

Elina Jokinen

TARGETED THERAPY
SENSITIVITY AND
RESISTANCE IN SOLID
MALIGNANCIES

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DEPARTMENT OF ONCOLOGY AND RADIOTHERAPY;
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ELINA JOKINEN

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AND RESISTANCE IN SOLID
MALIGNANCIES**

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Abstract

Cancer is a major global killer and a challenge for the healthcare worldwide. Earlier cancer has been treated with surgery, radiation, chemotherapy and hormonal therapy. Unfortunately the efficiency of these therapies has shown to be limited and this has raised an enthusiasm for development of new, targeted cancer therapies that are based on activated oncogenes. The challenge of the targeted therapies is therapy resistance, *de novo*, adaptive and acquired. This work investigated targeted therapy sensitivity and resistance in lung cancer, breast cancer, colorectal cancer, and melanoma cell lines.

The results of this study indicate that in some non-small cell lung cancer cell lines, dual PI3K and MEK inhibition is a more efficacious treatment than inhibition of either solely. It was also showed that the maximal effect of the dual inhibition can be achieved with alternative dosing schedules that are potentially more tolerable in clinical use. Furthermore, by combining ABT-263, entinostat or dasatinib to the dual PI3K and MEK inhibition, the efficiency of the therapy can be increased. Bcl-xl downregulation is a major determinant of the apoptotic response to the triple inhibitor treatment.

The current work showed that cancer stem cells can mediate resistance to targeted therapies. Since these cells follow the stochastic model, concurrent therapy with a targeted agent and a stem cell targeting drug might be needed for maximal therapeutic efficiency.

This study also showed that Gö6976 acts as a potent inhibitor of mutant *EGFR* despite the presence of T790M, the most important mechanism of acquired resistance for EGFR tyrosine kinase inhibitors in lung cancer, both *in vitro* and *in vivo*.

Keywords: cancer stem cells, carcinoma, drug resistance, molecular targeted therapy

Jokinen, Elina, Herkkyys ja resistenssi kohdennetuille hoidoille kiinteissä kasvaimissa.

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta, Kliinisen lääketieteen laitos, Syöpätaudit ja sädehoito; Oulun yliopistollinen sairaala

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Tiivistelmä

Syöpä on yksi johtavia kuolemanaiheuttajia ja tauti on maailmanlaajuinen haaste terveydenhuololle. Perinteiset syöpähoidot käsittävät kirurgian, sädehoidon, kemoterapian ja hormonaalisen hoidon, mutta näiden rinnalle on noussut uusia, aktivoituneiden onkogeenien signaalien estoon perustuvia hoitoja. Tämä työ tutki kohdennettuja syöpähoitoja ja näihin hoitoihin liittyvää resistenssiä keuhko-, rinta- ja paksusuolen syövän sekä melanooman solulinjoissa.

Tulokset osoittavat, että joissakin ei-pienisoluisen keuhkosyövän solulinjoissa yhdistetty PI3K- ja MEK-esto aiheuttaa tehokkaamman vasteen kuin kummankaan signaalireitin esto yksistään. Tässä työssä näytettiin myös, että maksimaalinen vaste yhdistetylle PI3K- ja MEK-estolle voidaan saavuttaa vaihtoehtoisilla annostelutavoilla, jotka ovat voisivat olla paremmin siedettyjä kliinisessä käytössä kuin kahden lääkkeen jatkuva annostelu. Tämä tutkimus osoitti lisäksi, että kaksoiseston tehokkuutta voidaan lisätä yhdistämällä hoitoon kolmas lääkeaine, ABT-263, entinostaatti tai dasatinibi. Bcl-xl proteiinilla on keskeinen rooli apoptoottisen vasteen määrittäjänä näille kolmen lääkkeen käsittelyille.

Tämä työ osoitti, että syövän kantasolut voivat välittää resistenssiä kohdennetuille syöpähoidoille. Nämä solut noudattavat niin kutsuttua stokastista mallia, joten parhaan vasteen saaminen saattaa edellyttää että hoito kohdentuu sekä erilaistuneisiin että kantasolutyyppiisiin syöpäsoluihin.

Tässä tutkimuksessa osoitettiin lisäksi, että Gö6976 toimii mutatoituneen EGFR:n estäjänä, huolimatta kehittyvää keuhkosyövässä resistenssiä välittävästä T90M mutaatiosta, sekä *in vitro* - että *in vivo* -malleissa.

Asiasanat: karsinooma, lääkeresistenssi, molekulaarisesti kohdennettu hoito, syövän kantasolut

*It's a magical world, Hobbes, ol' buddy...
Let's go exploring!*

Calvin

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August 2014,

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Abbreviation

AC	adenocarcinoma
AKT	murine thymoma viral oncogene homolog 1
ALDH1	aldehyde dehydrogenase 1
ALK	anaplastic lymphoma kinase
Bcl-2	B-cell lymphoma 2
Bcl-xl	B-cell lymphoma-extra large
BH	Bcl-2 homology domain
BSA	bovine serum albumin
CI	combination index
CRC	colorectal cancer
CSC	cancer stem cell
cPARP	cleaved poly ADP ribose polymerase
ECL	enhanced chemiluminescence
ECM	extra cellular matrix
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EMT	epithelial to mesenchymal transition
EML4	echinoderm microtubule-associated protein like 4
ERK1/2	extracellular signal-regulated kinase 1/2
ER	estrogen receptor
Grb2	Growth factor receptor-bound protein 2
IC	inhibitory concentration
IGF-R1	insulin like growth factor receptor 1
HDAC	histone deacetylase
HER2	human epidermal growth factor receptor 2
HER3	human epidermal growth factor receptor 3
HER4	human epidermal growth factor receptor 4
HGFR	hepocyte growth factor receptor
Hh	Hedgehog
K-Ras	kirsten rat sarcoma viral oncogene homolog
LCC	large cell carcinoma
Mcl-1	induced myeloid leukemia cell differentiation protein
MEK	mitogen-activated protein kinase kinase
MTS-assay	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) based cytotoxicity assay

mTOR	mammalian target of rapamycin
NSCLC	non-small cell lung cancer
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol (4,5)-bisphosphate
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKC	protein kinase C
PR	progesterone receptor
PTEN	phosphatase and tensin homolog
PVDF	polyvinylidene fluoride
Raf	murine leukemia viral oncogene homolog
Rb	retinoblastoma
RTK	receptor tyrosine kinase
RTKI	receptor tyrosine kinase inhibitor
SCC	squamous cell carcinoma
SCLC	small cell lung cancer
Shc	Src-homology
siRNA	small interfering RNA
SNP	single-nucleotide polymorphism
SOS	Son of Sevenless protein
TGF- α	transforming growth factor alpha
TKI	tyrosine kinase inhibitor
TNBC	triple-negative breast cancer
TP53	tumor protein p53
Wnt	wingless-related integration site

List of original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:

- I Jokinen E, Laurila N & Koivunen JP (2012) Alternative dosing of dual PI3K and MEK inhibition in cancer therapy. *BMC Cancer* 12(612).
- II Jokinen E & Koivunen JP (2014) Bcl-x1 downregulation is a major determinant of response to dual PI3K and MEK inhibition. Manuscript.
- III Jokinen E, Laurila N, Koivunen P & Koivunen JP (2014) Combining targeted drugs to overcome and prevent resistance of solid cancers with some stem-like cell features. *Oncotarget*. In Press.
- IV Taube E, Jokinen E, Koivunen P & Koivunen JP (2012) A novel treatment strategy for EGFR mutant NSCLC with T790M-mediated acquired resistance. *Int J Cancer* 131(4): 970-979.

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1 Introduction

Cancer is a major challenge for healthcare around the globe. The disease is one of the major killers worldwide and effective approaches to cure cancer have not yet been found. Cancer is of a complex nature that is characterized by unregulated cell growth enabled by DNA alterations that promote unlimited growth and proliferation and, on the other hand, hinder the mechanisms that control cell division. In healthy cells, DNA mutations occur constantly and they are repaired, or cells with damaged DNA are driven to apoptosis. However, these mechanisms are impaired in cancer. Accumulation of promoting mutations is needed before cancer can develop (Harris & McCormick 2010).

Intracellular signalling has a key role in the development and progress of cancer. The cell signaling cascades are activated by molecules, such as growth factors, that bind to cell surface receptors. The receptors in turn mediate a signal to downstream effectors, which transmit the signal ultimately to the nucleus. Mutations in the cell surface receptors or intracellular signal transmitters may promote cancer by hyperactivating or repressing the function of a pathway. Cellular components known to be frequently altered in cancer include the epidermal growth factor receptor (EGFR), the anaplastic lymphoma kinase (ALK) and their downstream signaling cascades phosphatidylinositol 3-kinase - murine thymoma viral oncogene homolog 1-mammalian target of rapamycin (PI3K-Akt-mTOR) and rat sarcoma viral oncogene homolog- murine leukemia viral oncogene homolog -mitogen-activated protein kinase kinase- extracellular signal-regulated kinase (Ras-Raf-MEK-ERK) pathways (Lemmon & Lemmon 2010).

Solid tumors include abnormal tissues that develop from the epithelial (carcinoma) or mesenchymal tissue (sarcomas). Benign tumors are local and cannot send metastases. Instead, malignant tumors can invade neighboring tissues and form metastases. The most common solid cancer types include lung cancer, breast cancer, prostate cancer, colorectal cancer and melanoma. Traditional treatments for solid malignancies include surgery, radiation therapy and chemotherapy (Roberts *et al.* 2007). However, the effect of surgery and radiotherapy is only local and they cannot cure spread cancer that is at an advanced stage. In addition, though chemotherapy is systemic, it has some limitations such as primary and secondary resistance. Metastatic cancer is still challenging to treat. The use of traditional therapies is limited due to the side effects. In the recent decades there has been an enthusiasm for development of new drugs for cancer cell mutations that are typical specific subsets of cancer.

These molecularly targeted inhibitors are designed to block activated cell surface receptors or mediators of the intracellular signaling pathways involved in cancer promoting cellular functions such as proliferation and growth. The benefits of the targeted therapies were thought to be less side effects and better efficacy as their mechanisms of action were somewhat better understood than the mechanisms of the traditional chemotherapies. Today, it is possible to design a molecule for blocking a biological key activity, such as those therapies targeting activated EGFR, which are already in clinical use.

Despite the promising potential of the molecularly targeted cancer therapies, some challenges need to be overcome. Similarly to chemotherapy, resistance to the treatments is a major issue also with targeted therapies. Some patients are unresponsive to the therapy already at the beginning of the treatment, having the so called primary or *de novo* resistance, and from the patients who initially respond to the therapy, even dramatically, virtually all ultimately develop a resistance. The mechanisms mediating resistance to targeted therapies and strategies to overcome them have been eagerly studied, however, they are not yet fully understood. Today, it has become more and more possible to relate resistance changes to genetic mutations. Known factors causing therapy resistance include gene amplifications, point mutations that affect i.e. drug binding, constant activation of downstream signal transmitters, and activation of parallel intracellular pathways (Sartore-Bianchi *et al.* 2009, Bonomi *et al.* 2007, Pao *et al.* 2005, Garraway & Jänne 2012).

The role of cancer stem cells (CSCs) has been speculated as one explanation for the developing therapy resistance. The cell type is capable of initiating and sustaining growth of a tumor. CSCs are undifferentiated cancer cells that have potential to turn into more differentiated cancer cells. CSCs are highly tumorigenic and it is thought that CSCs might be responsible for formation of new tumors (Lapidot *et al.* 1994, Bonnet & Dick 1997, Al-Hajj *et al.* 2003). It has been proposed that the inability of the current therapies to target the CSCs might at least partially explain patient relapses (Bao *et al.* 2006, Creighton *et al.* 2009). CSCs have been hypothesized to originate either from normal stem cells, more differentiated progenitor cells that have acquired stem cell traits through accumulation of genetic or epigenetic changes or even from fully differentiated cells that have gone through a partial reprogramming (Baccelli & Trump 2012). It is possible that the CSCs mediate the so-called adaptive resistance where the cells stay in a non-dividing stage during the treatments and survive, and start normal proliferation after termination of the therapy.

2 Review of the literature

2.1 Cancer

Cancer is one of the leading causes of deaths worldwide, approximately one eighth of all global deaths are caused by cancer (Garcia *et al.* 2007). It has been estimated that in 2012 there were 14.1 million cancer incidences and 8.2 million cancer deaths. (Cancer Research UK 2014). These numbers are continuously growing, mostly due to aging and growth of the world population and increase of cancer causing behavior such as smoking. In the western countries, cancers of prostate, colorectum and lung are the most common ones with males whereas breast, colorectal and lung cancers are the most typical female cancer types (Jemal *et al.* 2011).

Surgery was the first strategy used to treat cancer and radiation therapies were started at the beginning of the 20th century. Chemotherapy was introduced as a new cancer treatment in the 1940s. Unfortunately, the success of these treatments has been somewhat disappointing. Modern medicine studies the possibilities of molecularly targeted cancer therapies that are based on oncogenic alterations in specific cancer types. Originally the main goal of the so-called personalized cancer medicine was to find new effective agents. However today, the focus has shifted towards the search of the most effective action mechanisms and designing molecules to target them (Roberts *et al.* 2007, Harris & McCormick 2010).

2.2 Cancer genetics

Cancer can arise as a result of mutational events in oncogenes, tumor-suppressor genes, and microRNA genes. Usually accumulation of such alterations in a variety of genes is required for development of a malignant tumor. (Croce 2008). The cancer causing DNA mutations occur in originally normal cells. The DNA mutations may be caused by insertions or deletions of segments of the DNA, rearrangement of DNA segments, gene amplifications or reduction in gene copy number (Stratton *et al.* 2009). The most frequently occurring genetic variation in humans are stable substitutions of single bases in the DNA, the single-nucleotide polymorphisms (SNPs). For instance, SNPs in matrix metalloproteinase 9 have been related to occurrence and metastases of lung cancer (Hu *et al.* 2005). Cancer

cells may also have acquired new DNA segments from exogenous sources such as viruses like human papilloma virus (Talbot & Crawford 2004) or carry epigenetic changes that have altered the chromatin structure and gene expression through altering the methylation status of cytosine residues (Stratton *et al.* 2009). Additionally, somatic mutations in the mitochondrial DNA have been identified in many human cancers (Chatterjee *et al.* 2006).

