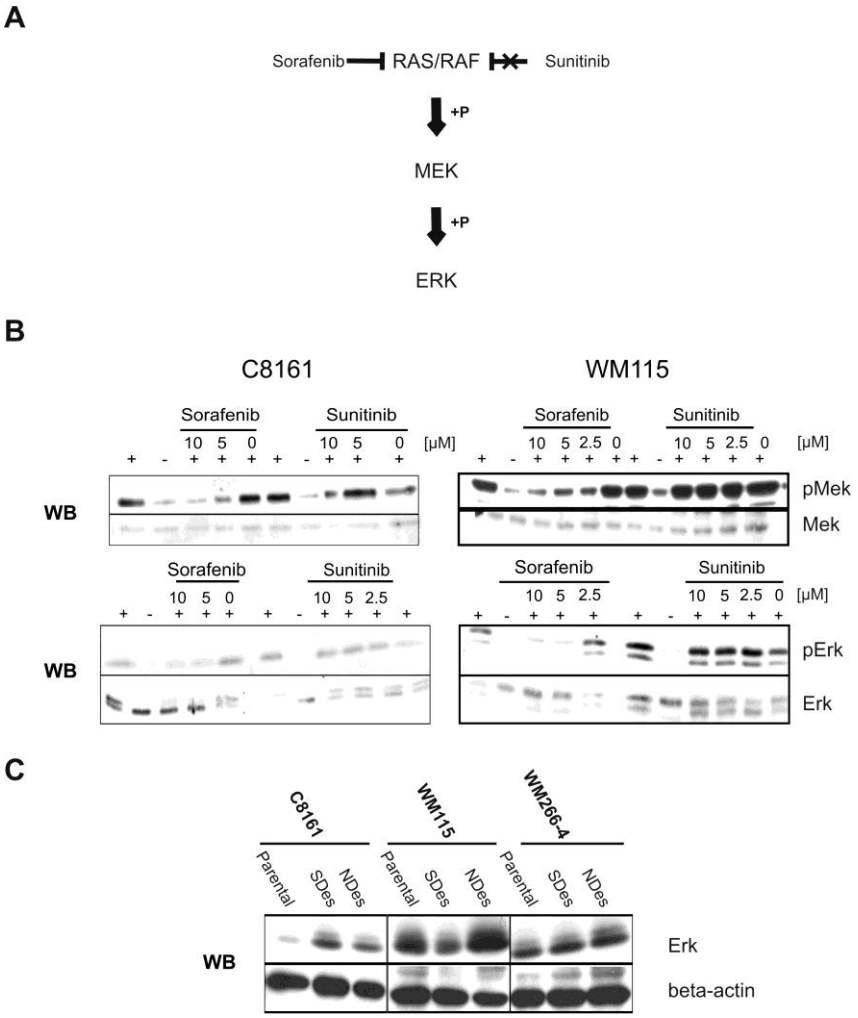


Figure 30: Sorafenib but not Sunitinib inhibited the Ras/Raf/Mek/Erk pathway. (A) Sorafenib, developed as a Raf-1 inhibitor, inhibits Raf-1 and putatively the phosphorylation of its downstream targets Mek and Erk. In contrast, Sunitinib has not yet been shown to inhibit Raf-1, Mek or Erk phosphorylation. (B) Sorafenib but not Sunitinib inhibited the phoshorylation of Mek and Erk in a dose-dependent manner in WM115 and C8161. Mek and Erk antibodies were used as a loading control. (C) The gene- (chapter IV.5.1) and protein-expression of Erk is increased in the highly Sorafenib desensitized cell lines C8161^{NDes} and WM115^{Ndes} compared to the respective parental cell lines. In WM266-4NDes, which displayed only a minor degree of Sorafenib desensitization, no altered expression compared to the parental cell lines was observed. Interestingly, Erk expression was as well elevated in C8161^{SDes} cells.

However, since gene-expression of MAPK3/ERK was increased in the Sorafenib desensitized melanoma cell lines (chapter IV.5.1), we also validated this observation on the protein-level (*Figure 30 C*). The protein-expression of Erk was elevated in the Sorafenib desensitized cell lines C8161^{NDes} and WM115^{NDes}, which demonstrated a high degree of Sorafenib desensitization. In contrast, no expression change was monitored for WM266-4NDes.

Interestingly, an increased Erk protein-expression was monitored for the Sunitinib desensitized cell line C8161^{SDes}. Summarized, the increased expression of the survival factor Erk in the Sorafenib desensitized cell lines might antagonize the inhibitory effects of Sorafenib on Ras/Raf/Mek/Erk signaling.

IV.6.2. Effects of Sorafenib and Sunitinib on JNK signaling

The role of the JNK pathways in regulating survival or cell death is controversially discussed. Initially, JNK was described as a stress response mediator of cell death [199]. However, recent findings demonstrated that JNK is a supporter of melanoma cell survival [200]. Moreover, our Sorafenib desensitization based gene-expression analysis identified the JNK signaling activators MAP3K7 and MAP3K11 as candidate genes that trigger Sorafenib insensitivity. Furthermore, a role of Sorafenib in the regulation of JNK signaling was postulated (*Figure* 31 *A*). To test this postulate in terms of Sorafenib resistance formation we monitored the phosphorylation levels of JNK1 and JNK2 in parental- and Sorafenib desensitized melanoma cell lines. Cells were treated with various Sorafenib and Sunitinib concentrations for 24 h. Western-blotting of total cell lysates and subsequent fluorescence detection was performed to evaluate the inhibitory effect of JNK1/2 phosphorylation of both drugs (*Figure* 31 *B*).

In the parental cell lines C8161, WM115 and WM266-4, Sorafenib decreased JNK1 phosphorylation in a dose-dependent manner. This effect correlated with the primary Sorafenib sensitivity. In C8161 cells, the inhibitory effect was visually detectable down to a Sorafenib concentration of 2.5 µM. In WM115 cells, a Sorafenib concentration of 5 µM was necessary to inhibit JNK1 phosphorylation. In WM266-4 cells, the inhibitory effect was detectable from Sorafenib concentrations of > 7.5 µM. Moreover, Sunitinib induced the phosphorylation in the primary Sunitinib insentive cell line C8161, while in the respective sensitive cell lines WM115 and WM266-4 a decreased phospho-level of JNK1 was observed.

In the Sorafenib desensitized cell lines, the ratio of phosphorylated JNK1 and JNK1-expression was approximately 2-fold higher that in the parental cell line. However, when these cell lines were treated with Sorafenib, its effect matched the one of the parental cell lines. In contrast, the effect of Sorafenib on JNK2 phosphorylation was different between the parental and Sorafenib desensitized cell lines. In parental C8161 cells, Sorafenib induced the JNK2 phosphorylation down to a concentration of 2.5 µM. On the contrary, a Sorafenib concentration of 10 µM was necessary to induce JNK2 phosphorylation in C8161^{NDes} cells.

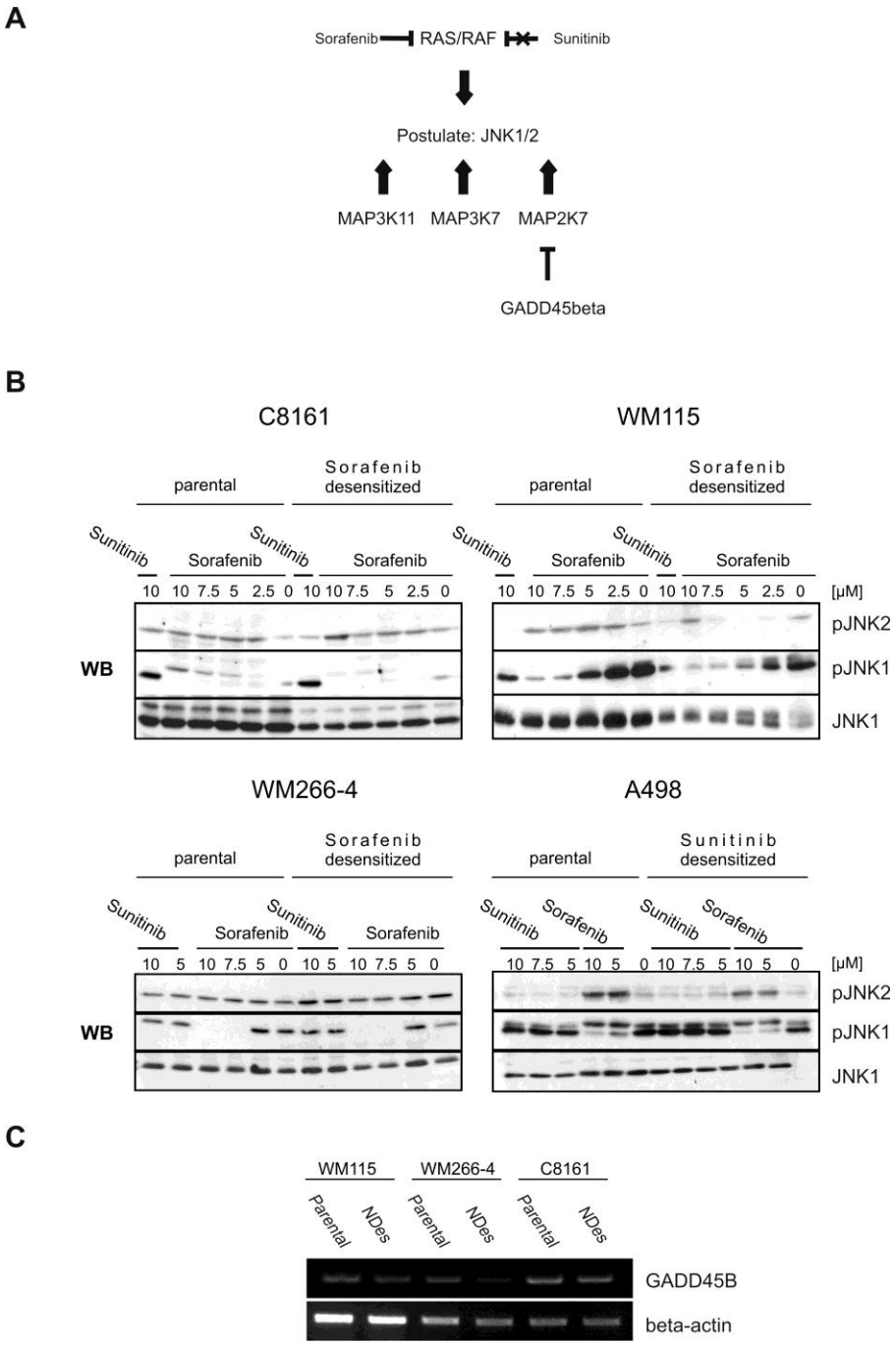


Figure 31: Sorafenib inhibited JNK1 and affected JNK2 phosphorylation. (A) Sorafenib putatively affects JNK signaling. JNK1/2 are activated by various MAP kinases. GADD45B is a repressor of signaling by inhibition of MAP2K7. (B) JNK1 phosphorylation is inhibited by Sorafenib. This effect is similar in parental and Sorafenib desensitized cell lines. JNK2 phosphorylation is differentially affected upon Sorafenib treatment when comparing parental- and Sorafenib desensitized cell lines. (C) GADD45B gene-expression is downregulated in Sorafenib desensitized- compared to their respespective parental cell lines.