Some DNA changes give cancer cells a growth advantage; these are called driver mutations. The rest are called passenger mutations for they do not confer the cell any growth advantage but were present when the driver mutations occurred (Stratton *et al.* 2009). There are around 350 human protein-coding genes that are known to play a role in the development of cancers (Futreal *et al.* 2004). However, it has been suggested that altogether there are approximately 2000 human genes that have cancer causing potential (Touw & Erkeland 2007).

2.2.1 Oncogenes

Proto-oncogenes are normal genes that, when hyperactivated, have cancer causing potential. These genes are involved in promotion of cell growth and proliferation and have potential to induce unlimited cell division. Proto-oncogenes encode proteins that can be classified as growth factors, growth factor receptors, transcription factors, chromatin remodelers, signal transducers, or regulators of apoptosis. Proto-oncogenes can be activated as a result of alterations, such as gene fusion or amplification. Activation turns them into oncogenes that overproduce gene products that promote cancer development and progression (Croce 2008).

2.2.2 Tumor suppressor genes

Tumor suppressors are genes that are involved in controlling cellular growth and division. In normal cells the function of these genes prevents cancer formation. They serve as negative regulators of cellular growth and proliferation by acting as cell cycle gatekeepers or by promoting apoptosis. Cancer development requires circumvention of these growth limiting programs; hence dysfunction of the tumor suppressors is crucial for tumor development. Loss or reduction of the function of tumor suppressors enables formation of cancer cells. It is thought that alteration of both alleles of a tumor suppressor gene is necessary for tumorigenesis. The best-known tumor suppressor genes are the *retinoblastoma 1*

(*RBI*) and tumor protein p53 (*TP53*) genes which both encode proteins (Rb and p53) that have central roles in controlling cell division. Alterations of the *TP53* gene are the most commonly seen mutations in cancers (Knudson 2002).

2.2.3 MicroRNA genes

MicroRNA genes do not encode proteins but are short RNA strands that regulate gene expression. MicroRNAs can anneal to messenger RNAs (mRNA) and block translation of the protein or cause degradation of the mRNA. Deletion or down-regulation of microRNAs is involved in the pathophysiology of some cancers such as lung cancer and colorectal neoplasia (Calin *et al.* 2002, Michael *et al.* 2003, Takamizawa *et al.* 2004).

2.2.4 Cancer epigenetics

The diversity of phenotypes within a cancer cell population cannot be solely explained by classic genetics. DNA methylation, addition of a methyl group to cytosine preceding guanine, is an important mechanism of controlling gene activity. One of the first epigenetic alterations found in human tumor cells was their low level of DNA methylation compared to normal cells (Feinberg & Fogelstein 1983). It has been suggested that DNA hypomethylation contributes to cancer development through creation of chromosomal instability, loss of imprinting and transposable element reactivation. In turn, hypermethylation has been linked to inactivation of tumor suppressor genes. Amplifications of histone methyltransferase genes have been identified in solid tumors (Esteller 2008).

Histone acetylation, adding of an acetyl group to histone lysines, is generally linked with transcriptional activation. Acetylation is a known post-translational modification of the tumor suppressor gene p53. Histone acetylation and methylation directly affect multiple nuclear processes such as gene transcription and repair and replication of the DNA (Esteller 2008).

2.3 Cancer biology

According to Hanahan & Weinberg (2011) there are six distinct biological capabilities that are acquired during tumor development: sustained proliferation signaling, downregulated growth suppressors, apoptosis resistance, replicative immortality, angiogenesis induction and activated invasion and metastasis.

Together these features enable tumor formation and progression as well as dissemination of metastases.

Sustained proliferation is a fundamental feature of cancer cells. Most often the cell proliferation is initiated by growth factors that bind to receptors located on the cell's surface. Activation of these receptors triggers multi-step signaling processes that ultimately leads to division of the cell. In normal cells proliferation is a tightly regulated process. However, in cancer cells proliferation promoting signaling is unregulated, i.e. through activation of parallel signalling pathways or disruption of negative feedback signalling, hence leading to uncontrolled cell growth and division (Hanahan & Weinberg 2011).

The so-called tumor suppressor genes are involved in limiting cell growth and division. In cancer cells, the growth suppressors are evaded which enables unlimited cell growth and proliferation (Hanahan & Weinberg 2011).

Apoptosis is initiated by inner or outer cell signals that activate a complex signaling cascade. Cancer cells carry DNA damages, the reason lying behind the hyperproliferation, and there is also imbalance in cell signaling due to over activated oncogene signaling. In normal cells this kind of stress would trigger apoptosis through complex inner cell signaling routes. However, cancer cells are resistant to programmed cell death (Hanahan & Weinberg 2011).

Normally there is only a limited number of times a cell can divide. Two distinct phases have been related to this limitation, namely, senescence and crisis that usually results in apoptosis. After repeated cell division cycles cells enter the senescence phase and those that overcome this barrier face the crisis phase. At this point most of the cells die, though rarely there are some that survive after the crisis phase and exhibit potential for unlimited proliferation. This transition is referred to as immortalization. The two barriers of proliferation, senescence and crisis/apoptosis, are recognized as important anticancer defenses (Hanahan & Weinberg 2011).

Angiogenesis is needed by the tumor tissue for distribution of nutrients and oxygen and for evacuation of carbon dioxide and metabolic wastes. During tumor progression, angiogenesis is almost always activated and continuously promoted. This produces continuously new blood vessels maintaining the growing tumor. Today it is known that angiogenesis initiates already at an early phase of tumor growth (Hanahan & Folkman 1996).

Progression of carcinomas is often followed by local invasion and formation of distant metastasis. Often the cells associated develop altered shape and changes in cell-to-cell and cell-to- the extracellular matrix (ECM) attachments. It is known

that some aggressive carcinomas harbor alterations in genes related to cell-to-cell and cell-to-ECM attachments. Typically those related to cell migration are upregulated and those promoting cytoskeleton are downregulated (Cavallaro & Christofori 2004).

Two enabling characteristics underlie the described hallmarks of cancer, namely, genomic instability and tumor-associated inflammation. Tumor formation and cancer progression require multiple cancer promoting mutations in the cell's DNA. Genome instability generates the genetic changes enabling the rise of the hallmark capabilities. Inflammation of malignant and premalignant lesions promotes cancer i.e. by supplying bioactive molecules such as growth factors and angiogenic factors. Furthermore, creation of the so-called tumor microenvironment by recruitment of seemingly normal cells also contributes to the acquisition of the hallmark capabilities.

Additionally, two emerging capabilities have been added to the list: reprogrammed energy metabolism and evaded immune destruction. Cancer cells are known to prefer glycolysis with their energy metabolism, even in aerobic conditions, clearly differing from healthy cells. Normally cancer growth is prevented by the immune system. Therefore, cancer needs to overcome this barrier. It is known that cancer cells are able to block some components of the immune system that are meant to prevent cancer formation. Hence, beside the six core hallmarks, reprogrammed energy metabolism and evaded immune destruction are distinct and characteristic features of cancer that are not yet widely studied but offer an important field for research (Hanahan & Weinberg 2011). (Fig. 1.)

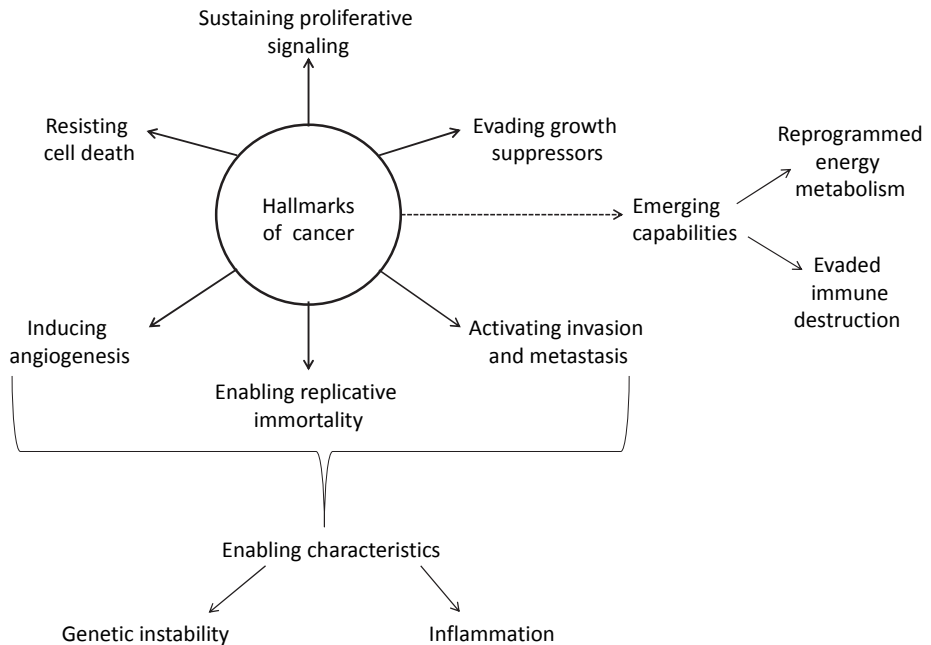


Fig. 1. Hallmarks of cancer. Modified from Hanahan & Weinberg 2011.

2.4 Cancer signalling

Constitutive activation of oncogenic signaling pathways occurs in a very high frequency in all human cancers. It is thought to be a central factor behind the cancer hallmarks such as apoptosis resistance and cell cycle progression (Hanahan & Weinberg 2011).

2.4.1 Receptor tyrosine kinases

Receptor tyrosine kinases (RTKs) are a family of receptors located on the cell surface. They are the key regulators of many important cellular processes including proliferation, differentiation, cell survival and migration (Blume-Jensen & Hunter 2001, Ullrich & Schlessinger 1990). Structurally RTKs consist of an extra-cellular ligand binding domain, a transmembrane domain and an intracellular catalytic domain. Tyrosine kinase receptors are activated when ligands (hormones, growth factors or cytokines) bind to the extracellular domain.

This induces receptor dimerization and autophosphorylation of the intracellular tyrosine kinase domain. The phosphorylated tyrosine kinase domain further phosphorylates downstream targets thus activating a signal transduction cascade (Lemmon & Schlessinger 2010). Normally RTK functions are tightly regulated. However, they bear oncoprotein potential and aberrant RTK activation has an important role behind several human cancers. This has led to an enthusiasm for development of RTK-based targeted molecular cancer therapies and many have already reached clinical use (Gschwind *et al.* 2004). EGFR, human epidermal growth factor receptor 2 (HER2) and ALK are examples of widely studied RTKs that malfunction in many cancers. Fig. 2 presents a simplified model of EGFR signaling as an example of RTK signaling.

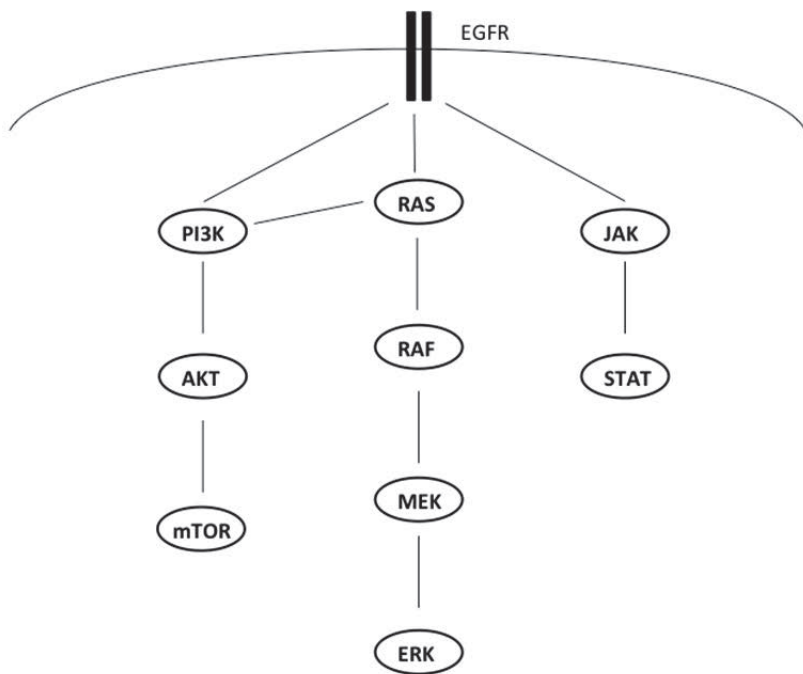


Fig. 2. RTK signaling from EGFR.

2.4.2 PI3K-Akt-mTOR pathway

Members of the PI3K lipid kinase phosphorylate convert phosphatidylinositol (4,5)-bisphosphate (PIP₂) to a second messenger, phosphatidylinositol (3,4,5)-trisphosphate (PIP₃). There are three subclasses of PI3K isoforms, I-III, of which

the class I has been related to cancer (Vanhaesebroeck *et al.* 2010). Class I PI3Ks are composed of p110 catalytic subunit, coded by *PIK3CA*, *PIK3CB*, *PIK3CD*, and *PIK3CG* genes, and p85 regulatory subunit. Activation of the PI3K signaling is caused by ligand binding to receptors such as insulin like growth factor receptor 1 (IGF-R), EGFR, or HER-2 (Ogita & LoRusso 2011). Activated PI3K produce phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which can activate downstream targets such as Akt, which further activates mTOR (Engelman 2009).

The PI3K-Akt-mTOR signaling pathway has an important role in cell survival and proliferation, and constitutive activation of the PI3K-Akt-mTOR pathway is common in many solid malignancies. The hyperactivation of the pathway is mostly caused by RTKs, somatic mutation in specific signaling components of the pathway such as *PIK3CA* or loss of the *phosphatase and tensin homolog (PTEN)* tumor suppressor gene. *PIK3CA* mutations are frequently detected in endometrial, breast, and urinary tract cancers while amplification of the gene is commonly seen in squamous cell cancers of head and neck and lung and gastric cancer. Genetic *PTEN* loss recurrently occurs in glioblastoma and endometrial and prostate cancers (Banerji *et al.* 2012).

2.4.3 Ras-Raf-MEK-ERK pathway

The Ras-Raf-MEK-ERK signaling pathway is involved in the regulation of cell cycle progression, differentiation and apoptosis and constitutive activation of the cascade is seen in many cancer types. Ras is a small GTP binding protein that activates several cell signaling pathways including the Raf-MEK-ERK and PI3K-Akt-mTOR cascades. Ligand binding to receptor leads to activation of the Src-homology/Growth factor receptor-bound protein 2/Son of Sevenless (Shc/Grb2/SOS) coupling complex which in turn stimulates the conformational change of Ras from inactive to active state (Roberts & Der 2007).

Four Ras isoforms have been identified: H-Ras, N-Ras, K-Ras 4A and K-Ras 4B which each have different potential to activate different downstream pathways (Yan *et al.* 1998). Ras proteins have an intrinsic GTPase activity, required for inactivation of the GTP bound active state, which is markedly increased by binding of GTPase proteins such as NF1 (Schubbert *et al.* 2007).

Raf kinase, downstream target of Ras, activates MEK1/2, which in turn catalyzes the activation of ERK1/2. Activation of ERK1/2 further phosphorylates a series of downstream targets that are involved in various cellular functions such

as proliferation, differentiation, cell survival and angiogenesis. ERK1/2 hyperactivation occurs very frequently in solid malignancies (Roberts & Der 2007).

Hyperactivation of Ras-Raf-MEK-ERK signaling can occur through various mechanisms, i.e. overactivation of RTKs or genetic alterations of the pathway members (Roberts & Der 2007). Activating Ras mutations or loss of NF1 cause activation of the pathway at the level Ras. Ras mutations are commonly found in pancreatic, colorectal, lung, endometrial and ovarian cancers and NF1 mutations are seen in sarcomas and colorectal cancers. Activating mutations in the Raf genes is another level where genetic constitutive activation occurs repeatedly while MEK1/2 or ERK1/2 mutations are rarely seen. Raf mutations frequently occur in melanoma and colorectal and thyroid cancers (Schubbert *et al.* 2007).

2.4.4 Bcl-2 family

Bcl-2 family members are mitochondrial proteins involved in the regulation of apoptosis and responsible for controlling the permeabilization of the outer membrane of mitochondria. The first characterized member of the protein family, the Bcl-2 encoded by proto-oncogene *BCL-2*, was originally found in human B-cell lymphomas. The Bcl-2 proteins have up to four conserved Bcl-2 homology (BH) domains: BH1, BH2, BH3, and BH4 (Bakhshi *et al.* 1985, Tsujimoto *et al.* 1985).

Upregulation of pro-apoptotic Bcl-2 proteins such as BIM is often linked to apoptosis while anti-apoptotic proteins such as induced myeloid leukemia cell differentiation protein (Mcl-1) and B-cell lymphoma-extra large (Bcl-xl) promote cell survival. The general balance between anti- and pro-apoptotic mediators is crucial for the determination between cell survival and apoptosis. The Bcl-2 related molecules have an important role in development, tissue turnover and defence against pathogens. However, impaired function of these proteins has been related to cancer since they have potential to inhibit cell death (Youle & Strasser 2008).

2.5 Cancer stem cells

The cancer stem cell model suggests that a cancer cell population originates from a small subset of undifferentiated cancer cells with tumor initiating capability and stem cell properties. CSCs have an unlimited self-renewal potential,

differentiation capability and high tumorigenic capacity. A high proportion of stem cells within a tumor has been related to poor patient outcome (Ginestier *et al.* 2007, Dalerba *et al.* 2007, Charafe-Jauffret *et al.* 2010) and resistance to traditional therapies (Bao *et al.* 2006, Creighton *et al.* 2009).

CSCs were originally identified in leukemia (Lapidot *et al.* 1994, Bonnet & Dick 1997) and breast cancer (Al-Hajj *et al.* 2003), but have since been also found in other human tumor types including colon, skin and lung cancer (Eramo *et al.* 2008, Schatton *et al.* 2008, Magee *et al.* 2012). CSCs have been speculated to originate either from normal stem cells, more differentiated progenitor cells that have acquired stem cell traits through accumulated of genetic or epigenetic changes or even from fully differentiated cells that have gone through a partial reprogramming (Bacelli & Trump 2012).

2.5.1 Hierarchical and stochastic cancer stem cell models

Two competing models are used to explain the heterogeneity of tumor cells. The hierarchical model proposes that there are two distinct cell subsets within the cancer cell population: CSCs that are able to form new tumors and more differentiated cancer cells that cover majority of the total cell population but are not tumorigenic. However, there is growing evidence for a stochastic model where cancer cells are thought to be plastic and able to change between the stem cell and differentiated state. Therefore each malignant cell would have CSC potential and the cell's state is determined by intrinsic and environmental factors (Nguyen *et al.* 2012, Gupta *et al.* 2011). (Fig. 3.)

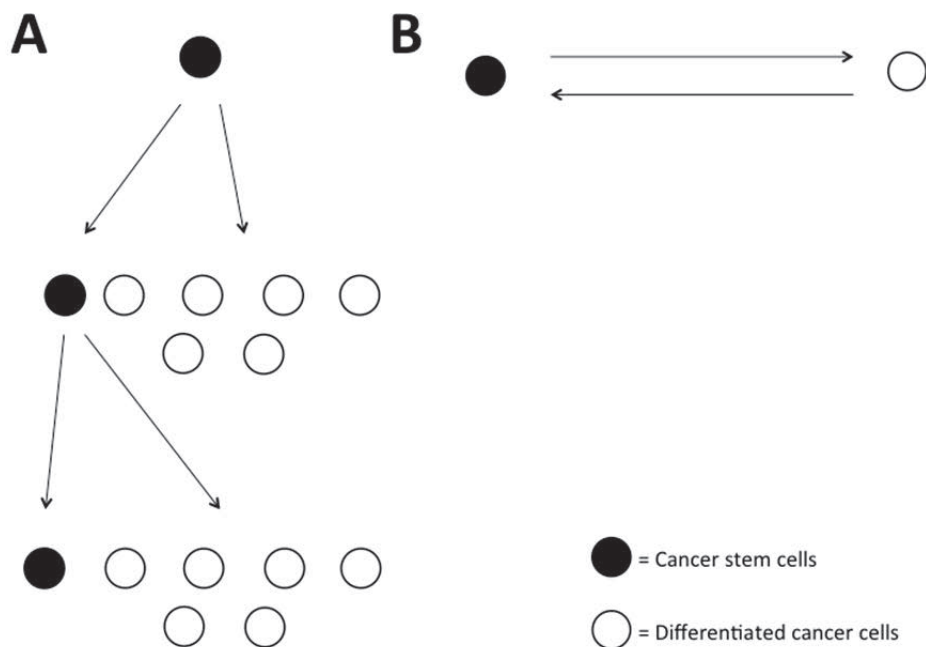


Fig. 3. Hierarchic vs. stochastic cancer stem cell model. (Modified from Magee *et al.* 2012.) The hierarchical CSC model (A) suggests that tumors comprise of two distinct types of cancer cells: differentiated and stem cell-like, the latter only being able to form new tumors whereas the stochastic CSC model (B) proposes that all cancer cells are able to change between the differentiated and stem cell state.

2.5.2 Markers used for cancer stem cell identification

High tumorigenicity in xenograft models is used as the golden standard for identification of CSCs since a universal marker has not yet been found. However, various cell surface markers are also used in CSC research. These include high CD44 and low CD42 expression (breast cancer), elevated CD133 expression levels (glioblastoma) and high aldehyde dehydrogenase 1 (ALDH1) expression or activity (various solid tumors) (Singh *et al.* 2003, Ginestier *et al.* 2007, Shipitsin *et al.* 2007, Huang *et al.* 2009). Additionally, many studies have also linked epithelial to mesenchymal transition (EMT) to the stem cell phenotype. It is thought that the EMT enables migration of cells from the primary tumor to a new site and formation of metastases. Hence, EMT proteins such as E-cadherin and vimentin are also used for identification of CSCs (Mani *et al.* 2008, Morel *et al.*

2008). Table 1 summarizes selected markers used to identify CSCs in selected solid tumor types.

Table 1. Selected CSC markers in specific solid tumor types.

Cancer type	CSC markers	Reference
Lung cancer	ALDH+, CD133+, CD44+	Jiang <i>et al.</i> 2009, Oudela <i>et al.</i> 2012
Breast cancer	CD44+/CD42-, ALDH+	Shipitsin <i>et al.</i> 2007, Ginestier <i>et al.</i> 2007
Melanoma	ABCB5+, CD133+, CD166+, CD34+, ALDH+	Frank <i>et al.</i> , 2005, Klein <i>et al.</i> , 2007; Hendrix <i>et al.</i> 2003, Luo <i>et al.</i> 2012
Colorectal cancer	ALDH+, CD133	Huang <i>et al.</i> 2009, Elsaba <i>et al.</i> 2010

2.5.3 Intracellular signalling pathways related to the cancer stem cell phenotype

In CSCs and normal stem cells many of the same pathways are activated, including wingless-related integration site (Wnt), Notch and Hedgehog (Hh). Notch signaling is involved in the regulation of cell-to-cell communication during embryonic development, apoptosis, cell differentiation and proliferation (Artavanis-Tsakonas *et al.* 1999). The pathway is also known to cross-talk with PI3K-Akt-mTOR signaling (Artavanis-Tsakonas *et al.* 2006, Meurette *et al.* 2009). During embryogenesis the Hh pathway regulates tissue polarity, and patterning and stem cell maintenance (Ingham & McMahon 2001). Hyperactivation of the pathway is known to be tumorigenic (e.g. Hahn *et al.* 1996). Wnt pathway plays a critical role with cell fate determination and regulation of organ development during embryogenesis (Grigoryan *et al.* 2008). Wnt signaling also has a central role in controlling tissue self-renewal in adults (Clevers 2006). The use of these pathways enable the cells to undergo self-renewal and differentiation (Takebe *et al.* 2011). Furthermore, activation of the Ras-Raf-MEK-ERK signaling pathway has been linked to the EM transition of CSCs (Mulholland *et al.* 2012). Also PI3K-Akt-mTOR signalling has been related to the CSC phenotype (Dubrovska *et al.* 2009, Hadt *et al.* 2012).

2.6 Solid tumors

Solid tumors are usually the result of abnormal growth that arises from the epithelial tissue. Solid malignancies were previously classified by histology, but in the recent years molecular classification has also become more relevant since

the use of genetic markers often helps to be predictive for specific therapeutic benefit. However, in many cases, such as in kidney cancer, morphological pathological classification is still important because of the lack of good predictive genetic markers.

2.6.1 Lung cancer

Lung cancer is one of the leading causes of cancer related deaths in the western world (Siegel *et al.* 2013). The disease can be divided into two subtypes based on their histology: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), the latter accounts 85 % of the cases. NSCLC is further divided into adenocarcinoma (AC) squamous cell carcinoma (SCC) and large cell carcinoma (LCC). SCC and AC cover a vast majority of the diagnosed cancer incidences. Tobacco smoking is a typical risk factor causing lung cancer and is strongly related to SCLC and SCC type NSCLC, although the AC subtype of NSCLC is frequently also seen with non-smoking patients. Typically mutated genes seen with lung cancer patients include p53, EGFR, kirsten rat sarcoma viral oncogene homolog (K-Ras) and ALK (Herbst *et al.* 2008).

EGFR

EGFR (HER1 or ErbB1) is an eagerly studied member of ErbB/HER family. The receptor family consists of four structurally related members, the other three of which are HER2 (ErbB2, c-Neu), human epidermal growth factor receptor 3 (HER3, ErbB3) and human epidermal growth factor receptor 4 (HER4, ErbB4). There are 13 ligands that are known to bind to the HER family receptors. HER2 has no known ligands, EGFR binds primarily epidermal growth factors and transforming growth factor alpha (TGF- α) whereas neuregulins preferentially bind to HER3 and HER4. Ligand binding to the HER family receptors activates multiple downstream signal routes such as Ras-Raf-MEK/ERK, PI3K-Akt-mTOR and JAK-STAT cascades which are involved in the regulation of cell survival and proliferation (Eccles 2011, Roengvoraphoja *et al.* 2013).

Alterations of EGFR have been identified in a variety of malignancies (Wang 2007 *et al.*¹, Wang 2007 *et al.*², Marks *et al.* 2008, Wang *et al.* 2009, Cheng *et al.* 2011). With lung cancer, mutated *EGFR* is found with 10-30 % of the patients. The most common activating *EGFR* mutations are deletions in exon 19 and

L858R point mutation, which alter the tyrosine kinase domain of the receptor (Zhang *et al.* 2006, Yun *et al.* 2007).

K-Ras

K-Ras belongs to the Ras fprotein amily, which has two additional members, N-Ras and H-Ras. The Ras-Raf-MEK-ERK cascade signals downstream from the EGFR. The pathway is included in key cellular functions such as growth and proliferation. Alterations of the *KRAS* gene are frequently found in lung cancer. Approximately 30 % of NSCLC tumors harbour *KRAS* mutations, though in SCLC they are rare. *KRAS* mutations rarely co-exist with *EGFR* or *ALK* mutations but *KRAS* and *BRAF* double mutations have been found in NSCLC (Imilienski *et al.* 2012). Hence, activating *KRAS* mutations are known to predict resistance to EGFR inhibitors (Pao *et al.* 2005, Ebenhard *et al.* 2005). Most activating single point KRAS mutations occur in codons 12, 13 and 61. A majority of *KRAS* mutations in the lung are smoking related are G→T transversions occurring in codon 12 (90 % of patients) or 13 (Herbst *et al.* 2008).