In parental- and Sorafenib desensitized WM115 cells, this effect was even more pronounced. In parental WM115 cells, JNK2 phosphorylation was induced starting from a Sorafenib

concentration of 2.5 µM. In WM115^{NDes} cells however, JNK2 phosphorylation was decreased by Sorafenib concentrations ranging from 2.5 µM to 7.5 µM. Only when treated with 10 µM Sorafenib, a minor increase in JNK2 phosphorylation was observed. Note that there was no significant change of the JNK2 phosphorylation-levels of parental and Sorafenib desensitized WM266-4 cell lines. It is further noteworthy that similar effects of JNK1 and JNK2 phosphorylation were observed for the kidney cell lines A498 and A498^{SDes}. Here, JNK1 phosphorylation was inhibited by Sorafenib but not Sunitinib. Phospho-JNK2 levels were increased upon Sorafenib, whereas the phospho-level increase was less distinct in the Sunitinib desensitized cell line. Furthermore, Sunitinib had no effect on JNK2 phosphorylation. Finally, the expression of the MAP2K7 repressor GADD45B was decreased in the Sorafenib desensitized melanoma- compared to their parental cell lines, suggesting a role of GADD45B and MAP2K7 in the differential regulation of JNK2.

IV.6.3. Reduction of JNK signaling genes sensitizes melanoma cell lines to the cytotoxic effects of Sorafenib

Sorafenib treatment affected the JNK signaling pathway and the gene-expression of MAP3K11 and MAP3K14 was increased in Sorafenib desensitized- compared to respective parental cell lines. Hence, we postulated a role of MAPK8/JNK1, MAPK9/JNK2 and its activators MAP3K11 as well as MAP3K14 in triggering Sorafenib insensitivity. Furthermore, as the MAP2K7 repressor GADD45B was downregulated in the Sorafenib desensitized melanoma cell lines, also MAP2K7 was postulated as a candidate for Sorafenib insensitivity, although gene-expression analysis revealed a slight downregulation of MAP2K7 in the Sorafenib desensitized cell lines. To analyze the additional Sorafenib induced apoptosis (AdA) upon candidate gene-reduction compared to the control siRNA, apoptosis was determined using PI-staining after 72 h, flow-cytometry and evaluation of the subG1-peak. Gene knock-downs ranged between 50 % and 90 %, as estimated by scanning densitometry while the knock-down efficacy was similar in parental and the respective Sunitinib or Sorafenib desensitized cell lines.

In parental C8161 cells, MAPK8/JNK1 reduction did not have any effect on Sorafenib sensitivity. In contrast, C8161^{NDes} cells demonstrated an increased rate of Sorafenib induced

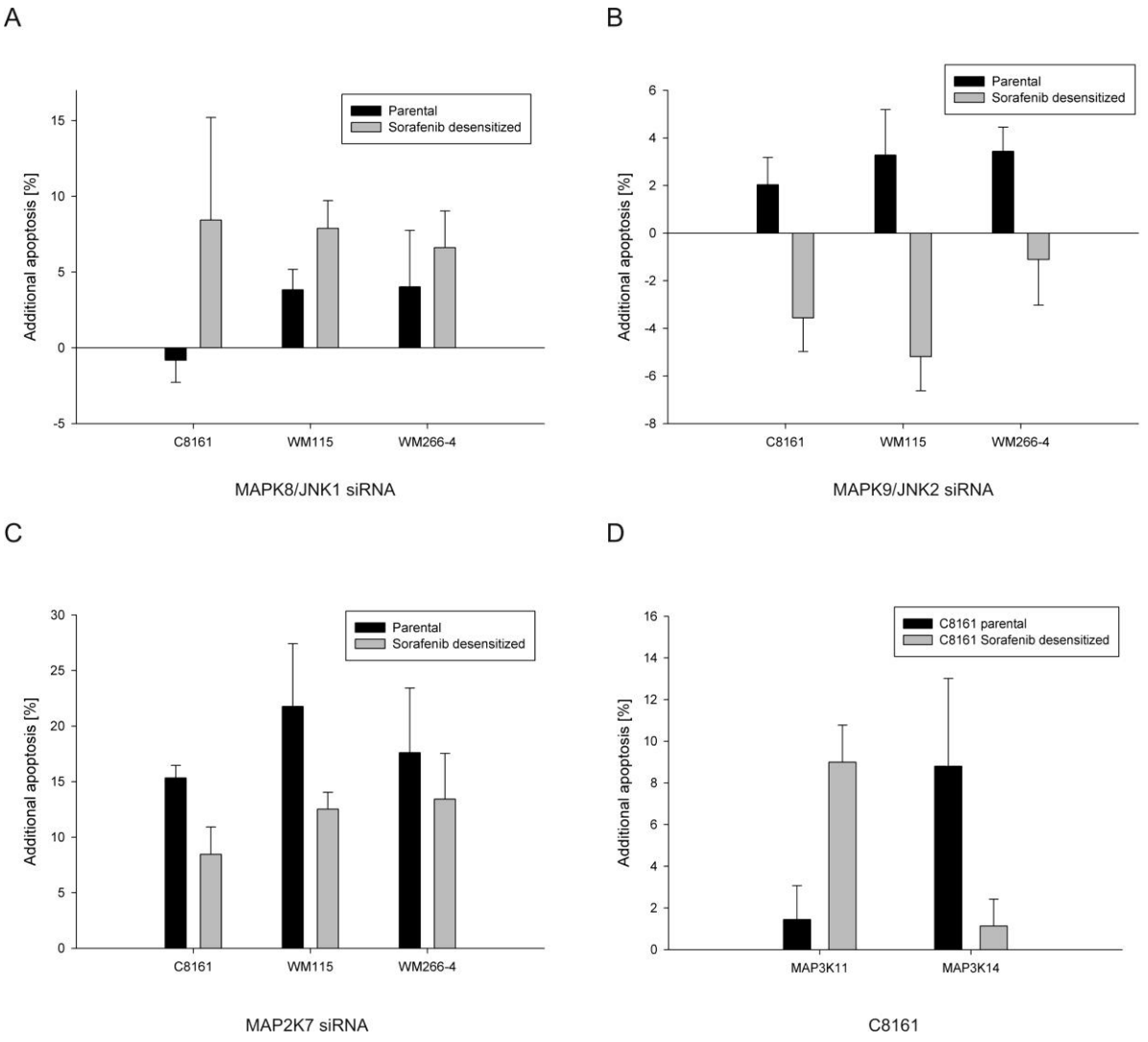


Figure 32: Reduction of JNK signaling genes triggers Sorafenib sensitivity. (A) Reduction of MAPK8/JNK1 increased the Sorafenib sensitivity of melanoma cell lines in terms of apoptosis induction. This effect was higher in the Sorafenib desensitized cell lines in correlation with the degree of desensitization. (B) MAPK9/JNK2 reduction resulted in a minor increase of the apoptosis inducing effect of Sorafenib. In contrast, the apoptosis inducing effect was reduced after JNK2 reduction in Sorafenib desensitized cell lines. (C) MAP2K7 reduction resulted in an increased apoptosis rate upon Sorafenib treatment. In line with the comparably decreased MAP2K7 gene-expression in Sorafenib desensitized cell lines, this effect was stronger in the parental melanoma cell lines. (D) In $C8161^{NDes}$ cells, MAP3K11 reduction led to an increased apoptosis rate upon Sorafenib treatment, while no effect was observed in parental C8161 cells. Reduction of MAP3K11 increased the Sorafenib apoptosis inducing effect in parental C8161 cells, while no effect was monitored in $C8161^{NDes}$ cells.

apoptosis (8.44 % ± 6.7 %; *Figure 32A*). Similar effects upon JNK1 reduction were monitored for the parental and Sorafenib desensitized cell lines WM115 and WM266-4 (AdA^{WM115}: 3.8 % ± 1.3 %; $AdA^{WM115NDes}$: 7.9 % ± 1.8 %; $AdA^{WM266-4}$: 4.1 % ± 3.7 %; $AdA^{WM266-4NDes}$: 6.6 % ± 2.4 %). The effect of MAPK9/JNK2 reduction on the cytotoxic effects of Sorafenib differed between the parental- and Sorafenib desensitized melanoma cell lines (*Figure 32 B*). In parental C8161, WM115 and WM266-4 cells JNK2 reduction resulted in a minor increase of Sorafenib sensitivity (ADA^{C8161}: 2.0 % ± 1.1 %; AdA^{WM115}: 3.8 % ± 1.3 %; $AdA^{WM266-4}$:

3.3 % ± 1.9 %) . In contrast, MAPK9/JNK2 reduction resulted in a decreased cytotoxic effect of Sorafenib in the Sorafenib desensitized cell lines C8161 and WM115 ($AdA^{C8161NDes}$: -3.6 % ± 1.4 %; $AdA^{WM115NDes}$: -5.2 % ± 1.4 %). In WM266-4^{NDes}, no significant change of apoptotic cell appearance was monitored.

MAP2K7 reduction resulted in an overall increased apoptosis rate of the melanoma cell lines (*Figure 32 C*). By comparison, this effect was higher in the parental- than in the Sorafenib desensitized cell lines. In case of the parental cell lines, the effect was highest for WM115-, followed by WM266-4 and C8161 cells (ADA^{C8161}: 15.3 % ± 1.1 %; AdA^{WM115}: 21.8 % ± 5.6 %; $AdA^{WM266-4}$: 17.6 % ± 5.6 %). The lowest AdA-value was monitored in $C8161^{NDes}$ cells (8.5 % ± 2.4 %). Moreover, $WM115^{NDes}$ and WM266-4^{NDes} cells demonstrated AdA-values of 12.5 % ± 1.5 % and 13.4 % ± 4.1 %, respectively. Next to MAPK7-, MAP3K11 reduction led to an increased Sorafenib induced apoptosis effect in $C8161^{NDes}$ cells (9.0 % ± 1.8 %), while no effect was observed C8161. An antagonistic pattern was monitored for the knock-down of MAP3K14. Here, parental C8161 cells displayed increased Sorafenib sensitivity upon MAP3K14 reduction (8.8 % ± 4.2 %), while in $C8161^{NDes}$ cells, the MAP3K14 had no effect on Sorafenib sensitivity.