ALK

Fusion of the *EML4* (echinoderm microtubule-associated protein-like 4) and *ALK* (anaplastic lymphoma kinase) genes is seen in 2-7 % of NSCLC tumors. As a result of the translocation, the ALK kinase is constantly activated causing hyper activation of the downstream targets, such as Akt and ERK 1/2, which are important regulators of proliferation and cell survival (Kwak *et al.* 2010).

2.6.2 Breast Cancer

Breast cancer is the most frequently occurring female cancer and a major cause of female cancer deaths in the western world. Though, thanks to advances in the disease screening and therapy development, mortality of the disease has significantly reduced in the past years even with the incidence rates continuously growing (Siegel *et al.* 2013). Breast cancers are divided into six biological subtypes: luminal A, luminal B, HER2-enriched, basal-like, normal breast and claudin-low, luminal A being the most common subtype (Eroles *et al.* 2012). In routine clinical practice, breast cancers are split into three prognostic and predictive subtypes (ER+, HER2+, and triple negative) based on the expression of

estrogen receptor (ER) and progesterone receptor (PR) and amplification of epidermal growth factor receptor 2 (HER2) (Eroles *et al.* 2012).

Steroid receptors

Majority of the breast cancer patients are ER and/or PR positive (70 % of all cases) (Eroles *et al.* 2012). The PR expression is under control of the functional oestrogen-ER-complexes (Anderson & Clarke 2004). Estrogen induced carcinogenesis has been suggested to involve formation of genotoxic metabolites, formation of DNA damaging active radicals. ER positive cancers also remain dependent on growth stimulation of tissue by estrogens.

HER2

HER2 belongs to the HER receptor family. The receptor binds several ligands, however no HER2 specific ones have been identified. HER2 gene amplification and protein overexpression are found in 15-30 % of breast cancer cases. Recently seven activating HER2 mutations in non-amplified HER2 breast cancer patients were identified, including G309A, D769H, D769Y, V777L, P780ins, V842I, and R896C (Bose *et al.* 2013). Activated HER2 signals through multiple downstream pathways, such PI3K-Akt-mTOR and Ras-Raf-MEK-ERK that regulate cell growth and proliferation. HER2 positive status is linked to an aggressive breast cancer phenotype (Taneja *et al.* 2010).

Triple-negative breast cancer

Triple-negative breast cancers (TNBC) do not express ER, PR or HER2 and they cover around 10-20 % breast cancers. The tumor type is typically aggressive and TNBC has been associated with poor prognosis. At the time of diagnosis TNBC patients are often younger than breast cancer patients in average (Boyle 2012). It has been proposed that BRCA1 loss may have an important role in TNBC (de Ruijter *et al.* 2011). Interestingly, around 30-35 % of triple-negative tumors have overexpression of androgen receptors (Niemeier *et al.* 2010, Park *et al.* 2010).

2.6.3 Melanoma

Skin cancers are divided to basal cell carcinoma, squamous cell carcinoma and melanoma. Melanoma, the most aggressive type of skin cancers, covers over 75 % of skin cancer death even though it represents only less than 10 % of all the skin cancer cases. The impact of the disease has been speculated to grow in the coming years (Kwong & Davies 2014). Activating mutations of the *BRAF* and *NRAS* genes are the most typically seen genetic alterations in melanoma. Increasing understanding of the most frequent alterations occurring in melanoma has led to improved clinical treatment results. Clinical trials with vemurafenib and dabrafenib, inhibitors of V600 mutant BRAF, have provided promising results in the clinic. However, due to high relapse rates of single inhibitor treatments, combinatorial approaches are now in the focus of current melanoma clinical research (Chapman *et al.* 2011, Hauschild *et al.* 2012).

2.6.4 Colorectal cancer

Colorectal cancer (CRC) is the third most frequently occurring cancer in both men and women (Siegel *et al.* 2013). Colorectal carcinogenesis requires 6-10 genetic mutations, which give the cancer cells growth advantage to normal cells. This process requires time what probably explains why CRC is related to high age (Treanor & Quirke 2007). Development of CRC is associated to western lifestyle since i.e. high consumption of red meat and animal fat and excessive alcohol consumption are known risk factors for CRC (Hamilton *et al.* 2010). Until recently, chemotherapy has been the routine treatment in advanced CRC cases. However, discoveries of typically altered genes in CRC such as *KRAS*, *NRAS* and *BRAF* have encouraged the development of molecularly targeted therapies and led to improved patients outcomes (Patel & Karapetis 2013). Also signaling through growth factor pathways such as the EGFR has been shown to have an important role in development and progress of CRC (Markowitz & Bertagnolli 2009). Majority of CRCs develop from premalignant lesions that are called adenomas. In over 70 % of CRC cases the disease arises from sporadic mutations in adenomatous polyps (Hardy *et al.* 2005).

2.7 Cancer therapy in solid malignancies

Traditionally cancer has been treated with surgery, radiation and chemotherapy. However, in the recent decades a new approach has been to develop molecularly targeted therapies that are based on specific oncogenic mutations.

2.7.1 Surgery

Surgery is the oldest cancer treatment and for long it has been also the only known method that may cure patients. Even today it is still the most successful curative treatment strategy for solid malignancies. However, the challenge in curative surgery is that the disease has to be detected at an early stage. With cancers that are more difficult to detect, such as pancreas cancer, the disease is often already developed to an advanced stage by the time of diagnosis, hence surgery is no longer a realistic option (Roberts *et al.* 2007).

2.7.2 Radiotherapy

Radiation as a cancer treatment is based mainly on the DNA and cellular membrane damage it causes to the exposed tissue. The DNA repair mechanisms of cells are usually able to correct most of the DNA damage caused by radiation, especially if only one of the DNA strands is broken. By dividing the radiation exposure into smaller doses, less damage is caused to normal tissue even though the outcomes remain the same. DNA repair in cancer cells is not as effective as in normal cells, thus by using fractionation of radiation doses, it is possible to avoid normal tissue destruction. If both the DNA strands are damaged, probability for cell death increases. Usually cells carrying the damaged DNA are not destroyed until the next mitosis. Sometimes cell death will not occur until after a few passages. DNA alterations caused by radiation are not always sufficient to result in apoptosis. In some cases these mutations might even be carcinogenic (Roberts *et al.* 2007).

Around 50 % of all cancer patients are treated with radiation at some point of the disease. Most typically radiation therapy is used as an adjuvant therapy with other therapy forms such as before and after surgery. Combining radiotherapy with chemotherapy in some clinical situations has significantly improved the outcomes gained with the treatment like in rectal and lung cancer. A modern

research approach is to combine radiation therapy with target based biologic drugs and to search sensitizing effects (Roberts *et al.* 2007).

2.7.3 Chemotherapy

Traditional chemotherapeutic agents target mainly dividing cells. Hence, the treatment is more cytotoxic to cells that proliferate rapidly. The effect of chemotherapeutic agents is based on a variety of mechanisms such as inhibition of enzymes involved in DNA and RNA synthesis, DNA/RNA damage, inhibition of mitosis through affecting the microtubule or cellular membranes damage (Roberts *et al.* 2007).

The drawback of chemotherapy is that it is highly toxic also to normal cells; especially fast dividing cells such as hematopoietic cells of the bone marrow are sensitive to some chemotherapeutic agents. However, usually healthy tissue is repaired more rapidly than tumor tissue. Typically chemotherapy is used in combination with other treatment strategies such as surgery in local cancers. While chemotherapy is regarded curable i.e. in testis cancer and lymphom, metastatic cancer is still a challenge to treat (Roberts *et al.* 2007).

2.7.4 Hormone based therapies

Hormone based therapies are typically used to treat cancers that arise from tissues that are under hormonal control, such as breast and prostate cancer. The first hormone based therapies were surgical removal of endocrine glands, which is still fairly common. For example breast cancer has been traditionally treated with ovary removal. Other typically used hormone based therapies include antiestrogens such as tamoxifen for premenopausal breast cancer patients and antiandrogens such as bicalutamide for prostate cancer patients (Roberts *et al.* 2007). ER/PR status is used as a guide to treat breast cancer patients with endocrine therapy (Rastelli & Crispino 2008). Aromatase inhibitors are used to treat Er+/Pr+ breast and ovarian cancer in post-menopausal women. The aromatase enzyme catalyzes conversion of estrogen from testosterone. Glucocorticoids, that are typically used to treat leukemia and lymphomas, can also be used as a hormonal therapy to decrease the levels of estrogens/androgens in breast or prostate cancer patients. (Roberts *et al.* 2007).

2.7.5 Targeted cancer therapies

In the past years, there has been an enthusiasm for development of gene based targeted cancer therapies. As these therapies are developed to specifically target an action mechanism, it is thought that they are better understood than the older chemotherapeutic agents. The benefits of the molecularly targeted treatments can be based on better predictability of the efficiency with patients as well as reduced side effects compared to the traditional therapies. Typically, the biological activity of molecularly targeted therapies is based on specific oncogenic alterations. For example *HER2* amplified breast cancers have been shown to respond to PI3K inhibitors (Faber *et al.* 2009) while *BRAF* mutant melanomas (Solit *et al.* 2006) and triple-negative breast cancers are repressed by MEK inhibitors (Hoeflich *et al.* 2009). In clinical practice, HER2 antibody and BRAF inhibitors are used to treat breast cancer and melanoma, respectively.

EGFR, HER2 and ALK receptor tyrosine kinase inhibitors and antibodies

Current therapies used against EGFR overactivation can be divided to either EGFR antibodies or TKIs. Monoclonal antibodies, such as cetuximab, block the extracellular domain of the receptor. Cetuximab is bound to EGFR with high affinity, followed by receptor internalization and degradation that ultimately results in downregulation of cell surface EGFR expression (Kim *et al.* 2001). Erlotinib and gefitinib are widely used small molecule inhibitors that block the intracellular ATP binding site of the EGF receptor. Especially patients with EGFR mutations have shown to be highly responsive for these drugs (Jänne & Johnson 2006).

Trastuzumab, a HER2-binding monoclonal antibody, was the first oncogene-targeting therapy for solid malignancies. The compound binds to the subdomain IV of the extracellular domain, which is close to the receptor's transmembrane domain. In turn, pertuzumab, another HER2 antibody used in the clinic blocks dimerization of the receptor and hence also prevents the signal transmission (Tai *et al.* 2010). The TKI lapatinib binds to ATP-binding site of both HER2 and EGFR, preventing autophosphorylating and downstream signaling of the receptor. Unlike trastuzumab, lapatinib is able to inhibit the truncated HER2 and EGFR forms which contain only the intracellular domain. Importantly, lapatinib has also been shown to be able to cross the blood-brain barrier and target brain metastases (Moy *et al.* 2007). With breast cancer patients, HER2 positivity is used as a

predictive factor for suitability for treatments with trastuzumab (Jahanzeb 2008, Murphy & Morris 2012), pertuzumab and lapatinib (Murphy & Morris 2012).

Several ALK inhibitors have shown growth inhibition potential with cell lines with *ALK* gene translocations. Crizotinib (PF-02341066) was the first developed inhibitor to block human ALK signaling. It inhibits ATP-competitively ALK and MET tyrosine kinases and blocks the tyrosine phosphorylation of activated ALK kinase (Christensen *et al.* 2007, Zou *et al.* 2007). Furthermore, crizotinib has been shown to cause tumor shrinkage in NSCLC patients with *EML4-ALK* fusions genes (Kwak *et al.* 2010). Additionally, several other ALK inhibitors are currently tested in the clinic for treatment of NSCLC (Casaluce *et al.* 2013). The diaminopyrimidine TAE684 is a small molecule drug inhibiting the ALK kinase. The compound binds ATP competitively and blocks receptor phosphorylation and its downstream signaling (Galkin *et al.* 2007, Koivunen *et al.* 2008). Despite the promising results of preclinical studies, TAE684 did not proceed to the clinic due to its toxicity.

PI3K-Akt-mTOR pathway inhibitors

Several small molecule inhibitors targeting the PI3K-Akt-mTOR pathway have been developed, including dual PI3K-mTOR inhibitors, PI3K inhibitors, Akt inhibitors and mTOR inhibitors. Allosteric, rapalog analog mTOR inhibitors, were the first to enter clinical trials from PI3K pathway inhibitor but they showed activity only in limited number of solid tumor types. Limited activity of the rapalog is suggested to result from various feedback loops leading to mTORC2-IRS-1 mediated hyperactivation of PI3K-Akt (O'Reilly *et al.* 2006). Rapalog induced PI3K-Akt hyperactivation led to development upstream inhibitors, which could overcome hyperactivation and potentially have more clinical activity. Furthermore, recently newer generation of inhibitors have entered clinical trials including isoform specific PI3K inhibitors and catalytic inhibitors of mTORC1/2.

Currently, only the rapalog inhibitors of the PI3K-Akt-mTOR pathway have entered the clinic for the treatment of renal cell, neuroendocrine, and breast cancers. Multiple clinical trials are underway to test efficiency of dual PI3K-mTOR, PI3K and Akt inhibitors in various solid malignancies. However, based on early phase clinical trials, their single agent clinical activity seems to be modest in unselected solid malignancies. The drugs appear to be more active in tumors bearing genetic alterations in *PIK3CA* or *PTEN* but no clear correlation between genotype and response have been established (Brachmann *et al.* 2009).

Ras-Raf-MEK-ERK pathway inhibitors

Large efforts have been done to develop Ras inhibitors, but so far clinical trials have not proved to be successful. Various TKI Raf inhibitors have been developed, sorafenib being the first to enter the clinic in the treatment of renal and hepatocellular cancers (Wilhelm *et al.* 2006). Even though sorafenib is a potent inhibitor of wild-type and mutant B-Raf, it hasn't shown clinical activity in B-Raf driven cancers such as melanoma (Eisen *et al.* 2006). Later, inhibitors preferentially targeting mutant B-Raf, such as vemurafenib and dabrafenib, have been developed. These inhibitors have significant clinical activity in B-Raf mutant melanoma and have been approved for this indication (Joseph *et al.* 2010).

MEK inhibitors are classed into ATP competitive and ATP non-competitive agents. Most of the today known MEK inhibitors fall into the latter category, hence are not directly competing for the ATP-binding, but bind to a unique allosteric binding site adjacent to the ATP binding site. For this reason non-ATP-competitive MEK inhibitors have high specificity for the target (Wallace *et al.* 2005). CI-1040 was the first allosteric MEK1/2 inhibitor to enter the clinical trials. CI-1040 showed to be well tolerated but possessed insufficient antitumor activity in patients (Rinehart *et al.* 2004). Most of next generation MEK inhibitors share similar chemical structure but are more potent inhibitors of the target.

Dual PI3K and MEK inhibition

Even though PI3K-Akt-mTOR and Ras-Raf-MEK-ERK are the most commonly altered signaling pathways in solid malignancies, the clinical efficiency of single pathway inhibitors have generally been disappointing (Britten *et al.* 2012, Bedard *et al.* 2012, LoRusso *et al.* 2012). Cancers can be *de novo* dependent concurrently on these two parallel pathways and cross-signaling of the two pathways is also evident (Faber *et al.* 2009, Chandarlapaty *et al.* 2011). Furthermore, multiple *in vivo* and *in vitro* studies have shown that PI3K-Akt-mTOR and Ras-Raf-MEK-ERK pathways regulate each other's activity through feedback mechanisms, such as PI3K-Akt-mTOR activation via ERBB receptors following MEK inhibition (Turke *et al.* 2012). The interaction of the parallel PI3K-Akt-mTOR and Ras-Raf-MEK-ERK signaling pathways is thought to explain the inefficiency of single inhibition treatments. Preclinical models have shown that dual targeting with PI3K and MEK inhibitors is efficacious in various cancer models and genotypes. Activity is also seen in genotypes that have been difficult to treat, such as Ras

mutant tumors and basal like breast cancer (Hoeflich *et al.* 2009, Wee *et al.* 2009, Sos *et al.* 2009, Faber *et al.* 2009).

Drugs targeting cancer stem cells

CSCs are shown to be resistant to current targeted therapies, radiation therapy and most chemotherapeutic agents. However, some agents are known to induce CSC specific toxicity *in vitro*. Such agents include microbial- or plant-derived biomolecules, small molecule inhibitors that target central components of CSC signaling pathways and compounds that specifically target molecules on the cell surface of CSCs. Moreover, some traditionally and widely used drugs, such as metformin have shown CSC toxicity (Naujokat & Steinhart 2012). For example, the ionophore salinomycin was shown in a high-throughput screening to selectively target breast cancer CSCs both *in vivo* and *in vitro* (Gupta *et al.* 2009). The compound has also been shown to be able to overcome the ABC transporter mediated drug resistance mechanism (Fuchs *et al.* 2009). Activation of embryonic stem cell pathways, such as Notch, Wnt and Hedgehog, have also been related to the stem cell phenotype. Hence, blocking these pathways could be one alternative to target the CSCs. For example, blocking of γ -secretase-mediated Notch cleavage has been under the scope and some agents have already entered the clinic (Takebe *et al.* 2010).

Bcl-2 inhibitors

A vast majority of all tumors have dysfunctional p53 pathway, and overexpression of anti-apoptotic/pro-survival proteins such as Bcl-2 or Bcl-x1 is fairly common (Amundson *et al.* 2000). This has led to an increased interest into the development of therapeutic agents, BH3 mimetics, that bind to the BH3 binding domain of Bcl-2-like pro-survival molecules, and inactivate them (Cory *et al.*, 2003; Fesik, 2005). Since the BH3 domain has been shown to preferentially bind certain pro-survival proteins (Chen *et al.*, 2005), using BH3 mimetics increases the likelihood of specific targeting the Bcl-2-like proteins, which are central for maintaining some tumor types. This way reduced cytotoxicity to healthy cells is enabled (Adams & Cory 2007).

2.8 Targeted therapy resistance

Resistance to targeted therapies is a significant problem with current cancer treatments. Different types of targeted therapy resistance are divided to primary (*de novo*) resistance and acquired (secondary) resistance.

2.8.1 Primary resistance

Some cancers have been found to be unresponsive to targeted therapies even though the targeted oncogene is present. This type of initial resistance to targeted therapies is referred to as primary resistance. Initial unresponsiveness to targeted therapies has been found to be fairly frequent. Typically primary resistance is caused by constitutively activated signal transduces downstream the drug target, which covers most of the patients that do not response to the used therapy (Sartore-Bianchi *et al.* 2009, Bonomi *et al.* 2007, Pao *et al.* 2005). For example approximately 30% of patients with activating *EGFR* gene mutations do not response to EGFR TKI therapy. Known mechanisms behind primary resistance to EGFR TKIs include insertions in *EGFR* and *HER2* exon 20 (Wu *et al.* 2008, Wang *et al.* 2006) and genetic alterations in non-*EGFR* related genes, such as *KRAS* (Raponi *et al.* 2008).

2.8.2 Acquired resistance

Virtually all cancers develop secondary resistance to targeted therapies even after promising initial responses. Known mechanisms behind acquired resistance include secondary mutations in the drug target gene that affect drug binding, activation of parallel signaling pathways and amplifications of the target gene or another oncogenes (Garraway & Jänne 2012). For example, two resistance mechanisms for acquired resistance for EGFR TKI therapies in NSCLC have been identified. The more common of these is the T90M secondary mutation that is seen in approximately 50% of all patients with acquired resistance (Kobayashi *et al.* 2005, Hammerman *et al.* 2010). The mutation increases the affinity of the mutant EGFR for ATP restoring it to the level of wild-type EGFR (Yun *et al.* 2008). Another known mechanism for EGFR TKIs mechanism is amplification of the gene encoding the MET receptor, which causes resistance through kinase switching (Engelman *et al.* 2007²).

With ALK rearranged cancers, several secondary mutations, such as L1152R, causing resistance to ALK TKI crizotinib, have been identified. The genetic alterations are known to affect drug binding or activate parallel signaling pathways such as the EGFR downstream signal cascade (Sasaki *et al.* 2011, Yamaguchi *et al.* 2014). A widely studied approach for overcoming acquired resistance is combined inhibition of multiple targets of the same or different signaling pathways (Abrams *et al.* 2010, Nazarian *et al.* 2010, Katayama *et al.* 2011, McCubrey *et al.* 2012). As an example dual ALK and EGFR inhibition has been suggested as an approach against cancers with acquired ALK TKI resistance (Sasaki *et al.* 2011).

3 Aims of the present study

In the recent decades, new molecularly targeted therapies have been eagerly studied to find more efficient approaches for cancer treatments. The mechanism of the targeted therapies is often based on specific oncogenic mutations in certain tumor types and many of these types of drugs have already entered clinical practice. In some cases, the action mechanisms as well as positive and negative predictive factors for targeted therapies are well understood. However, the clinical benefit of a single targeted therapy is commonly limited to a specific cancer genotype, and often seems to work only in some cancer types. The major limitation for clinical efficacy of the use of targeted cancer drugs is therapy resistance.

This study investigates targeted therapy sensitivity and resistance in solid tumor types. More specifically, the main aims of the study are:

1. To study sensitivity of PI3K and MEK co-inhibition in solid tumors.
2. To study apoptosis resensitization in PI3K and MEK co-inhibition sensitive cancer cell lines.
3. To gain more understanding of CSC mediated adaptive resistance to targeted therapies
4. To investigate new agents targeting T790M mediated resistance in EGFR mutated NSCLC.

4 Materials and methods

Methods used in the original publications are summarized in Table 2. Details of the used methods are described below starting from chapter 4.1.

Table 2. Summary of the methods used in original publications.

Method	Publications
MTS cytotoxicity/cell growth analysis	I, II, III, IV
Western blot analysis	I, II, III, IV
Colony formation	III
PathScan analysis	I
Generation of acquired resistance	III, IV
Mouse xenografts	III, IV
DNA extraction, PCR and sequencing	IV
Retroviral expression and Ba/F3 models	IV

4.1 Cell lines and inhibitors

The cell lines used for the studies are presented in Table 3 and Table 4. The NSCLC cell lines were kind gifts from Dr. Pasi Jänne (Dana-Farber Cancer Institute, Boston, USA), the breast and colorectal lines from Dr. Peppi Koivunen (Oulu University, Oulu, Finland) and the melanoma cell lines were purchased DSMZ GmbH (Braunschweig, Germany). The H3122 Luc cell line was generated by a retroviral transfection with a firefly luciferase plasmid with puromycin resistance (MSCV-Luc- Puro, a gift from Dr. Andrew Kung, Dana-Farber Cancer Institute, Boston, USA). The cell lines were cultured in RPMI-1640 supplemented with 5 or 10% fetal bovine serum and 100 IU/ml penicillin and streptomycin. All the cell culture reagents were purchased from HyClone (Logan, UT).

Table 3. Cell lines used in the original publications.

NSCLC		Breast cancer		CRC		Melanoma	
Cell Line	Genotype	Cell Line	Genotype	Cell Line	Genotype	Cell Line	Genotype
A549	K-Ras	MCF-7	ER+	HCT116	K-Ras	SK-MEL-1	B-Raf
H358	K-Ras	T-47-D	ER+			COLO-800	B-Raf
H441	K-Ras	SKBR3	HER3			Sk-Mel-30	N-Ras
HCC827	EGFR	BT474	HER3			IPC-298	N-Ras
PC-9	EGFR						
Ma-1	EGFR						
H3122	ALK						
H2228	ALK						
H1437	MEK						
A431	ND						
H1581	ND						

Table 4. Acquired cell lines used in studies III and IV.

Cell line	Genotype
HCC827GR	EGFR+c-MET amp
PC-9ER	EGFR+T790M
H1975	EGFR+T790M

Inhibitors used in the original publications are presented in Table 5. All the inhibitors were dissolved in DMSO to a final concentration of 10mM and stored at -20°C . The drug solutions for the experiments were prepared from a 10mM stock solution immediately before use. Further dilutions were made in the cell culture medium.

Table 5. Chemical agents used in the original publications.

Drug	Target	Mecanism	From
Erlotinib	EGFR	Kinase domain inhibition by ATP binding blockage	Alexis Biochemicals; Lausen, Switzerland
Gö6976	PKC	Kinase domain inhibition by ATP binding blockage	LCLabs and Calbiochem
Gö6986	PKC	Kinase domain inhibition by ATP binding blockage	Sigma-Aldrich; St. Louis, MO
Staurosporine	PKC	Kinase domain inhibition by ATP binding blockage	Sigma-Aldrich
UCN-01	PKC	Kinase domain inhibition by ATP binding blockage	Sigma-Aldrich
Crizotinib	ALK	Kinase domain inhibition by ATP binding blockage	LCLabs and Calbiochem; La Jolla, CA
TAE684	ALK	Kinase domain inhibition by ATP binding blockage	a gift from Dr. Nathanael Gray, Dana-Farber Cancer Institute, Boston
Lapatinib	HER2	Kinase domain inhibition by ATP binding blockage	Alexis Biochemicals
Tamoxifen	ER	Receptor blockage	Sigma-Aldrich
AG1024	IGF1R1	Kinase domain inhibition by ATP binding blockage	Alexis Biochemicals
Sunitinib	Multi-TKI	Kinase domain inhibition by ATP binding blockage	Alexis Biochemicals
Dasatinib	Multi-TKI	Kinase domain inhibition by ATP binding blockage	LCLabs and Calbiochem
Sorafenib	Multi-TKI	Kinase domain inhibition by ATP binding blockage	LCLabs and Calbiochem
CI-1040	MEK	Kinase domain inhibition by ATP binding blockage	Alexis Biochemicals
ZSTK474	PI3K	Kinase domain inhibition by ATP binding blockage	Alexis Biochemicals
PI-103	PI3K+mTOR	Kinase domain inhibition by ATP binding blockage	Alexis Biochemicals
Vemurafenib	B-Raf V600E	Kinase domain inhibition by ATP binding blockage	LCLabs and Calbiochem
Salinomycin	Stem Cells	Action as a potassium ionophore (proposed)	LCLabs and Calbiochem
Abamectin	Stem Cells	Unknown	Sigma-Aldrich
Ly-2157299	TGF- β R1	Kinase domain inhibition by ATP binding blockage	Axon Medchem BV, Groningen, Netherlands
SD 208	TGF- β R1	Kinase domain inhibition by ATP binding blockage	Axon Medchem BV

Drug	Target	Mecanism	From
NVP-XAV939	WNT	Stimulation of β -catenin degradation by axin stabilization	Axon Medchem BV
DBC	Notc	γ -Secretase inhibition	Axon Medchem BV
Vismodegib	Hedgehog	Receptor (Smoothened) antagonist	Axon Medchem BV
Etoposide	Chemo	Prevention of re-ligation of the DNA strands	LCLabs and Calbiochem
Cisplatin	Chemo	DNA crosslinking	Sigma-Aldrich
ABT-263	Bcl-2 family	Blockage of the BH3 binding site	Selleck Chemicals LLC Houston TX, USA
Entinostat	HDAC	-	LCLabs and Calbiochem
5-AZA	Methylation	Inhibition of DNA methyltransferases	Sigma-Aldrich

4.2 MTS cytotoxicity/cell growth assay (I, II, III, IV)

Cells were plated onto 96-well plates with three to six parallel wells for each treatment, the experiments being replicated at least three times. The inhibitor treatments were started after 24-48h, and the plates were developed 72h later using an MTS reagent mix [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt], (Promega; Madison, WI) supplemented with phenazine methosulfate (Sigma-Aldrich; St. Louis, MO) according to the manufacturer's guidelines. The absorbances were read on a plate reader (Athos Labtec Instruments; Salzburg, Austria) at a wavelength of 488nm. The data were displayed graphically using GraphPad Prism (GraphPad Software; La Jolla, CA), with the absorbance in the non-treated wells as the reference value (100%). For study I the combination index (CI) was calculated using Calcosyn software (BIOSOFT, Cambridge, UK), and a 3.3:1 ratio of the PI3K inhibitors to the MEK inhibitor was used in the CI analysis.