V. Discussion

V.1. Cell response upon Sunitinib and Sorafenib treatment

V.1.1. *In vitro* Sunitinib and Sorafenib sensitivity is cancer type independent

The receptor tyrosine kinases and their downstream signaling pathways, which are inhibited by Sunitinib or Sorafenib, appear to be active in many tumors, suggesting that Sunitinib and Sorafenib may have a broad spectrum of activity [72]. Thus, we tested randomly picked cell lines from various cancer tissue origins in regard to the apoptosis inducing- and proliferation inhibiting effects, i.e. sensitivity, of both drugs. Our phenotypic screen revealed that the *in vitro* sensitivities of both drugs are not determined by the cancer type. However, this finding was surprising for the kidney carcinoma cell lines CAKI1 and CAKI2, which showed only low sensitivies for both drugs, since these have been already approved for the treatment of RCC. This might be explained by a major drawback of the *in vitro* cell system itself, as it excludes the inhibition of angiogenesis, which is one important mode action of both drugs [78,91]. Nevertheless, while excluding angiogenesis, the screen supports the postulate that both drugs exhibit their anti-cancer effects in a broad spectrum of cancer types. Furthermore, the pool of tested cell lines met two requirements, which were essential for subsequent gene-expression analyses: (1) the cell line pool demonstrated a wide range of Sunitinib and Sorafenib sensitivities, which is an inevitable variable for a cancer type independent but sensitivity based gene-expression analysis. (2) The pool consisted of cell lines, which demonstrated a higher, lower and similar susceptibility for either of both drugs, which is essential for the comparison of the drug-specific mode of action. Hence, the cell line pool presents an excellent model system to determine the pro-apoptotic and anti-proliferative effects of both drugs in a contrastable manner.

V.1.2. Comparision of the transcriptional cell responses upon Sunitinib or Sorafenib treatment

Sunitinib and Sorafenib target and thereby inhibit multiple tyrosine kinases. With the increasing number of kinase targets, the mode of action of both drugs is higly complex. Thus,

we based the comparative analysis of both drugs on the gene-expression changes in correlation to the above mentioned drug-dependent phenotypes (apoptosis induction), postulating that these indirect effects might be the superior option to determine the different sensitivities for both drugs. Furthermore, genes with differential gene-expression changes in primary sensitive and insensitive cell lines might be surrogate-marker candidates.

In general, our gene-expression analysis revealed genes, the expression-profiles of which correlate with the primary Sunitinib or Sorafenib sensitivity. For both drugs, gene-groups were identified, in which genes were downregulated in the sensitive- and upregulated in the insensitive cell lines upon drug treatment, while other groups demonstrated an antagonistic expression pattern. In case of Sunitinib treatment, the trigger, defining down- or upregulation was defined by a Sunitinib LD_{50}-value of approximately 12-13 µM. Interestingly, a similar LD_{50}-value defined the threshold of up- or downregulated genes for the Sorafenib treatment as well, indicating that in *in vitro* settings, cell lines can be classified by sensitivity independently of drug and cancer type.

Upon Sunitinib treatment, downregulated genes (in sensitive cell lines) constitute the majority of the identified genes, arguing that Sunitinib induces cell death rather by down- than upregulation of multiple genes. These downregulated genes are mostly involved in the modulation of apoptosis/survival. As an example, the signaling integrator AKT1 can be named. In mammals, no modified or mutated AKT genes have been found so far. However, a number of studies have discovered gene amplifications in human cancers [169], suggesting that downregulation of AKT1 has a major impact on the cell survival effect in the cells upon Sunitinib treatment. Moreover, multiple studies over the last decade have conclusively shown that AKT is a critical factor for cell survival [201]. AKT1 has been shown to activate IKKA and thereby activate NFκB signaling [202-203]. The involvement of NFκB signaling is further supported by our expression analysis, since IKKA, ATM, PKD1 and CAV1, which are all positive regulators of NFκB [204-206], were downregulated in the primary Sunitinib sensitive cell lines. The importance of AKT1 and CAV1 was further demonstrated by clinical studies of Campell, Jasani *et al* [207], which correlated the co-expression of AKT1/CAV1 or mTOR/CAV1 with decreased primary kidney cancer- and mRCC patient survival. Hence, since FRAP1, which encodes for mTor, was also downregulated upon Sunitinib treatment the AKT1/CAV1/FRAP1 downregulation might explain the clinical benefit of Sunitinib in mRCC patients. An additional function of Akt is the phosphorylation/activation of the pro-survival cAMP-response element binding protein (CREB) transcription factor [208]. Next to AKT1, two

other genes identified in our screen (ATM and MAPK11) were shown to modulate CREB activity [209-210], suggesting a potential role of CREB activation in the modulation of the apoptotic threshold within cells upon Sunitinib treatment. Interestingly, three genes that were downregulated upon Sunitinib treatment (PTPNR2, GRK5 and PKD1) are associated with diabetes, which might explain a side-effect, the decrease blood in glucose levels and the remission of type I and II diabetes in patients, of Sunitinib [211-212].

Summarized, as these genes are downregulated in the Sunitinib sensitive cell lines a shift of the apoptotic threshold as an underlying mechanism of the Sunitinib induced cytotoxic effects can be suggested. In contrast, the antagonistic expression pattern of the primary Sunitinib insensitive cell lines suggested an antagonistic cell response in terms of the apoptotic threshold. The exact underlying mechanism, whether a cell line is Sunitinib sensitive or insensitive, remains elusive. Nevertheless, the identified genes might account as potential surrogate markers for the Sunitinib treatment of patients. However, it is noteworthy that the expression fold-changes of the identified genes mostly ranged between three and four suggesting that the gene-expression alteration of a single gene is not sufficient to modulate the Sunitinib induced apoptotic threshold. Moreover, our data suggest that a network of expression alterations might trigger the underlying mechanism of the cytotoxic effect of Sunitinib. Consequently, a single gene might be of only limited use as a surrogate marker. Hence, rather the expression of various genes associated with AKT-, NFκB- or CREB signaling should be the fundament for further *in vivo* studies to determine surrogate markers for Sunitinib.

Upon Sorafenib treatment, down- or upregulated genes (in sensitive cell lines) displayed a similar contribution in terms of gene-quantity. Comparing the transcriptional responses of Sunitinib and Sorafenib, only a single gene, PLAUR, demonstrated a similar gene-expression change indicating a completely different mode of action of both drugs. Moreover, while Sunitinib predominantly decreased the gene-expression of pro-apoptotic genes in the Sunitinib sensitive cell lines, Sorafenib treatment primarily led to a gene-expression increase rather than a decrease of the respective genes. Like for Sunitinib, the genes identified upon Sorafenib treatment can be associated with intra-cellular survival/apoptosis signaling. Positive regulators of survival like PAK4 [213], PINK1 [214], CDC2 [215] and PRKAA2 were upregulated in the Sorafenib insensitive and downregulated in the respective sensitive cell lines. Moreover, positive regulators of apoptosis like SLK, MEKK1 and the putative tumor suppressor LATS2 were upregulated in the Sorafenib sensitive and downregulated in the Sorafenib insensitive

cell lines. Summarized, the differential expression of these genes induced by Sorafenib treatment correlated with the determined Sorafenib induced phenotype, suggesting their role in the modulation of the apoptotic threshold. Surprisingly, also genes like CAMKIV, BCL2L and FGF2, which represent negative regulators of apoptosis [216-217], displayed increased expression in the sensitive cell lines upon Sorafenib treatment. This is in contrast to the observed Sorafenib dependent phenotype and indicates a fine balance of a complex network of genes that determines Sorafenib sensitivity. Furthermore, similar observations were made in terms of NFκB signaling. In the Sorafenib sensitive cell lines, positive regulators of NFκB signaling like PRKAA2 [218] and PKCZ [219] were down- and negative regulators like PTPN4 [220] as well as MEKK1 [221] were upregulated upon Sorafenib treatment. Contrarily, Sorafenib treatment also resulted in an upregulation of MAPK6 and TBK1, which are both activators of NFκB signaling [183,222]. Whether the regulation of positive or negative regulators genes eventually results in the activation or inhibition of the NFκB signaling is indicated by the altered expression of another gene upon Sorafenib treatment, the plasminogen activator urokinase receptor PLAUR. The expression of PLAUR was downregulated in all cell lines of the model system. Various studies have shown that NFκB signaling promotes PLAUR expression [223-224] indicating that Sorafenib treatment triggers the inhibition of NFκB signaling. Moreover, the Plaur/Plau system functions in an anti-apoptotic manner. In PLAU -/- mice for example, T241 fibrosarcoma implanted cells exhibited decreased proliferative and increased apoptotic indices, suggesting that alterations in host expression of these components may affect the balance between tumour cell death and proliferation [225]. Moreover, anti-Plau antibodies that block the binding of PLAUR to PLAU, substantially decreased the level of phosphorylated Erk and apoptosis was promoted, demonstrating that endogenous Plau is a major determinant of Erk activation and protection from apoptosis [226]. Additionally, a positive correlation of the pro-survival AKT/PI3K pathway and PLAUR expression was shown in glioblastoma cells [227], which support the crucial role of PLAUR in regard of Sorafenib induced apoptosis. Note that Sunitinib also induced the downregulation of genes, which act as activators of NFκB signaling and consequently, PLAUR expression was decreased in all Sunitinib sensitive cell lines but U118, while expression was not changed in the Sunitinib insensitive cell lines. These findings indicate an overlaying mechanism of Sunitinib and Sorafenib to modulate the survival/apoptosis ratio. However, upon Sorafenib treatment, the gene-expression of PLAU, the ligand for PLAUR, was also decreased, while the respective effect was not observed upon Sunitinib treatment, suggesting a potentiated impact of the

PLAUR/PLAU system due to the treatment with Sorafenib. Finally, PLAUR expression is associated with breast cancer. Targeting the PLAUR/PLAU expression results in increased apoptosis in rat breast cancer [228] and inhibited invasion in breast cancer cell lines [229]. The HER2 and ESR1, for which overexpression is a frequent characteristic of breast cancer, were also downregulated upon Sorafenib treatment, which argues for an underlying mechanism for the observed clinical benefit of Sorafenib in breast cancer patients [230]. Concluded, as the expression of the identified genes was altered in correlation to Sorafenib sensitivity, a shift of the apoptosis/survival ratio as an underlying mechanism of the cytotoxic effects of Sorafenib is suggested. Hence, those identified genes might be potential Sorafenib specific surrogate-markers. By direct comparison, there is almost no correlation of the identified genes between Sorafenib and Sunitinib treatment. Besides, since both drugs alter the expression of genes involved in similar signaling pathways such as NFκB- or AKT signaling, a converging mechanism can be assumed.