4.3 Western blot analysis (I, II, III, IV)

The cells were plated onto 6-well plates and drugged 1-2 days later for the desired time, after which they were lysed in RIPA buffer (1% Igepal CA-630, 20 mM Tris-HCl pH 8.0, 137 mM NaCl, 10% glycerol, 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM sodium orthovanadate, 10 μ g/mL Aprotinin, 10 μ g/mL Leupeptin, and 10 μ g/mL Pepstatin). Protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad; Hercules, CA) and the concentrations in individual samples were equalized before adding 3x Laemmli buffer to a final concentration of 1x. Equal amounts of protein were run on 7.5%-12% SDS-PAGE

gels, transferred to polyvinylidene fluoride (PVDF) membranes, probed with the antibodies and developed using the enhanced chemiluminescence (ECL) chemiluminescence system (Millipore; Billerica, MA) for detection on radiographic films, which were scanned to an electronic format.

Primary antibodies used in the studies are presented in Table 6. All the primary antibodies were used as 1:1000 dilutions in 5% bovine serum albumin (BSA), except the CD44 antibody in 5% milk powder. The secondary antibodies used include Anti-Mouse IgG HRP-linked antibody (for ALDH1 and CD44) and Anti-Rabbit IgG HRP-linked antibody. All antibodies were from Cell Signaling Technology, (Danvers, USA) except for ALD1 (BD Transduction Laboratories, Franklin Lakes, USA).

Table 6. Primary antibodies used in studies I-IV.

Primary antibody	Phosphorylation site
ALDH1	-
CD44	-
phospho-Akt	S473
Akt	-
phospho-ERK1/2	T202/Y204
ERK1/2	-
phospho-ALK	T1096
ALK	-
phospho-S6	Thr389
S6	-
phospho-4E-BP1	Thr37/46
4E-BP1	-
phospho-EGFR	Y1068
EGFR	-
PARP	-
cleaved PARP	-
phospho-HER2	Y1221/1222
HER2	-
phospho-HER3	Y1289
HER3	-
Bcl-xl	-
Mcl-1	-
BIM	-
β -actin	-

4.4 Colony formation assay (III, IV)

For the colony formation, assays 300-1300 cells were plated on 24-well plates, allowed to attach for one day, and treated with inhibitors for 7 days. At least two parallel wells were used for each treatment. After 7 days, the drugs were withdrawn and the cells were allowed to proliferate and form colonies for several weeks. The cell culture media was changed when needed. After differences in the growth of colonies had appeared, the cells were washed with PBS, fixed with methanol and dyed with 0.005% crystal violet (Merck; Darmstadt, Germany), a chromatin-binding stain. The plates were imaged using a digital camera.

4.5 Generation of acquired resistance (III, IV)

For generation of acquired resistance, H3122 cells were plated on 6-well plates, three replicate wells per treatment. The cells were exposed to 1 μ M crizotinib, 3.3 μ M ZSTK474, 0.1 μ M salinomycin or their combination two days after plating. Drug containing media was replaced weekly with a fresh, drug containing media and the plates were visually inspected under phase contrast microscope. At three months of exposure to the drugs, growing colonies were present only in the crizotinib treated wells when one of three wells were fixed and stained as for colony formation assays. One of the crizotinib resistant wells (H3122CR cells) was trypsinized and the cells were transferred to non-drug containing media. After a single passage in non-drug media cells were 1) analysed with western blot and MTS-assays, 2) plated on 96-well plates on average one cell per well to generate clones, or 3) let to grow in non-drug media and analysed with MTS assay for presence of resistance every three passages. Drug combination treated wells were followed for additional three months, medias being changed and visually inspected weekly. After six months, the experiment was terminated since no growing colonies or surviving cells were seen in the combination plates.

4.6 Mouse xenograft models (III, IV)

For the xenograft experiments in study I firefly luciferase reporter (MSCV-Luc-Puro) PC9-resistant cells with I firefly luciferase reporter were used. 1×10^6 cells were injected s.c. into both flanks of Hsd Athymic Nude Foxn1 female mice (Harlan Laboratories, Boxmeer, Netherlands), and the bioluminescence of the tumors was visualized with IVIS Spectrum (Caliper Life Sciences, Hopkinton,

MA) after an i.p. injection of 15 mg/kg D-luciferin Firefly (Caliper Life Sciences; Hopkinton, MA) prior the start of the therapeutic treatment (day 0) and on days 8, 12 and 18 of the treatment. Gö6976 or the vehicle was administered i.p. once a day on five days a week. The region of interest in the tumors (n = 8 for Gö6976, n = 5 for vehicle) was determined by means of Living Image software (Caliper Life Sciences; Hopkinton, MA).

For *in vivo* studies in paper III H3122 Luc cells were treated with 0.1 μ M TAE684 for 14 days and let to recover for 1 day before injection. TAE-untreated H3122 Luc cells were used as controls. After trypsination, the cells were counted using trypan blue exclusion, and the cells were diluted with PBS to 100,000 or 10,000 cells/0.2ml and immediately injected on mice. Female NOD.Cg-Prkdc(scid)Hr(hr)/NCrHsd mice (Harlan Laboratories; Boxmeer, Netherlands) were anesthetized with fentanyl/fluanisone/midazolam and 0.2ml of the cell solution was injected on both flanks. Mice were anesthetized with isoflurane, injected intraperitoneally with 15 mg/kg D-luciferin Firefly and imaged weekly using the IVIS Spectrum *in vivo* imaging system with Living Image software (PerkinElmer; Waltham, USA). All the animal experiments were performed according to protocols approved by the Provincial State Office of Southern Finland.

4.7 Pathscan analysis (I)

The PathScan analysis was carried out with the PathScan® RTK Signaling Antibody Array kit (Cell Signaling Technologies, Danvers, MA) according to the manufacturer's guidelines. In brief, cells were plated on 6 cm diameter plates and drugged the following day for 24 h. Whole cell lysates were collected, protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) and the protein concentrations were equalized. The lysates were applied to nitrocellulose membranes and incubated over night, washed, exposed to the secondary antibodies, developed with ECL and imaged with a Fujifilm LAS-3000 Luminescent Image analyser and the ImageReader LAS-3000 program.

4.8 siRNA knock down (II)

For the knock down studies *BCL2L1* small interfering RNA (siRNA) (Dharmacon smart pool) was used at 25nmol/l concentration and transfected with

DharmaFECT transfection reagent (both from Dharmacon, Lafayette, CO). The experiments were performed according to the manufactures protocol. In short, the cells were plated on 96 (for MTS) or 6 well (for western blot) plates in non-antibiotic media. After 24-48h the media was changed to media containing siRNA+lipid or lipid (controls), prediluted in Opti-MEM (Gibco/Invitrogen, Carlsbad, CA). The following day, the media was changed to normal media. The incubation was continued for the desired time (total of 24-96h) and before the assays.

4.9 DNA extraction, PCR and sequencing (IV)

DNA was extracted from cell lines using Blood & Cell Culture DNA Mini Kit (Qiagen, Valencia, CA). Exon 20 of the EGFR gene was amplified with polymerase chain reaction (PCR) using Taq-polymerase (Fermentas, St. Leon-Rot, Germany). PCR products were sequenced using ABI3130xl Genetic Analyzer (Applied Biosystems; Carlsbad, CA). All the cell lines were analysed by STR analysis (IdenticeLL, Århus, Denmark) to rule out cross-contamination.

4.10 Retroviral expression and Ba/F3 models (IV)

The following retroviral vectors were used: EGFR ex19 del +/- T790M in JP1520 backbone, EGFR L858R +/- T790M in JP1536 backbone (gifts from Dr. Pasi Jänne) and MSCV-Luc-Puro, (a gift from Dr. Andrew Kung). 293T cells were transfected with retroviral expression vectors and packaging plasmids (pCL-Eco, kind gifts from Dr. Pasi Jänne) using the Fugene 6 reagent (Roche Diagnostics; Mannheim, Germany). Retroviral supernatants were collected 48 hr later, filtered and applied to the target cells in the presence of polybrene (Sigma-Aldrich). The target cells were then selected with puromycin (Sigma-Aldrich) for 72–96 h, beginning 48 h after the infection. In the Ba/F3 models that followed the puromycin treatment regime, the cells were transferred to media without WEHI-3B supplementation to select for transformed, IL-3 independent clones. The Ba/F3 cells are dependent on IL-3 supplementation but lose the dependency when transformed with oncogenes.

5 Results

5.1 Dual PI3K and MEK inhibition in cancer treatment

In study I, dual PI3K inhibition was examined with ZSTK474 (PI3K inhibitor), PI-103 (PI3K + mTOR inhibitor) and CI-1040 (MEK inhibitor).

5.1.1 Single agent PI3K or MEK inhibition in non-small cell lung cancer cell lines

The effects of the inhibitors alone were first studied in the NSCLC lines A549 (*K-Ras* mutant), HCC827 (*EGFR* mutant) and H3122 (*EML4-ALK* translocated), representing the three most frequent oncogenic genotypes of the disease, for establishing concentration frames for the target inhibition. When the cells were treated with increasing concentrations of the inhibitors, downregulation of pAkt and pS6, downstream targets of PI3K, was seen in western blots with 3.3 μ M ZSTK474 and with 1 to 3.3 μ M PI-103. At these concentrations, also a major reduction in the number of viable cells was seen in the MTS cytotoxicity assay. CI-1040 induced complete inhibition of ERK1/2, an immediate downstream target of MEK, at 1 μ M concentration. Only the H3122 cell line showed marked reduction in cell viability in the MTS assays in response to increasing concentrations of CI-1040.

5.1.2 Dual PI3K and MEK inhibition

A panel of NSCLC cell lines (n=12) were tested for their sensitivity to dual PI3K and MEK inhibition. When compared to single agent treatments, two cell lines, H3122 and H1437, showed increased cytotoxicity for dual inhibition, with no significant differences between ZSTK474 and PI-103.

The NSCLC cell lines (H3122, H1437) showing major synergy upon dual inhibition in addition with a breast (MDA-MB231) and a colorectal cancer line (HCT116) previously identified to be sensitive to dual inhibition, were further studied with western blots for cell signaling in response to the PI3K and MEK blockage. Activation of parallel pathways was noted in response to single inhibitor treatments, and dual inhibition was able to overcome this single inhibitor-induced stimulation of parallel pathway activation. The cell lines were

further analysed for cleaved poly ADP ribose polymerase (cPARP), a widely used apoptosis marker. When dual inhibition with either ZSTK474 or PI-103 was administered, marked PARP cleavage was seen in the H3122 line but not in the other lines tested.

5.1.3 Alternative dosing of dual PI3K and MEK inhibition

When alternative dosing of dual PI3K and MEK blockage was experimented with MTS cytotoxicity assay, two of the four dual inhibition sensitive cell lines (MDA-MB231 and H1437) showed similar cytotoxicity to continuous dual blockage with continuous PI3K (72h) and short (15 min) MEK inhibition treatment. Instead, with the H3122 and HCT116 lines, both the PI3K and MEK inhibitors needed to be administered throughout the treatment period for maximal cytotoxicity.

Next western blot was used to investigate intracellular signaling in response to alternative dosing of the dual inhibition. No or only minor recovery of pAkt downregulation was noted when PI3K inhibition was administered for 15 min at the beginning the 6h period, whereas pERK1/2 recovered completely in 6 h when the cells were treated with the MEK inhibitor for 15 min. H3122, the only cell line identified to induce apoptosis in response to dual PI3K and MEK blockage, was further used to analyse whether alternative dosing could also result in apoptosis.

Marked cPARP levels were seen when cells were treated for 15 min with dual blockage and then continued with sole PI3K or MEK inhibition for 48 h.

5.2 Bcl-xl downregulation is a major determinant of response to dual PI3K and MEK inhibition

In study II, the effect of combining dual PI3K and MEK blockage with inhibition of a third target was further studied in HCT116 (colon cancer), MDA-MB231 (breast cancer) and H1437 (NSCLC) cell lines.

5.2.1 ABT-263, entinostat and dasatinib increase the cytotoxicity of dual PI3K and MEK inhibition.

HCT116 (colon cancer), MDA-MB231 (breast cancer) and H1437 (NSCLC) cell lines were chosen for the studies. These cell lines are known to be sensitive to

dual PI3K and MEK inhibition but to have limited apoptotic response to the therapy. The cells were first addressed to treatments were dual PI3K and MEK inhibition was tested in combination with a panel of other small molecule inhibitors. Of the 21 tested drugs, only three, BH3 mimetic ABT-263, histone deacetylase (HDAC) inhibitor entinostat, and multikinase inhibitor dasatinib were noticed to decrease cell viability in MTS assays compared to sole dual blockage, suggesting increased cytotoxicity.

Next MTS cytotoxicity assay was used to further explore whether concurrent dual blockage is needed for maximal cytotoxicity of the treatments or if combination with single PI3K or MEK inhibition is sufficient. In the HCT116 line, ABT-263 decreased cell viability when combined with a PI3K inhibitor (ZSTK474) but not with a MEK inhibitor (CI-1040). Conversely, when HCT116 was treated with entinostat or dasatinib, concurrently with either ZSTK474 or CI-1040, decreased cell viability was seen. In the MDA-MB231 line, marked increase in cytotoxicity was seen with concurrent administration of ABT-263 or dasatinib with ZSTK474 or CI-1040. Increased cytotoxicity was also seen with co-administration of entinostat and ZSTK474 but not with CI-1040. In the H1437 line, entinostat was the only agent noted to increase cytotoxicity in combination with either ZSTK474 or CI-1040 while no marked change was seen with ABT-263 or dasatinib combinations.