Looking forward, it is essential that these *in vitro* data upon Sunitinib or Sorafenib treatment are validated in an *in vivo* model system. Further, this *in vivo* model system would presumably help to reduce the long list of identified genes for an advanced discrimination of a beneficial or failing therapy upon treatment with both drugs. Moreover, the cause for the transcriptional response upon Sunitinib and Sorafenib treatment remains elusive. Our RTK protein-array data, in which Sunitinib and Sorafenib treatment displayed similar changes of the phosphorylation-levels, indicate that RTK phosphorylation-levels are not likely the cause for the dissimilar gene-expression pattern. Since Sunitinib and Sorafenib target multiple other kinases, a comparative phosphoproteomics analysis using mass spectrometry might fill these gaps of knowledge.

V.1.3. Establishment of Sunitinib and Sorafenib desensitized cell lines

Drug resistance remains a major factor in limiting the effectiveness of cancer therapy, resulting in an overall poor prognosis for patients. To date, there is no knowledge about resistance mechanisms against multi-targeted kinase inhibitors such as Sunitinib or Sorafenib. Drug resistance might arise from the heterogeneity of the tumor [231-232], whereas the underlying mechanisms can be manifold. Therefore, it is generally assumed that due to the instability of the cancer cell genome resistance is formed by selection of cancer cells that

exhibit genetic alterations, which compensate the cytotoxic or cytostatic effects of the drug. To test this postulate, we established model-systems of parental and Sunitinib or Sorafenib desensitized cancer cell lines, as for both indications the clinical and pre-clinical benefit of Sunitinib was shown. Notably, we did not use resistant clones as polyclonal desensitized cell lines represent the superior option to mimic resistance formation in patients.

In accordance with these hypotheses, our study demonstrates the existence of cell sub-populations within a parental cell line population, which can be selected by Sunitinib or Sorafenib treatment. These sub-populations demonstrate higher Sunitinib LD_{50}-values, indicating genetic alterations, whereas the degree of desensitization varies. Primary more sensitive cell lines display a higher degree in desensitization and vice versa, which might be a limitation of the selection procedure. Further, upon Sorafenib treatment, other sub-populations out of the parental cells are selected. These Sorafenib selected sub-populations display not only remarkably higher LD_{50}-values against Sorafenib but also a decrease in Sunitinib sensitivity. This effect was also observed in the Sunitinib desensitized cell lines, which demonstrate less sensitivity against Sorafenib than the respective parental cell lines. This decrease was by trend less distinct, when compared to the Sunitinib selected sub-populations, which suggests the existence of multiple sub-populations within the parental population, which are specifically selected by either Sunitinib or Sorafenib. Those sub-populations, while their genetic backgrounds differ, might exhibit converging mechanisms to compensate Sunitinib induced cytotoxic effects. Since the selected sub-populations displayed varying degrees of desensitization for Sunitinib and Sorafenib, they compose an well suited model system to identify the underlying mechanisms of drug resistance formation in a drug contrastable manner.

V.1.4. Correlation of Sunitnib resistance and short term drug response

The aim of this study was to gain insights into the molecular understanding of resistance mechanisms against Sunitinib in cancer therapy before the clinical reality of resistance formation emerges. By correlating Sunitinib desensitization and changes in gene-expression, we provide first evidence of strong correlative gene-expression alterations and Sunitinib resistance formation within a kidney carcinoma and melanoma model system. Regarding Sunitinib resistance, most of the upregulated genes identified in the expression analysis were

cancer type specific and can be generally associated with survival or resistance mechanisms. Moreover, treatment with either of the drugs led to downregulation of genes, which are involved in survival processes. Comparing the Sunitinib cell response and the potential resistance genes, only one gene displayed an antagonistic expression pattern in kidney carcinoma or melanoma cell lines. In Sunitinib desensitized kidney cells, DAPK3 was upregulated, while in primary Sunitinib sensitive cells, DAPK3 was downregulated upon Sunitinib treatment, suggesting that differential DAPK3 expression impairs Sunitinib´s mode of action in a pro-apoptotic manner. This finding is surprising as DAPK3 is a mediator of apotosis [233] in an ERK-DAPK3 interplaying manner [234], suggesting that Sunitinib lessens its own pro-apoptotic effect. However, another function of DAPK3 is the interaction with AT-4 [235] via its leucin-zipper domain, which is located at the C-terminus. ATF-4, a member of the ATF/CREB family, seems to function as a negative regulator of transcription by interfering with ATF1/2 [236]. Accordingly, it is conceivable that these negative activities can be modulated by DAPK3 [237]. In case of Sunitinib treatment with decreased DAPK3 levels this would imply a transcriptional inhibition of the respective downstream target genes, while in Sunitinib desensitized kidney cell lines, an antagonistic effect can be suggested. The importance of transcriptional regulation of ATF/CREB family members in terms of Sunitinib sensitivity is further supported by three other genes, PRKX, CTNNB1 and TYRO3, which demonstrate elevated expression in sub-populations selected from kidney- and melanoma cell lines. Since Sunitinib treatment causes the downregulation of AKT1, ATM and MAPK11, which are activators of CREB [208-210], a bypassing mechanism of those genes to promote Sunitinib resistance is indicated. The functions and relevance in Sunitinib sensitivity of PRKX, CTNNB1 and TYRO3 are further discussed in the next chapter.

Other interesting antagonistic correlations of potential resistance genes and genes downregulated upon Sunitinib treatment can be attested for NFκB signaling and angiogenesis. Upon Sunitinib treatment, seven genes, which are known positive regulators of NFκB signaling, are downregulated, suggesting the inhibition of this pathway and thus a change of the apoptotic threshold as a mechanism of Sunitinib to induce apoptosis. In line with this observation, five genes that are as positive regulators of NFκB signaling were upregulated in the Sunitinib desensitized kidney sub-populations. Combined, these data highlight the particular importance of the NFκB signaling pathway as a key regulator of Sunitinib in- and sensitivity. Additionally, the CRE-binding protein (CBP) was shown to cooperate with NFκB for synergistic transcriptional activation [238]. Hence, a converging mechanism of NFκB and

CREB in terms of Sunitinib resistance formation is indicated. Furthermore, these findings would suggest a clinical benefit of a combinatorial treatment of Sunitinib and proteasome inhibitors like PS-341 that block NFκB activation and thus may prevent or delay the formation of Sunitinib resistance in kidney carcinoma. A growth inhibiting effect of PS-341 has already been shown *in vitro* and xenografts as single- or combinatorial treatment with temozolomide for melanoma [239-241]. However, only IRAK1, a positive modulator of NFκB signaling, was elevated in the melanoma desensitized sub-populations. Hence, no role of NFκB signaling in Sunitinib resistance or a potential benefit of a combinatorial therapy of Sunitinib and NFκB inhibitors to prevent Sunitinb resistance formation is indicated. Another mechanism for Sunitinib resistance in melanoma might be exhibited by STK39/SPAK. SPAK reduction using siRNA was shown to increase the sensitivity of HeLa cells to TRAIL induced apoptosis [242]. Conversely, upregulation of STK39/SPAK might inhibit apoptotic events in the cells. Moreover, WNK2 and WNK4 are upregulated in the Sunitinib desensitized melanoma sub-populations, of which the activation of SPAK has been shown [243], indicating that overregulated SPAK is also strongly activated in those cells. Another group of genes, which are upregulated in kidney- and melanoma desensitized sub-populations, are dual-specificity phosphatases, which dephosphorylate various MAPKs [244], indicating that deregulation of MAPK signaling is a major condition in Sunitinib resistance formation.

While the used *in vitro* cell lines are per se of marginal use for the study of angiogenesis, our apoptosis correlated gene-expression analysis nevertheless identified genes that exhibit roles in angiogenesis. Upon Sunitinib treatment, three positive regulators of angiogenesis (SRPK1, CDC2L5 and ITGB3) were downregulated in primary sensitive cell lines, suggesting their role in the underlying mechanism of Sunitinib to inhibit angiogenesis. In contrast, seven promoters of angiogenesis (CSF1, VEGFA, SHB, EPHA2, TBK, C-MYC and NRP1) were upregulated in the Sunitinib desensitized cell lines, suggesting that induced angiogenesis might be an additional factor for Sunitinib resistance *in vivo*. However, this remains purely speculative as our in vitro model lacks the feature to study angiogenesis and hence no correlation of gene-expression and phenotype can be made. Nevertheless, these findings point towards a further fruitful analysis of angiogenesis on a functional level in an *in vivo* model. In melanoma, angiogenesis is also a hallmark of melanoma progression and anti-angiogenic agents have been infrequently tested in patients with advanced melanoma [245]. Hence, the increased expression of MMP2, which was observed in the Sunitinib desensitized melanoma

sub-populations, might be of interest, since interaction of MMP2 and ITGB3 facilitate vascular invasion on the endothelial cell surface [246].