5.2.2 ABT-263, entinostat and dasatinib increase apoptotic response to dual PI3K and MEK inhibition.

Next it was investigated with western blot if the cytotoxic response seen in MTS assays occurs through apoptosis. In HCT116 and MDA-M231 cell lines, marked PARP cleavage was seen when ABT-263 was combined with ZSTK474 and/or CI-1040. In H1437, PARP cleavage was only seen when ABT-263 was combined with dual PI3K and MEK blockage. The HCT116 line responded to entinostat treatments analogously to ABT-263 with detectable PARP cleavage when entinostat was co-administered with ZSTK474 and/or CI-1040. In the H1437 line, PARP cleavage was detected with entinostat and ZSTK474 combination but not with other treatments tested. No apoptosis was seen in the MDA-MB231 line with entinostat combinations. Dasatinib was only able to induce marked PARP cleavage in H1437 cell line when combined with dual PI3K and MEK blockage.

5.2.3 *Bcl-xl* downregulation or blockage is a major determinant of apoptotic response to PI3K and MEK dual blockage.

HCT116 and H1437 lines were analysed further with western blot for alterations in the expressions of Bcl-2 related proteins in response to treatments. In both cell lines correlation between BIM upregulation with MEK inhibition was seen. Though surprisingly in H1437, BIM upregulation was absent when ZSTK474 was co-administrated with MEK inhibition. In HCT116 line, Mcl-1 upregulation was noted with ABT-263 treatments and in response to combination treatments whereas upregulation of Mcl-1 was seen when dual PI3K and MEK blockage was combined with ABT-263, entinostat, or dasatinib. In H1437, Mcl-1 downregulation correlated with PI3K inhibition. In general, in both cell lines Bcl-xl downregulation was seen with drug combinations, which had been seen to cause apoptosis. As an exception, Bcl-xl downregulation was not seen in HCT116 line treated with apoptosis inducing ABT-263 combinations. Though, since ABT-263 is an indirect Bcl-xl inhibitor, this was expected.

5.2.4 *Knockdown of Bcl-xl* expression increases the cytotoxicity of dual PI3K and MEK blockage

Next, the HCT116, MDA-MB231, and H1437 cells were exposed to Bcl-xl specific siRNA knockdown pool or corresponding treatment without siRNA. First the downregulation of Bcl-xl was confirmed with western blot, then the cells were examined for responses to sole and combined PI3K and MEK inhibition. When compared to the control cells, increased cytotoxic response to single and combined PI3K and MEK inhibition in MTS assay was noticed in the MDA-MB231 cell line but not with HCT116 and H1437. Instead, when compared to the control cells, increased levels of cPARP were detected in *BCL-XL* knockdowns in response to both sole and combined PI3K and MEK inhibition in the HCT116 and MDA-MB231 lines. In H1437 no marked increase in PARP cleavage after *BCL-XL* knock down was seen.

5.3 Cancer stem cell-like cells mediated resistance to targeted cancer therapies

Adaptive resistance mediated by cancer stem cell-like cells was investigated in study III.

5.3.1 Adaptive resistance to ALK inhibition in H3122 cell line

H3122, an ALK–translocated and dependent NSCLC cell line, was selected as an initial model for studying adaptive drug resistance. H3122 cells were first treated with the ALK inhibitor TAE684 for 7 days, after which the drug was withdrawn and the cultures were analysed with phase contrast microscopy. TAE684 induced marked cell death, a decline in cell numbers and a disappearance of the papillary structure. The drug was withdrawn, the remaining cells started to re-proliferate almost right away. After 14 days of re-growth, the cells were re-challenged with TAE684, with similar response to the initial drug challenge.

5.3.2 Cells mediating the adaptive resistance in H3122 express stem cell marker ALDH1

To study whether the cells showing adaptive resistance would bear a CSC phenotype, the cells were studied for CSC markers. ALDH1 expression was low in untreated H3122 cells, but the ALK inhibitor treatment with TAE684 induced it gradually, with a marked extent from 12 h onwards. A similar increase in ALDH1 expression was seen also with crizotinib, another unrelated ALK inhibitor, suggesting that the phenomenon is related to ALK inhibition rather than to any specific inhibitor. When TAE684 was withdrawn for 14 days, ALDH1 expression was comparable to the initial, low level. Re-challenging with TAE684 after regrowth again resulted in similar induction of ALDH1.

5.3.3 Tumorigenicity of TAE684-treated, ALDH1-positive cells in xenografts

Next the tumorigenicity of the ALDH1 positive cells was investigated *in vivo*. 10,000 or 100,000 of either non-treated control or TAE684-treated, luciferase reporter-incorporated H3122 cells were injected into mice on both flanks. All the mice injected with 100,000 cells rapidly generated tumors, with a similar rate of occurrence. Instead, increased tumorigenicity of the TAE684-treated cells was seen with the mice injected with 10,000 cells since 40% of these mice developed tumors whereas none of the mice injected with control cells had tumors during the 9-week follow-up period.

5.3.4 Pharmacological screening for agents targeting ALDH1-positive H3122 cells

The colony formation assay was used to study whether a combination of pharmacological agents could target cancer stem-like cells *in vitro*. H3122 cells were treated with TAE684 in combination with pharmacological agents (drugs known to target stem cells or related pathways, or traditional chemotherapeutic agents) for 7 days, and then the drugs were withdrawn. Significantly fewer colonies were seen when TAE684 was combined with ZSTK474 (PI3K inhibitor), ABT-263 (BH3 mimetic) or salinomycin (CSC specific drug). ZSTK474 possessed this activity also as a single agent, whereas ABT-263 and salinomycin had no activity solely.

Since ZSTK474, ABT-263 and salinomycin inhibited colony formation in combination with TAE684, the effect of these combinations on ALDH1 expression was investigated next. When the H3122 cells were treated with salinomycin or ZSTK474, no marked increase in ALDH1 expression was seen, while TAE684 induced high ALDH1 expression. When TAE684 was combined with salinomycin, or ZSTK474, marked inhibition of ALDH1 induction was seen. ABT-263 treatment alone or combined with TAE684 did not alter the ALDH1 expression, so ZSTK474 and salinomycin combinations were used in the further experiments.

5.3.5 Effect of combination treatments on cell signalling in H3122

Next it was studied how Akt and ERK1/2 signaling activity, (immediate PI3K and MEK downstream effectors), alter in H3122 cells treated with TAE684 according to different time schedules. A 5-hour treatment with TAE684 induced a notable downregulation of phosphorylated Akt and ERK1/2, but after 5 days the phosphorylation of Akt and ERK1/2 increased again. This effect could be prevented by a 5-h treatment with ZSTK474. When the H3122 cells were treated with salinomycin for 5 days, no change in either Akt or ERK1/2 was seen.

The effect of combining ALK inhibitor with PI3K inhibitor or salinomycin was studied also in short-term assays. It was seen that combination of TAE684 and ZSTK474 had enhanced cytotoxicity in a short-term assay (72h MTS) as well whereas salinomycin+TAE684 combination did not.

5.3.6 Stem cell markers in targeted therapy-sensitive cell line models

Since TKI treatment of the ALK-translocated NSCLC H3122 cells induced high expression of ALDH1 *in vitro* and increased tumorigenesis *in vivo*, the generality of this phenomenon in targeted therapy-sensitive cancer cell lines was screened next. One ALK translocated, an EGFR mutant NSCLC line, two HER2-amplified and two ER positive breast cancer lines, two B-Raf mutant and two N-Ras mutant melanoma lines were exposed to TAE684, erlotinib, lapatinib, tamoxifen, vemurafenib and CI-1040, respectively, for 7 days and analysed for the expression of ALDH1 and CD44. The ALK translocated H2228 was the only line to respond to targeted therapy by upregulation of ALDH1 expression. Similarly to H3122, CD44 expression was seen in the melanoma lines, with some increase in SK-MEL-30 and a marked increase in COLO-800 in response to treatment.

5.3.7 Pharmacological screening for agents targeting ALDH1-positive H2228 cells

Since ALDH1 expression was increased in the H2228 cells in response to TKI, the cell line was further studied for response to combined inhibition of ALK or B-Raf with PI3K, or salinomycin. The colony formation assays indicated that formation of colonies was clearly reduced when the cells were treated with combinations in comparison to single agents. Markedly reduced formation of colonies were seen when TAE684 was combined with ZSTK474 or salinomycin.

Next the expression of ALDH1 or CD44 following the combination therapies was studied in H2228. Downregulation of ALDH1 was seen with ZSTK474 and combination of TAE684 and ZSTK474 but not with salinomycin combinations. Instead, CD44 expression was unaltered by single agents but markedly downregulated in response to TAE684 combined with ZSTK474 or salinomycin.

5.3.8 Effect of combination treatments on cell signalling in H2228

Intracellular signaling in response to short or long exposures to TAE684 was studied next in the H2228 cell line. A 5-hour treatment with TAE684 resulted in complete inhibition of pAkt signaling, while pERK1/2 levels remained unaltered. By 5 days, marked upregulation of pAkt and pERK/ERK was seen even though the cells had been subject to continuous TAE684 treatment. When the cells were

treated with TAE684 for 5 days and exposed to ZSTK474 for 5 h, complete downregulation of pAKT was seen.

It was further analysed with the H2228 cells whether combining ALK inhibitor with a PI3K inhibitor or salinomycin in a short-term (72h) assay would cause a similar response as in the colony formation experiments. TAE684 and salinomycin had no or limited activity alone while ZSTK474 showed concentration dependent cytotoxicity. Concurrent administration of TAE684 and ZSTK474 had an enhanced cytotoxicity in the short-term assay as well, but the combination of salinomycin with TAE684 did not alter the toxicity of the therapy.

5.3.9 Adaptive resistance as a factor in the development of acquired resistance

Since the data suggested that PI3K inhibition and salinomycin are able to inhibit adaptive resistance in H3122 cells, it was further investigated whether adaptive resistance could serve as a source for acquired resistance. H3122 cells were exposed to crizotinib with or without ZSTK474 or salinomycin to monitor the cells for the development of acquired resistance. After a three-month exposure to the drugs, growing colonies were seen amongst the crizotinib-treated cell whereas no growing colonies were seen in plates treated with crizotinib and ZSTK474 or salinomycin even after six months of exposure.

5.3.10 The resistance mechanism to ALK inhibitor crizotinib in H3122

When the resistance mechanism of crizotinib-treated H3122 cells (H3122CR) was studied further, it was seen that when compared to the original line, H3122CR cells showed a ~10 fold increase in IC50 concentrations in response to both crizotinib and TAE684. When exposed to increasing concentrations of crizotinib and TAE684 in both cell lines, no difference in ALK phosphorylation was noted. Moreover, downregulation of phosphorylated Akt and ERK1/2 was seen in both cell lines in response to either crizotinib or TAE684, however, complete blockage was seen only in with H3122. Since EGFR and HER2 have been linked to ALK TKI resistance, it was next studied whether these RTKs could be mediators of acquired resistance in H3122CR cells. No increase was seen in the levels of either phosphorylated or total EGFR or HER2 in the H3122CR cells relative to the control cells, but when exposed to afatinib, a dual EGFR and HER2 inhibitor, in combination with crizotinib, markedly increased cytotoxicity was evident.

Furthermore, cPARP was indicated only in H3122CR cells when they were exposed to both drugs, but not with single agent treatments.

5.4 Gö6976, a classical protein kinase C inhibitor, inhibits EGFR mutant non-small cell lung cancer with T790M-mediated acquired resistance.

Study IV set out to find novel kinase inhibitors for genetic subsets of NSCLC.

5.4.1 Gö6976 in non-small cell lung cancer cell lines

For identification of novel kinase inhibitors for treatment of NSCLC, a panel (n=8) of cell lines representing major oncogenic genotypes of the disease (K-Ras, EGFR and EML4-ALK) was exposed to increasing concentrations of various kinase inhibitors and analysed with an MTS growth/cytotoxicity assay. Upon exposure to Gö6976, a classical protein kinase C (PKC) inhibitor, *EGFR* mutant lines displayed a major effect on growth/cytotoxicity, a similar cytotoxicity that was seen with classical EGFR TKI erlotinib.

When the cell lines were further analysed with western blot for intracellular signaling pathway activity in response to Gö6976, no effect on the activity of EGFR signaling or its downstream targets Akt and ERK1/2 was seen with K-Ras and EML4-ALK translocated cell lines, which had also shown no cytotoxicity in the MTS assays. Instead, marked downregulation of phosphorylated EGFR, Akt and ERK1/2 was seen in the *EGFR* mutant cell lines (PC9 parental, HCC827 parental and H3255). Erlotinib seemed to have a more prominent effect on EGFR phosphorylation in the *EGFR* mutant lines than Gö6976, but similar levels of inhibition were seen in the downstream targets Akt and ERK1/2.

Mutant EGFR signals also through heterodimers of HER2 and HER3, so the activity of these dimer partners in response to Gö6976 and erlotinib was analysed in the EGFR mutant lines. As with *EGFR* phosphorylation, both inhibitors downregulated phosphorylated HER2 (PC9 parental, HCC827 parental lines) and HER3 (all the lines tested), though the effect was more pronounced compared to erlotinib.

5.4.2 Gö6976 in EGFR tyrosine kinase inhibitor sensitive and resistant models

When TKI-sensitive cell lines with the wild-type *ERBB*-family genes A431 (EGFR dependent) and H1819 (HER2 dependent) were exposed to erlotinib and Gö6976, no cytotoxicity was seen in MTS assays but moderate sensitivity to erlotinib was shown. Furthermore, in western blots no downregulation of EGFR/HER2, or downstream targets Akt and ERK1/2 in response to Gö6976 was seen, controversially to erlotinib.

Gö6976 was also shown to inhibit the growth of *EGFR* mutant cell lines with a known mutation causing acquired resistance to TKIs. PC9 resistant line with T90M *EGFR* secondary mutation showed similar sensitivity to Gö6976 as the parental cell line but was resistant to erlotinib. Contrary, HCC827 resistant cells, carrying c-mesenchymal epithelial transition factor (c-MET) amplification, showed no response to Gö6976 in the MTS assay. When further studied with western blot, PC9-resistant cells showed similar downregulation of phosphorylated *EGFR* as the parental cell line when response to erlotinib was markedly reduced. Moreover, downregulation of phosphorylated Akt and ERK1/2 levels was seen at tenfold lower concentrations with Gö6976 than with erlotinib. In the HCC827 resistant cells phosphorylated EGFR was downregulated in response to both inhibitors, but conversely to the parental line, expression levels of phosphorylated Akt and ERK1/2 remained unchanged.