Another interesting observation was made by the comparison of the RTK phosphorylation-levels of parental WM115 cell and the respective Sunitinib desensitized sub-population. Here, the phospho-levels of the IGFR1 and ErbB3 were more than two-fold upregulated. The IGF-1R is implicated in various cancers and its anti-apoptotic effects can cause resistance against chemotherapeutics and radiotherapy [247]. In the particular case of melanoma, IGF-1R antisense oligonucleotides have been shown to inhibit cell survival *in vitro* and growth of FO-1 melanoma xenografts in nude mice [248]. Next to the IGF-1R, ErbB3 is known as a poor prognosis factor in melanoma [249]. Next to the RTKs with increased phosphorylation-states, the EphA4 and FGFR3 demonstrated a two-fold decreased phosphorylation-level in $WM115^{SDes}$ cells. The EphA4 is suggested as a tumor suppressor in melanoma cells, as its expression is downregulated in aggressive melanomas compared to melanocytes [250]. The second RTK with monitored decreased phosphorylation was the fibroblast growth factor receptor 3 (FGFR3, fold-change: 2.7). An apoptotic effect of FGFR3 has been shown in chondrocytes, whereas the effect was chondrocyte- rather than an FGFR3-specific [251]. Moreover and in contrast to aforementioned observations, the FGFR3 accounts as an oncogene and thus displays an antagonistic function regarding the phenotype of $WM115^{SDes}$ cells. Interestingly, the RTKs that are direct targets of Sunitinib like the c-KIT, PDGFRs, c-Ret or VEGFR1 did not exceed phosphorylation two-fold changes. Nevertheless, it is noteworthy that their phosphorylation levels were upregulated as well (~1.5 fold), indicating a potential fine tuning in the emergence of Sunitinib resistance in melanoma. Summarized, these four RTKs might change the apoptotic threshold of the $WM115^{SDes}$ cells in an anti-apoptotic manner.

Concluded, the gene-expression data of the response to Sunitinib treatment and Sunitinib desensitized sub-populations suggest that differential expression of NFκB signaling members, angiogenesis related- and MITF related genes (*Chapter V.1.5*) plays a major role in Sunitinib sensitivity and resistance formation. Sunitinib resistance formation is likely based on the modulation of the apoptotic threshold by multiple factors rather than by mechanisms that antagonize Sunitinibs direct inhibitory effects, which correlates with the observed Sunitinib sensitivity dependent phenotypes. This was further supported by our phospho-array data, which did not display any correlation between the altered RTK phosphorylation states upon Sunitinib or Sorafenib treatment and drug sensitivity. Further, the modulation of the apoptotic

threshold is the most plausible explanation for the observed decreased Sorafenib sensitivity of the Sunitinib desensitized sub-populations, since this modulation is apparently a rather drug-unspecific effect. Accordingly, a sequential therapy of Sunitinib and Sorafenib might be an unpromising strategy to prevent Sunitinib resistance.

V.1.5. PRKX, TTBK2 and RPS6KA6/RSK4 trigger Sunitinib insensitivity

The serine/threonine kinases PRKX, TTBK2 and RSK4 as well as IRAK1 and DUSP4 demonstrated elevated mRNA- and protein expression in Sunitinib desensitized kidney carcinoma and melanoma cell lines suggesting their tissue independent but Sunitinib dependent role in Sunitinib resistance formation. As there was no correlation in the protein expression of these genes observed in the Sorafenib desensitized cell lines, a Sunitinib specific resistance mechanism is indicated.

To date, no association of PRKX, which is family member of cAMP-dependent kinases, with apoptotic signaling has been described so far. Nevertheless, PRKX is involved in renal development, regulating epithelial cell migration, uretic bud branching and induction of glomeruli formation [252], indicating its oncogenic potential. Moreover, PRKX is capable of auto-phosphorylation and activating CREB-dependent transcription *in vitro* [253], whereas the transcription factor CREB induces the expression of the microphthalmia-associated transcription factor (MITF) [254]. Mutations and/or aberrant expression of several MITF family member genes have been reported in papillary renal cell carcinoma (TFE3, TFEB) and melanoma (MITF) which suggests a role of the MIT-family in oncogenesis [135-137]. It is further suggested that de-regulation of MITF through amplification or other mechanisms may preserve a critical lineage survival function in melanoma [134]. As our expression analysis revealed the correlation of elevated PRKX expression and Sunitinib resistance formation, we consequently postulate PRKX´s role in this context. This postulate is supported by the monitored correlation between increased TYRO3- and β-catenin expression and Sunitinib resistance formation in melanoma. TYRO3 was identified as a regulator of MITF, the knock-down of which inhibited tumorigenesis *in vivo* [255]. β-catenin serves as a positive regulator of MITF expression in melanoma [256]. Unlike PRKX and TYRO3 however, β-catenin not only displayed a correlation of increased expression and Sunitinib- but also Sorafenib resistance formation, suggesting a common mechanism to avoid Sunitinib´s cytotoxic effects. Additional

evidence for PRKX´s crucial role in Sunitinib resistance formation was obtained in our knock-down studies. Here, the specific reduction of PRKX resulted in the Sunitinib sensitization of kidney carcinoma and melanoma cell lines. In the parental cell lines, this sensitization effect was only minor to moderate, depending on the endogenous PRKX expression. In the Sunitinib desensitized cell lines however, this effect in correlation to the elevated endogenous expression was strikingly higher, which indicates an expression based mechanism of PRKX to trigger Sunitinib resistance. This proposed mechanism is backed up by reduction of PRKX in Sorafenib desensitized cell lines. Here, PRKX reduction resulted in Sunitinib desensitization matching the effect in the parental cell lines, which was in line with the unchanged gene-expression.

Next to CREB, PRKX is shown to phosphorylate fetal and adult tau-protein [257], which is also a substrate of TTBK2. Like for PRKX, no association of TTBK2 with apoptosis or survival has been described so far. TTBK2 is one of two isoforms within the TTBK family, which belongs to the casein kinase 1 (CK1) group and functions by phosphorylating tau-protein on Serine 201 and 210 [258-259]. Since PRKX and TTBK2 are both phosphorylating the tau-protein, a redundant role is indicated. Like for PRKX, we observed a strong tissue origin independent correlation of TTBK2 expression and Sunitinib resistance formation and specific reduction of TTBK2 resulted in increased Sunitinib sensitivity. This effect was small in the parental cell lines and strikingly higher in the Sunitinib desensitized cell lines, correlating with the respective endogenous TTBK2 expression. Compared to the knock-down of PRKX-, TTBK2 reduction displayed an overall smaller effect on triggering Sunitinib sensitivity, which can be explained by a redundant TTBK1 mechanism. Surprisingly, in the Sorafenib desensitized cell lines the effect of TTBK2 reduction was in no correlation to the observed protein expression, suggesting a yet elusive mechanism of Sorafenib induced Sunitinib resistance formation.

The role of RSK4, a member of the RSK-family, in apoptotic processes is controversially discussed. RSK4, which is present in many tissues, participates in p53-dependent cell growth arrest and furthermore, an inhibitory role of RSK4 during embryogenesis was suggested [260-262]. On the contrary, aberrant expression of RSK4 has been observed in breast cancer, indicating an oncogenic role of RSK4 [263]. The Rsk4 protein appears predominantly cytoplasmic [260] and no translocation into the nucleus has been reported yet. Nevertheless, Rsk1 and Rsk2 have been shown to activate several proteins of the Ras/MAPK signaling cascade including Creb and Mitf, which might be a link to the above mentioned PRKX/MITF survival mechanism [147-151]. Our Sunitinib resistance correlating gene-expression data gave

further support for RSK4´s oncogenic role and drug resistance formation. Like for PRKX and TTBK2, a strong correlation of endogenous expression and the Sunitinib sensitizing effects was monitored. It is noteworthy that RSK4 expression was not elevated in C8161^{SDes}. Further, its reduction in parental and Suntinib desensitized C8161 equally triggered Sunitinib sensitization, suggesting a fine-tuning role of RSK4 in the formation of Sunitinib resistance. While PRKX, TTBK2 and RSK4 reduction triggered Sunitinib sensitivity, their reduction also resulted in an increased Sunitinib induced anti-migratory effect in A498 cells. In line with the data for Sunitinib induced apoptosis, the effect of the reduction was highest for PRKX, followed by TTBK2 and RSK4. Interestingly, there was no correlation between this effect and endogenous gene-expression in parental and Sunitinib desensitized A498 cells, which might be due to the direct effect of these genes on migration [264-265]. Nevertheless, these data indicate that PRKX, TTBK2 and RSK4 not only affect Sunitinib induced apoptosis but also function in triggering the tissue independent overall Sunitinib insensitivity.

Summarized, the correlation of the Sunitinib dependent phenotype, gene-expression and knock-down studies revealed a crucial role of PRKX, TTBK2 and RSK4 in triggering Sunitinib resistance formation. While the underlying mechanism remains elusive, our data suggest that transcriptional regulation of the three kinases might play an important role in Sunitinib resistance formation. Since PRKX, TTBK2 and RSK4 are not directly targeted by Sunitinib [76] (unpublished), development of specific or multi-targeted inhibitors for these genes for combinatorial therapy might circumvent or substantially delay Sunitinib resistance formation and enhance survival prognosis. These kinase-inhibitors might moreover boost the potential benefit of Sunitinib for the treatment of melanoma, where Sunitinib in combination with chemotherapeutics like Temodar® has yet failed to improve the overall progression free survival of patients.

V.1.6. Correlation of Sorafenib-, Sunitinib resistance and drug response

In order to compare the mode of actions of Sunitinib and Sorafenib, we also focused our attention, next to the transcriptional cell response, on the resistance mechanisms against Sorafenib. Disappointingly, the available kidney cell lines demonstrated primary low Sorafenib sensitivities and thus were not applicable for a contrastable gene-expression analysis with the Suntinib desensitization. Conversely, the melanoma cell line population,

with its primary antagonistic sensitivities against either of both drugs and varying degrees of Sorafenib desensitization marks an suited model-sytem to compare the fundamental resistance mechanisms of Suntinib and Sorafenib. Like Sunitinib, Sorafenib did not meet the primary endpoint of a clinical phase III trial, in which Sorafenib in combination with carboplatin and paclitaxel was tested in melanoma [266]. Nevertheless, today multiple clinical trials of Sorafenib in combinatorial therapy with agents like interleukin-2 are ongoing [86]. Thus, this study might give advanced knowledge, whether those clinical studies are reasonable.

The synoptic sight of the altered gene-expression patterns of Sorafenib- and Sunitinib desensitized sub-populations in comparison to the parental melanoma cell lines displays that the expression of only three genes, IRAK1, CSK and CTNNB1, which is an effector of canonical WNT signaling, are similary altered. The importance of the WNT pathway as a trigger of Sorafenib resistance is further supported by the expression of the receptor related tyrosine kinases (RYK), CSNK1A1, CSNK1E and CSNK1G2 that are also associated with the WNT pathway. Of particular interest in this context might be RYK. RYK mRNA is expressed in a broad range of mammalian tissues, both during development and in adults [267-268]. Furthermore, the observation that Ryk-deficient mice have a similar phenotype to mice deficient in EphB3 receptor tyrosine kinases suggests that RYK may interact with Eph-receptor signaling [269]. In line with this suggestion, the EPHB3 displays increased expression in the Sorafenib desensitized sub-populations. This is further supported by the increased expression of CD44, IKKB, the EGFR and PLAUR in Sorafenib desensitized sub-populations, since these are target genes of WNT/CTNNB1 signaling (http://www.stanford.edu/~rnusse/pathways/targets.html). Concluded, a similar mechanism of these genes in the formation of Sorafenib and Sunitinib resistance is suggested, since CTNNB1 is an inducer of MITF [256], acting as a transcription factor.

However, comparing just the melanoma cell lines in terms of functionality, most of the identified genes differ. Another interesting Sorafenib resistance mechanism might by based on altered centrosome- and spindle assembly, since PLK-1, AURKA, AURKB and BUB1, were identified in our screen. Specific reduction of PLK-1 using siRNA resulted in an increased apoptosis in both parental and Sorafenib desensitized cell lines, indicating its pro-survival role in melanoma. Consequently, we used Poloxin®, a PLK-1 inhibitor, in combination with Sorafenib and found a synergistic effect to induce apoptosis (data not shown), which supports the role of PLK-1 as a potential target for combinatorial therapy with Sorafenib. Additionally, the increased expression of NEK2, which is an activator of

CTNNB1, and CTNNB1 itself are associated to centrosome separation [188], indicating that CTNNB1 plays a crucial dual role in Sorafenib resistance formation by linking two cellular processes. A third mechanism might be reasoned in an activation of NFκB signaling, since 14 NFκB associated genes are upregulated in the Sorafenib desensitized sub-populations. Moreover, the expression of TTBK1 was increased in Sorafenib desensitized melanoma sub-populations, mirroring the TTBK1 expression observed in Sunitinib desensitized kidney cells. This increase in expression hints to a converging mechanism of Sorafenib and Sunitinib resistance, which is further supported by our knock-down experiments, where TTBK2 reduction resulted in altered apoptosis rates upon Sunitinib treatment in Sorafenib desensitized sub-populations compared to the respective parental cell lines. Thus, the transcriptional phenotype rather resembles the Sunitinib desensitized kidney- than the melanoma sub-populations and argues for a likewise modulation of the apoptotic threshold. As mentioned in *Chapter V.1.2*, primary Sunitinib and Sorafenib sensitive cells respond with the inhibition of NFκB signaling upon treatment with both drugs. Hence, a functional antagonism of the Sorafenib desensitized sub-populations is indicated. Further, it is noteworthy that the transcriptional response upon Sunitinib treatment resulted in the antagonistic expression of ten genes including CHUK/IKKA, which is in correlation to the decreased Sunitinib sensitivity of the Sorafenib desensitized sub-populations. As a consequence, we performed knock-down studies of the IKK-family member CHUK/IKKA, IKKB, IKKE and IKKG, as they are activators of NFκB signaling (reviewed by Michael J. May and Sankar Ghosh [270]). In our study, specific reduction of the IKK members resulted in increased Sorafenib sensitivity regarding apoptosis induction in correlation to the endogenous expression. However, the impact of the reduction was rather low (apoptosis increase of ~ 10 %) in comparision to the genes that trigger Sunitinib sensitivity like PRKX or TTBK2. This might be reasoned in redundant mechanisms of the IKK-family members and other above mentioned NFκB signaling activators. Hence, a future study of PS-341, an inhibitor of NFκB, in combination with Sorafenib might be necessary to study Sorafenib resistance formation in melanoma. However, the high quantity of the indentified genes and the associated mechanisms might be a major problem to overcome potential Sorafenib resistance formation by using Sorafenib in combination with single targeted compounds. Thus, the combination of multi-targeted kinase inhibitors with Sorafenib might be a superior strategy with potential clinical benefit. In particular this might be of interest for the combination of Sorafenib and Gefitinib, since multiple genes identified in our screen overlap with the cellular targets of

Gefitinib [271], including the aurora kinases A and B, the casein kinases epsilon, BUB1 as well as EGFR and IKKE. Hence, three of the above discussed potential mechanisms reasoning Sorafenib resistance formation in melanoma, might be inhibited by Gefitinib.

V.1.7. The role of JNK signaling in Sorafenib´s mode of action and resistance formation

The Ras/Raf/Mek/ErkK signaling cascade integrates cellular signals to diverse functions like regulation of cell cycle progression or apoptosis. Furthermore, Sorafenib but not Sunitinib was designed as a Raf-1 inhibitor. Hence, differential Ras signaling upon Sorafenib or Sunitinib treatment might explain the different sensitivies of both drugs, i.e. a different mode of action. Of particular interest in this signaling cascade might be Erk, since it was postulated as an regulator of NFκB DNA-binding activity [272]. The underlying mechanism remains elusive, but studies of Siwak *et al.* suggest an indirect mechanism by activation of cyto- and chemokines that are activators of NFκB [273-274]. In our study, we demonstrate, that Sorafenib but not Sunitinib inhibits the phosphorylation of Mek and Erk in a dose-dependent manner. Here, the inhibition of Mek and Erk was in no correlation to the primary Sorafenib sensitivity of the cell lines and thus giving no prognostic value in terms of Sorafenib sensitivity. However, the expression of Erk is increased in the Sorafenib desensitized melanoma sub-populations, indicating that Erk might be a modulator of Sorafenib resistance formation. Moreover, Erk has been implicated in the activation of Mitf, which links Sorafenib resistance- with Sunitinib resistance formation (see. *Chapter V.1.5*). Hence, a further detailed comparative analysis of Erk activation in parental and Sorafenib desensitized cell lines is of importance.

Next to Erk, c-Jun NH2-terminal kinase (Jnk) phosphorylation is associated to Sorafenib treatment [196]. Additionally, in human melanoma, the Erk signaling pathway upregulates Jnk, which might be the functional link of the putative inhibitory effect on Jnk signaling upon Sorafenib treatment. Activated Jnk in turn activates c-Jun and its downstream targets, including cyclin D1 and RACK1, which in turn enables protein kinase C (PKC) to phosphorylate and enhance Jnk activity [275]. The Jnk proteins are a subgroup of the MAPKs and comprise three isoforms, Jnk1, -2 and -3 [276]. The role of the Jnk pathway in cell death and survival is controversially discussed. In melanoma, however, Jnk supports survival by controlling cell cycle arrest and apoptosis [200]. Thus, we adressed the question whether

Sorafenib – compared to Sunitinib – may act as an inhibitor of Jnk signaling in melanoma and kidney cell lines. Indeed, Sorafenib inhibited the phosphorylation of Jnk1 in a dose-dependent manner. Unlike for the Erk phosphorylation, inhibition of Jnk1 correlated with the primary Sorafenib sensitivity, indicating its role in triggering Sorafenib sensitivity. In functional terms, our gene-expression analysis revealed the downregulation of cyclin D1 depending on the primary Sorafenib sensitivity upon Sorafenib treatment. Moreover, the ratio of pJnk1 and Jnk1 was approximatly 2-fold higher in the Sorafenib desensitized melanoma sub-populations, supporting its functional role as a trigger of Sorafenib insensitivity. The impact of Jnk1 in this context, however, is lessend by the fact that Sorafenib inhibits Jnk1 phosphorylation in the Sorafenib desensitized sub-populations in a similar degree to the inhibitory effects of Sorafenib in the respective parental cell lines. Furthermore, cyclin D1 expression was decreased in the Sorafenib desensitized melanoma sub-populations, suggesting a yet elusive regulation of its expression next to the above mentioned Jnk1 mechanism. However, since p19/1/c-fos, c-Jun expression and members of WNT signaling like CTNNB1 are elevated in the Sorafenib desensitized sub-populations, a Jnk dependent interaction on c-Jun promoter between phosphorylated c-Jun and β-catenin/TCF4, like described by Saadeddin *et al.* [277], is indicated. The role of Jnk1 was further elucidated by our knock-down studies. Here specific reduction of Jnk1 had an additive effect on Sorafenib induced apoptosis, which further hints that inhibition of Jnk1 is one mechanism of Sorafenib to exhibit its cytotoxic effects. This is further supported by two independent observations. First, the expression of growth arrest and DNA-damage-inducible beta (GADD45B), which is a repressor of Map2k7 [198], is decreased in Sorafenib desensitized sub-populations, indicating that Map2k7 is activated in these cells. Second, specific reduction of MAP2K7 and MAP3K11 that are activators of Jnk1 [197] elevated the cytotoxic effects of Sorafenib in correlation to the endogenous expressions. These findings further imply a functional crosstalk between Jnk- and NFκB signaling to trigger Sorafenib insensitivity. Thus, Jnk signaling adds a further level to the complex network of Sorafenib´s mode of action and resistance formation. Concluded, these mechanisms might be the major disadvantage of the treatment with Sorafenib as the formation of Sorafenib resistance might not be easily targeted by yet available anti-cancer drugs.

VI. Summary

Cancer is a fatal disease that accounts for more than 12 % of all human death. Over the past years, new insights into the molecular mechanisms of cancer led to the development of new therapeutic strategies for an advanced cancer treatment. Sunitinib and Sorafenib represent a new class small molecule multi-target kinase inhibitors, which already demonstrated clinical benefit for several cancer indications. However, in case of both drugs the mode of action remains elusive and drug-resistance formation emerges as a major drawback. Hence, it is pivotal to advance the understanding of the underlying mode of action of the drugs and of drug-resistance formation, which should help to develop advanced therapeutic strategies. Therefore, our study aimed to identify mode of actions and drug-specific resistance mechanisms of Sunitinib and Sorafenib on the transcriptional level in a contrastable manner.

Sunitinib treatment resulted in the downregulation of genes that are associated with Akt-, NFκB signaling as well as Creb activation, suggesting that a modulation of the apoptotic threshold is an important mechanism of Sunitinib to induce cancer cell death. In comparison, the gene-expression pattern upon Sorafenib treatment was similar on functional terms, as the expression of NFκB associated genes was also altered. Hence, a converging mechanism of both drugs to exhibit their cytotoxic effects can be suggested.

In this thesis, we provide first evidence of strong correlative gene-expression and Sunitinib resistance formation within a kidney carcinoma and melanoma cell line model system. Most of the identified upregulated genes were cancer type specific and can be generally associated with survival or resistance mechanisms. In contrast, the expression of PRKX, TTBK2 and RPS6KA6 was elevated in both kidney- and melanoma cell lines, suggesting their cancer type independent role as a regulator of Sunitinib insensitivity. Specific reduction of these genes, which led to Sunitinib sensitization, further demonstrated the crucial role of those genes to trigger Sunitinib resistance. The respective correlative gene-expression analysis regarding Sorafenib resistance revealed various genes, which can be categorized in with three major signaling pathways/mechanims: (1) centrosome and spindle assembly; (2) the Wnt/β-catenin pathway and (3) NFκB signaling, which suggest a converging mechanism of Sorafenib- and Sunitinib resistance formation. However, in comparision to the genes that trigger Sunitinib sensitivity like PRKX, the specific reduction of the those genes had only minor impact. This might reflect the complexity of the underlying cellular signaling pathways, suggesting that only inhibition of multiple targets might help to overcome Sorafenib resistance in melanoma.

Finally, we focused our attention on Raf/Ras/Mek/Erk/Jnk signaling, since Sorafenib but not Sunitinib was designed as a Raf-1 inhibitor. As expected, Sorafenib but not Sunitinib inhibited the phosphorylation of the Raf-1 down-stream kinases Mek and Erk. Moreover, Sorafenib but not Sunitinib inhibited the phosphorylation of Jnk1, which correlates with the primary Sorafenib sensitivity. Furthermore, knock-down studies of Jnk signaling (JNK1, MAP2K7) resulted in Sorafenib sensitization, suggesting its role in triggering Sorafenib resistance.

VII. Zusammenfassung

Krebserkrankungen sind die Ursache für 12 % aller Todesfälle. In den letzten Jahren wurden neue biologische Einblicke in die Krebsentstehung und Progression gewonnen, was zu der Entwicklung von neuen therapeutischen Stragtegien für eine verbesserte Krebsbehandlung führte. Sunitinib und Sorafenib representieren eine neue Klasse an Medikamenten, welche als Multi-Kinase Inhibitoren bezeichnet werden. Obwohl beide Medikamente schon erfolgreich für die Behandlung von verschiedenen Krebs Indikationen eingesetzt werden, ist das Verständins der Wirkmechanismen sowie auftretender Resistenzbildung lückenhaft oder unverstanden. Dieses Verständins ist jedoch essentiell für eine verbesserte Anwendung beider Medikamente wie z.B. kombinatorische oder sequentielle Therapien mit anderen Wirkstoffen. Daher korreliert die vorliegende Arbeit verschiedene, durch die Behandlung mit beiden Medikamenten induzierte oder durch Resistenzbildung vorliegende Genexpressionsmuster, um jenes Verständnis zu erweitern.

Die Behandlung von Krebszelllinien mit Sunitinib resultierte in einer verringerten Expression von Genen, welche sowohl mit Akt und NFκB Signalwegen als auch Creb Aktivierung assoziiert werden können. Die Funktionalität betreffend zeigte die Behandlung mit Sorafenib ähnliche Ergebnisse, da auch Diese die Verringerung NFκB assozierter Gene bedingte, sodass auf einen konvergierenden Mechasnismus beider Medikamente zu schließen ist.

Weiterhin zeigen wie in dieser Studie eine starke Korrelation von der Expression verschiedener Gene mit dem Grad der Sunitinib Desensibilisierung innerhalb eines Nierenkarzinom und Melanom Zelllinien Modellsystems. Die meisten dieser identifizierten Gene sind Krebstyp spezifisch und können mit Überlebens- oder Resistenzmechanismen assoziiert werden. Im Gegensatz hierzu sind die Gene PRKX, TTBK2 und RPS6KA sowohl in den Sunitinib desensibilisierten Nierenkarzinom als auch Melanom Zelllinien stärker exprimiert, was auf einen Krebs unspezifischen Mechanismus, welcher die Sunitinib insensitivität steuert, schließen läßt. Die spezifische Expressionsreduktion dieser Gene führte zudem zu einer Sunitinib Sensibilisierung, was ihre herrausragende Rolle im Bezug auf Sunitinib Resistenzbildung unterstreicht. Die respektive Genexpressionsanalyse mit Sorafenib zeigte ebenfalls eine Reihe an potentiellen Gene, welche für die Resistenzbildung verantwortlich gemacht werden können. Diese lassen sich funktionell in drei Gruppen unterteilen: (1) Gene, welche and der Zentrosomen- und Spindel Orientierung beteiligt sind;

(2) Gene, welche mit dem Wnt/β-catenin oder (3) NFκB Signalwegen assoziiert sind. Die spezifische Expressionsreduktion dieser Kandidatengene zeigte allerdings nur geringes Potential, die Krebszelllinien gegenüber Sorafenib zu sensibilisieren. Dies legt den Schluß nahe, dass die Inhibition multipler Prozesse innerhalb der Zelle nötig sind, um die Schwierigkeiten der Sorafenib Resistenz zu lösen.

Als letzten Vergleich legt diese Studie ihren Fokus auf die den Raf/Ras/Mek/Erk/Jnk Signalweg, da Sorafenib, im Gegensatz zu Sunitinib, als Raf-1 Inhibitor designed wurde. Erwartungsgemäß war nur Sorafenib in der Lage die „down-stream“ Kinasen Mek und Erk zu inhibieren. Zudem wurde die Phosphorylierung von Jnk1 nur durch Sorafenib inhibiert, während Sunitinib keinen Inhibitionseffekt zeigte. Die Rolle des Jnk Signalweges im Hinblick auf Sorafenib Resistenz wurde weiterhin durch Knock-down Experimente von JNK1 und MAP2K7 gezeigt, die zu einer Sensibilisierung der Krebszelllinien gegenüber Sorafenib führten.

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X. Appendix

X.1. Supplementary tables

Table 1: Data of the comparative gene-expression analysis of parental and Sunitinib desensitized (^{SDes}) melanoma cell lines.

Symbol	Expexted score (dEXP)	Observed score (d)	Numerator (r)	Denominator (s+s0)	Fold-change
CSK	-0.4	2.5	1.5	0.6	2.7
CSNK1G3	-0.5	1.8	1.1	0.6	2.1
CTNNB1	-0.8	2.0	1.5	0.7	2.7
DUSP10	0.2	1.5	1.5	1.0	2.6
DUSP4	1.4	1.7	1.5	0.9	2.8
DUSP6	0.6	1.6	1.8	1.1	3.5
EIF2AK4	-0.3	1.9	1.3	0.7	2.5
EPHB4	-0.3	2.1	1.6	0.8	2.9
ICK	0.0	2.0	1.8	0.9	3.3
IRAK1	0.0	2.6	1.6	0.6	2.9
MMP2	0.2	1.6	1.6	1.0	2.8
PPP5C	0.5	1.9	1.3	0.7	2.4
PRKCG	0.4	1.6	1.1	0.7	2.1
PRKCG	0.4	1.9	1.3	0.7	2.5
PRKX	0.4	1.7	1.9	1.1	3.6
PTPN4	0.6	1.6	1.5	1.0	2.8
RPS6KA6	0.6	3.2	2.2	0.7	4.6
STK39	0.2	1.9	1.6	0.8	3.2
STPK1	0.8	1.8	1.5	0.8	2.7
TRIB1	-0.2	1.7	1.0	0.6	2.0
TRIB2	-0.1	1.6	2.3	1.5	4.1
TTBK2	1.1	1.9	2.4	1.2	4.3
TTN	1.1	2.4	3.8	1.6	10.2
TYRO3	0.7	1.6	2.3	1.5	3.7
TYRO3	0.6	1.9	1.8	0.9	3.3
ULK1	1.3	2.2	4.3	1.9	15.5
WNK2	1.7	5.0	3.1	0.6	8.6
WNK4	1.8	1.7	1.3	0.8	2.5
CCNT2	-0.4	-4.4	-3.5	0.8	0.1
DCAMKL2	-0.4	-3.7	-2.9	0.8	0.1
HDAC7A	-0.1	-4.1	-4.4	1.1	0.0

Table 2: Data of the comparative gene-expression analysis of parental and Sunitinib desensitized (^{SDes}) kidney cell lines.

Gene name	Expected score (dEXP)	Observed score (d)	Numerator (r)	Denomintaor (s+s0)	Fold-change
CCL7	-0.71	3.91	7.14	1.83	147.43
PIM1	0.35	3.44	6.84	1.99	69.92
CXCL12	-0.52	3.09	5.22	1.69	55.52
BTC	-0.88	3.52	5.17	1.47	38.20
CDKN1B	-0.89	2.67	4.51	1.69	35.22
KSR1	0.01	4.29	4.83	1.13	28.54
MST1R	0.63	3.41	4.80	1.41	24.55
TTBK1	1.11	3.43	4.49	1.31	24.35
EphA10	-0.36	3.77	4.51	1.20	24.00
ATR	-0.95	2.68	4.32	1.61	22.89
FRAP1	-0.25	2.30	4.95	2.16	21.37
ROCK2	-0.01	2.22	5.14	2.31	21.37
TRIM28	0.95	3.80	4.39	1.15	19.18
MAST2	0.07	2.14	4.69	2.19	19.05
SPHK1	-0.87	2.48	4.33	1.75	17.50
EIF2AK2	0.41	2.44	3.64	1.49	16.60
Cd44	0.10	3.15	3.94	1.25	16.52
PRKAR1A	0.37	2.85	4.05	1.42	15.96
STK16	0.16	3.59	4.06	1.13	15.84
MTMR8	0.59	1.92	3.24	1.69	14.55
TIMP1	0.96	3.28	3.99	1.22	14.27
CSK	-0.47	3.56	3.71	1.04	13.97
MMP14	0.14	3.91	3.66	0.94	12.68
MAP3K11	0.13	2.44	3.48	1.43	11.84
CSNK1G2	-0.59	2.46	3.49	1.42	11.69
PLK1	0.43	2.76	3.59	1.30	11.33
ANXA2	-0.99	2.10	3.11	1.48	11.18
SIRPB1	0.74	2.15	3.30	1.54	11.09
CCNL2	-0.49	1.99	3.03	1.52	10.88
CDK4	-0.64	2.45	3.32	1.36	10.79
NUAK1	-0.02	2.28	3.19	1.40	10.49
E2F6	-0.40	2.54	3.26	1.28	10.09
CSNK1E	-0.59	2.89	3.26	1.13	9.95
CDK8	-0.64	3.43	3.27	0.95	9.88
AURKB	-0.96	2.70	3.49	1.29	9.75
THRA	0.33	3.18	3.21	1.01	9.59
MKNK1	0.16	2.23	3.00	1.35	9.56
CDH1	-0.19	2.45	3.15	1.29	9.38
BCAR1	0.23	2.01	3.06	1.52	8.79
DDR1	-0.46	1.92	2.91	1.52	8.59
PRKAR1B	0.37	2.13	3.11	1.46	8.58
MAPK3	-0.33	2.68	3.09	1.16	8.28
FASLG	-0.31	3.06	2.99	0.98	7.79
GAB1	-0.24	1.98	2.63	1.33	7.70
AURKA	-0.96	2.69	2.90	1.08	7.51
EEF2K	-0.37	2.18	3.50	1.61	7.45
PRKCB1	0.39	2.10	2.83	1.34	7.43
CCRK	-0.69	2.76	2.88	1.05	7.22
MAP3K7	0.86	2.18	2.79	1.28	7.20
PAK3	0.28	2.06	2.84	1.38	7.04
BUB1	-0.82	2.41	2.71	1.12	7.03
ADAM15	-1.28	3.08	2.77	0.90	7.02
CHUK	-0.13	2.30	2.64	1.15	6.93
CNNM1	-0.49	2.56	2.81	1.10	6.88
MCAM	0.08	2.19	2.67	1.22	6.66
CDC2L5	-0.62	3.10	2.69	0.87	6.33
IRAK1	-0.07	2.19	2.48	1.14	6.22
ADAM17	-1.27	2.20	2.53	1.15	6.17
Mapk1	-0.33	2.22	2.58	1.16	6.16
AKT1	-1.11	2.58	2.63	1.02	6.02
CCND3	-0.50	2.08	2.41	1.16	5.92
RARA	0.60	2.62	2.55	0.97	5.81
MAPK14	0.25	2.18	2.61	1.20	5.59
KIF1C	0.01	2.17	2.32	1.07	5.46
TGFBR1	-1.07	2.13	2.47	1.16	5.46
CAV2	-0.73	2.10	2.37	1.13	5.30
VIM	1.33	2.03	2.36	1.16	5.29
ADAM15	-1.29	2.44	2.41	0.98	5.27
ADAM12	-1.33	2.64	2.37	0.90	5.20
PLAUR	-1.16	1.97	2.71	1.37	5.17
PDK4	0.32	1.92	2.80	1.46	5.06
CTNNB1	-0.89	2.00	2.19	1.10	4.87
IKBKG	-0.10	2.01	2.09	1.04	4.57
CAMK4	-0.76	2.11	2.17	1.03	4.48
IGF2	-0.17	2.48	2.13	0.86	4.38
SYK	0.85	2.19	2.11	0.96	4.21
PPP1CA	0.43	2.12	2.03	0.96	4.00
MAP2K2	0.06	2.35	1.84	0.78	3.60
ITK	-0.05	2.13	1.82	0.85	3.49
SBK1	0.69	-3.73	-4.57	1.23	0.05

Table 3: Data of the comparative gene-expression analysis of parental and Sorafenib desensitized (^{NDes}) melanoma cell lines.

Gene name	Expected score (dEXP)	Observed score (d)	Numerator (r)	Denomintaor (s+s0)	Fold-change
PRKACB	0.24	0.96	2.75	2.87	6.19
CCNT1	-0.23	0.96	3.72	3.87	22.42
NGFB	-0.44	0.97	2.66	2.76	6.52
MAP3K11	0.10	0.97	2.96	3.05	7.46
TGFBR2	0.56	0.98	2.87	2.94	8.38
CAV2	-0.37	0.98	2.69	2.76	6.38
CTNNB1	-0.47	0.98	2.57	2.63	5.98
RYK	0.40	0.98	3.15	3.21	7.54
GAB1	-0.10	0.98	3.00	3.05	9.72
PTPN14	0.22	0.99	3.39	3.43	9.39
CAV1	-0.37	1.00	2.52	2.51	5.85
EEF2K	-0.18	1.01	3.55	3.53	7.83
AKT1	-0.62	1.01	2.76	2.74	6.58
ADAM12	-0.76	1.01	2.55	2.53	5.81
BCAR1	0.16	1.01	3.38	3.35	10.93
VIM	0.85	1.01	2.76	2.73	6.53
PDGFB	0.20	1.01	3.32	3.29	9.04
CCRK	-0.35	1.01	2.84	2.81	7.11
ADAM15	-0.73	1.01	2.65	2.61	6.07
p19/1(cfos)	0.17	1.02	2.87	2.81	8.04
Mapk1	-0.15	1.03	2.90	2.82	7.43
FBXL5	-0.14	1.03	3.07	2.98	9.77
IKBKG	-0.03	1.04	2.49	2.38	5.65
RARA	0.36	1.05	2.75	2.63	6.57
CDC2L5	-0.31	1.05	2.72	2.58	6.43
EPHB3	-0.15	1.05	3.34	3.17	11.89
CCND3	-0.24	1.06	2.84	2.68	7.42
FASLG	-0.14	1.07	2.87	2.69	7.19
CSNK1A1	-0.29	1.07	3.11	2.91	8.41
DAPK3	-0.22	1.08	3.20	2.97	10.80
NEK2	0.14	1.09	3.37	3.11	10.26
HDAC5	-0.07	1.09	2.97	2.72	7.73
AURKB	-0.50	1.12	3.51	3.13	9.99
MAPK3	-0.15	1.13	3.29	2.90	9.42
IRAK1	-0.02	1.14	2.93	2.58	7.83
CHUK/IKKA	-0.05	1.15	3.06	2.66	8.67
ADAM17	-0.72	1.15	2.98	2.59	7.76
BUB1	-0.42	1.15	3.09	2.68	8.65
MAP3K7	0.52	1.17	3.29	2.81	9.18
ATR	-0.12	1.17	4.83	4.12	63.74
E2F6	-0.18	1.18	3.56	3.02	12.21
ATR	-0.49	1.19	4.19	3.53	23.86
CDK8	-0.32	1.19	3.02	2.54	8.09
ROCK2	0.01	1.20	5.34	4.43	26.21
AURKA	-0.50	1.21	3.23	2.66	9.01
MAST2	0.07	1.26	5.24	4.16	25.26
Cd44	0.09	1.27	3.91	3.07	17.11
TIMP1	0.58	1.27	3.82	2.99	12.45
ADAM15	-0.72	1.30	3.03	2.33	8.18
FRAP1	-0.11	1.32	5.45	4.14	27.70
CDH1	-0.08	1.33	3.69	2.77	12.09
PLK1	0.26	1.34	3.98	2.97	13.96
CDK4	-0.32	1.36	3.90	2.86	14.08
STK16	0.12	1.38	4.02	2.92	15.68
PLAUR	0.75	1.38	3.53	2.55	11.29
CSNK1E	-0.29	1.39	3.67	2.64	12.29
CSNK1G2	-0.29	1.40	4.09	2.92	15.36
THRA	0.22	1.42	3.54	2.50	11.52
CXCL12	-0.25	1.42	5.15	3.64	63.71
PAP	0.19	1.42	4.75	3.35	31.31
PRKAR1A	0.24	1.43	4.48	3.13	20.00
NUAK1	0.01	1.43	3.88	2.70	14.07
MMP14	0.11	1.44	3.79	2.63	13.82
TTBK1	0.67	1.44	4.53	3.15	26.61
ANXA2	-0.52	1.45	3.90	2.70	15.35
SPHK1	-0.45	1.46	4.99	3.43	23.36
EphA10	-0.17	1.46	4.15	2.83	17.88
HDAC1	-0.07	1.47	3.72	2.54	13.02
MKNK1	0.12	1.49	3.70	2.48	12.84
TRIM28	0.57	1.56	4.55	2.91	21.34
MST1R	0.38	1.57	5.05	3.22	28.87
CCNL2	-0.23	1.59	3.92	2.46	15.12
PIM1	0.23	1.63	6.39	3.93	48.14
EIF2AK2	0.26	1.64	4.43	2.70	22.86
BTC	-0.46	1.65	5.45	3.29	46.74
CSK	-0.22	1.70	4.11	2.42	17.21
KSR1	0.03	1.91	5.19	2.71	34.62
CDKN1B	-0.46	2.01	5.51	2.74	49.98
CCL7	-0.35	2.44	8.04	3.30	205.32

X.2. Abbreviations

AdA	Additional (increased) formation of apoptotic cells
Ampr	Ampicilline resistence
ATP	Adenosintriphosphate
bp	Base pairs
BSA	Bovine serum albumin
°C	Degree celsius
Ca2+	Calcium Ions
cDNA	Complementary DNA
CHUK/IKKA	Conserved helix-loop-helix ubiquitous kinase
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamintetraacetate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid
ERK	Extracellular signal-regulated kinase
FCS	Fetal calf serum
GTP	Guanosintriphosphate
h	Hour
HEPES	N-(2-Hydroxyethyl)-piperazin-N‘-2-Ethansulfonic acid
IC_{50}	Half maximal inhibitory coefficient
JNK1	MAPK8, mitogen-activated protein kinase 8
JNK2	MAPK9, mitogen-activated protein kinase 9
kb	Kilobase
kDa	Kilodalton
LD_{50}	Half maximal lethal dose
µ	Micro
m	Milli
M	Molar
MAP	Mitogen-activated protein
MAPK	MAP kinase
min	Minute
MMP	Matrix metalloprotease
NDes	Sorafenib (Nexavar) desensitized sub-population, generally superscripted
PAGE	Polyacrylamide gel elektrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor

PDGFR	Platelet-derived growth factor receptor
PLAUR	Plasminogen activator, urokinase receptor
PLK-1	Polo like kinase 1
PRKX	protein kinase, x-linked
pSer	Phosphoserine
pThr	Phosphothreonine
PTP	Phosphotyrosine-specific phosphatase
PTP-MEG	Megakariocyte PTP
pTyr	Phosphotyrosine
pY P	phosphotyrosine
RNA	Ribonucleic acid
RPS6KA6/RSK4	ribosomal protein S6 kinase, 90kDa Polypeptide 6
RT	Room temperature
RTK	Receptor tyrosine kinase
SAM	Significance analysis of microarrays
SDes	Sunitinib (Sutent) desensitized sub-population, generally superscripted
SDS	Sodium dodecyl sulfate
SH	Src homology
SHP-1	SH2-containing PTP-1
SHP-2	SH2-containing PTP-2
siRNA	Short interfering RNA
Src	Homologue to v-src (sarcoma viral oncogene)
Tris	Tris(hydroxymethyl)aminomethan
TTBK2	Tau tubulin kinase 2
Vol	Volume
WB	Western Blot
WT	Wild type

X.3. Acknowledgements

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