The cells were next analysed with western blot for PARP cleavage to analyse whether the cytotoxic response to Gö6976 occurs through apoptosis. The PC9 parental line showed PARP cleavage in response Gö6976 or erlotinib, whereas marked PARP cleavage was only seen in the PC9-resistant line when the cells were exposed to Gö6976.

5.4.3 Gö6976 in EGFR mutant-transformed Ba/F3 models

Ba/F3 cells, which establish IL-3 independence when transformed with oncogenes, were used to further study the effect of Gö6976 on mutant *EGFR*. The Ba/F3 cells were transformed with *EGFR* ex19 deletion or L858R mutants with or without the T790M acquired resistant mutation and then analysed for cytotoxicity. Both the ex19 deletion and the L858R mutants showed high sensitivity to erlotinib, while their counterparts with T790M mutations were resistant to the inhibitor. When treated with Gö6976, the cells remained sensitive to the inhibitor

despite the presence of the T790M secondary mutation. Moreover, in both *EGFR* ex19 deletion mutant and L858R mutant cells with T790M *EGFR* phosphorylation was blocked with low concentrations of Gö6976 while the effect of erlotinib on *EGFR* phosphorylation was highly limited in both the cell lines.

5.4.4 Gö6976 structurally related inhibitors in NSCLC

Other inhibitors with similar structure to Gö6976 were studied for possible similar activity against mutant *EGFR*. A549 (*K-Ras* mutant), H3122 (*EML4-ALK*-translocated) and PC9 resistant (*EGFR* ex19 deletion+T790M mutant) cell lines were exposed to potent PKC family member inhibitors (bisindolylmaleimide I, Gö6983, staurosporine, and UCN-01) with similar chemical structure to Gö6976. All the tested cell lines showed sensitivity to staurosporine and UCN-01, but only a minimal response to bisindolylmaleimide I and Gö6983. However, unlike Gö6976, none of the inhibitors showed preferential cytotoxic activity in the *EGFR* mutant lines relative to the two *EGFR* wild-type cell lines tested.

5.4.5 In vitro generation of cell lines with acquired resistance to Gö6976 and erlotinib

PC9, a cell line known to generate T790M secondary mutation mediated acquired resistance, was exposed to gradually increasing concentrations of erlotinib or Gö6976 *in vitro* simultaneously. With both inhibitors, the majority of cells died during the first weeks, but large, morphologically senescent-looking cells survived and slowly divided despite the presence of increasing inhibitor concentrations. After four weeks, fast growing colonies of cells with a similar morphology to the original cell line had developed on the erlotinib-treated plate and had invaded the plate completely in a week. Instead, during the 12-week experiment the large, slowly dividing cells were the sole population surviving on the Gö6976-treated plate.

Clones of the surviving cells were collected and studied for responses to erlotinib or Gö6976. Erlotinib resistant clones showed sensitivity only to Gö6976, whereas Gö6976-resistant clones showed comparable sensitivity to the original cell line to both the inhibitors. Furthermore, when the erlotinib resistant cells were analysed with western blot, marked downregulation of phosphorylated *EGFR* and *ERK1/2* occurred at tenfold lower concentrations of Gö6976 than of erlotinib, and only minor downregulation of phosphorylated *Akt* was seen in response Gö6976

treatment. When exon 20 of the *EGFR* gene of the erlotinib and Gö6976-resistant clones was sequenced, the allele containing T790M was present only in the erlotinib-resistant clones.

5.4.6 Gö6976 in the T790M in vivo xenograft model

For analysis of the potential of Gö6976 in xenograft models, nude mice bearing tumor xenografts of PC9-resistant cells with the luciferase reporter gene (Luc) on both flanks were generated. The mice were treated five times a week for a total of 14 times with i.p. Gö6976 or vehicle. The tumors of the mice treated with Gö6976 showed a statistically significant tumor growth reduction on day 18 when compared to the vehicle-treated group. Moreover, a minor reduction in phosphorylated EGFR and ERK1/2 in the Gö6976-treated tumors in comparison to the vehicle treated ones, no difference in Akt phosphorylation was noted.

6 Discussion

Therapy resistance has shown to be a major challenge for the targeted cancer therapies. Some patients do not benefit from the therapy even though the cancer harbors the targeted oncogene and have the so-called primary or *de novo* refractory phenotype. On the other hand, a vast majority of the patients, that have originally responded well, ultimately develop an acquired resistance to the therapy. Some mechanisms, including mutations affecting drug binding and activation of alternative cell signaling routes, lying behind acquired targeted therapy resistance are known, however the details are still in many parts not understood. Additionally, a third type of therapy resistance has recently been characterized, namely, adaptive resistance where a portion of the cancer cells remain in a non-dividing state in the presence of a drug and start normal cell division again after removal of the drug. Understanding the mechanisms of resistance could lead to development of smarter therapies and, ultimately, to better patient outcomes.

6.1 Primary resistance

Targeting overactivated oncogenic pathways has shown to be a promising strategy for fighting cancer. However, it is known that even though the treated tumor would carry the targeted oncogene, not all tumors are responsive to the therapy. In addition, predictive markers for some therapies known to be efficient are not fully understood at the patient level.

Here, dual pharmacological inhibition of PI3K and MEK was investigated in NSCLC cell line models with specific oncogenic genotypes. This study showed that in some cancer cell lines dual PI3K and MEK inhibition therapy can be more efficient as a treatment strategy than inhibition of either alone which is in line with previous studies (Hoeflich *et al.* 2009, Wee *et al.* 2009, Sos *et al.* 2009). However, a majority of the NSCLC cell lines tested did not show additive effect with the dual blockage since only two of the twelve tested cell lines showed synergistic response to dual PI3K and MEK blockage. As noted also in previous studies (Sos *et al.* 2009), the response to dual inhibition was not associated with any specific oncogenic genotype. Though, the NSCLC cell lines identified here as dual inhibition sensitive were noticed to be more sensitive to MEK inhibitors than the other lines tested. In earlier studies MEK inhibition-sensitive cancer models, such as triple-negative breast or *KRAS* mutant colorectal cancers, have shown

increased cytotoxicity or reversal of resistance when MEK inhibitors have been combined with the PI3K-Akt-mTOR pathway inhibition (Hoeflich *et al.* 2009, Wee *et al.* 2009). It can be speculated that dual PI3K and MEK inhibition therapy could give additive efficacy to cancers that show dependency on MEK signaling. Therefore it is likely that response to dual PI3K and MEK blockage is more associated with feedback activation induced by the inhibition of one pathway on the other rather than any specific oncogenic genotype.

The current work identifies four cancer cell lines showing sensitivity to the concurrent PI3K and MEK inhibition. However, induction of apoptosis in response to the treatment was seen only in one of these cell lines. It is likely that cancer therapies, which are unable to induce cell death, are prone to have very limited clinical activity and therefore, induction of apoptosis should be the primary goal of a therapy in these cases. Hence, the mechanism determining whether dual PI3K and MEK blockage causes apoptosis was studied further. The results indicated that by combining a third agent to the dual blockage could induce apoptosis markedly. A Bcl-xl inhibitor ABT-263, HDAC inhibitor entinostat and a multikinase inhibitor dasatinib were shown to be able to markedly increase the efficiency of dual PI3K and MEK inhibition. It was identified that agents inducing apoptosis affected the expression of the anti-apoptotic protein Bcl-xl. Downregulation of Bcl-xl was linked to apoptotic response to dual PI3K and MEK blockage, but, on the other hand, apoptosis did not necessarily occur even though lower Bcl-xl levels were seen. In recent works, similar results have been identified with Bcl-xl linked to the apoptotic response following dual PI3K and MEK inhibition (Tan *et al.* 2013, Hata *et al.* 2014).

The current work also characterizes the mechanism of resistance in the NSCLC line H2228, which has ALK translocation but is unaffected by ALK inhibitors (Koivunen *et al.* 2008). This study showed that H2228 has high basal expression on CSC markers and combining stem cell targeted drugs to ALK inhibitor could markedly increase the therapeutic response. The results indicated that CSCs could be mediators of primary resistance in this cell line. To our knowledge, this is the first work to characterize this phenomenon.

6.2 Adaptive resistance

Adaptive resistance is used to describe a resistance mechanism where a subpopulation of the cells survives in the presence of the drug in a dormant or a slowly dividing state. After drug removal these cells start dividing normally.

CSCs have earlier been related to chemotherapy and radiotherapy resistance (Bao *et al.* 2006, Creighton *et al.* 2009) as well as to poorer patient outcomes (Ginestier *et al.* 2007, Charafe-Jauffret *et al.* 2010). The presence of CSCs offers a possible explanation to adaptive resistance and some studies have provided evidence for this theory (Shien *et al.* 2013). However, the mechanisms of adaptive resistance remain in most parts unrevealed. The current work investigated cancer stem cell-mediated adaptive resistance to targeted cancer therapies. It also characterized drug combinations targeting this form of resistance, and provides evidence that targeting adaptive resistance may prevent the later occurrence of acquired resistance. An understanding of the mechanisms behind resistance can provide clues for smarter therapeutic approaches, which can in turn increase the efficiency of cancer therapies.

The findings of this study show that exposure to targeted therapies may cause increased expression of CSC markers and increased *in vivo* tumorigenesis at least in some cell lines. This phenomenon was seen with three of the twelve cell lines studied. Though, there has not been found any general marker for CSC. Multiple markers, such as ALDH1, CD44 and EMT, have been linked to the phenotype. However, the results of this study showed some discordancy between ALDH1 and CD44 expression in the H2228 cells, as has been noted also previously (Magee *et al.* 2012).

The hierarchical CSC model suggests that the total tumor cell population comprises of two types of cells: CSCs, which are responsible for tumor initiation, and the differentiated cancer cells that cover a vast majority of the total cell population. Controversially, the stochastic model proposes that all cancer cells are able to change between the stem cell and differentiated state and that the cell's state is determined by factors from within and outside of the cell such as the presence of drugs. Based on the hierarchical model, effective cancer therapies would only be required to eliminate the CSCs, since they are solely responsible for tumor growth. Instead, following the stochastic model effective therapeutic response would only be reached by targeting both CSCs and the differentiated cancer cells (Gupta *et al.* 2011, Nguyen *et al.* 2012). The data of this study supported the latter proposal since the H3122 ALK TKI induced CSC phenotype was shown to be reversible and could be reinduced by re-exposure to the drug. Also the results of the drug combination studies with inhibitors targeting CSCs and more differentiated cancer cells reflect the stochastic model. It can be concluded that both the differentiated and stem-like cells need to be targeted simultaneously for maximal therapeutic efficiency.

The H3122 cells were screened with 27 selected pharmacological agents that were either targeting central cell signaling pathways CSCs or cytotoxics. PI3K inhibitor and stem cell-targeting drug salinomycin were identified to inhibit adaptive resistance. These agents were also shown to inhibit the expression of the CSC marker ALDH1, supporting the idea that CSCs are general mediators of adaptive resistance. Though, since sole PI3K inhibitor treatment is also cytotoxic in H3122 cells, it cannot be ruled out that increase of acute cytotoxicity would not partially be involved in inhibiting adaptive resistance. Instead, salinomycin is non-cytotoxic solely indicating that it may target CSCs specifically. The compound has earlier been identified in a chemical screen to be selective for CSCs (Gupta *et al.* 2009). The PI3K-Akt-mTOR signaling pathway has also been linked to the CSC phenotype (Dubrovska *et al.* 2009, Hardt *et al.* 2012). In the H3122 and H2228 cells PI3K-Akt-mTOR signaling activity increased in long-term ALK TKI exposures after initial downregulation.

In summary, the current work provides evidence of CSC-mediated resistance to targeted cancer therapies and brings more knowledge of the mechanisms of this phenomenon. It was shown that longer exposures to targeted therapies can cause arousal of the CSC phenotype and this phenomenon was proved to be reversible. Furthermore, this research also characterized drug combinations targeting this form of resistance. The results of this study provide evidence to the stochastic model of the CSCS, hence it can be concluded that combining TKIs and CSC targeting agents might be needed for more efficacious cancer therapies.

6.3 Acquired resistance

Acquired resistance to molecularly targeted therapies majorly limits the efficiency of the treatments. Virtually all patients ultimately develop resistance to targeted therapies, even after significant initial responses. The most important mechanisms of acquired resistance are thought to be mediated by secondary mutations that affect the drug binding or activate parallel cell signaling pathways, the presence of highly tumorigenic CSCs that are not targeted by the used therapies, or enhanced drug removal (Engelman & Jänne 2008).

In *EGFR* mutated NSCLC, treatment with first generation EGFR inhibitors such as erlotinib results in initial response followed by development of acquired resistance 9-13months after initiation of the therapy. T790M secondary mutation is the most important resistance mechanism for EGFR TKIs present seen in over 50% of the patients (Kobayashi *et al.* 2005, Hammerman *et al.* 2009). There has

been an eager search for agents circumventing the T790M mediated resistance but so far no clinical active agents are available. Irreversible EGFR TKIs such as HKI-272 and PF00299804 have shown activity against the T790M mutation in preclinical models, but unfortunately their activity in a clinical setting has been very limited (Kwak *et al.* 2005, Engelman *et al.* 2007¹, Wong *et al.* 2009, Jänne *et al.* 2011)

The current work identified Gö6976, a well-characterized classical PKC inhibitor, as an inhibitor of mutant EGFR with no significant inhibitory activity with the wild-type EGF receptor. Moreover, it was shown that the inhibitory activity of Gö6976 is not limited by the T790M-mediated acquired resistance mutation. Other PKC inhibitors assayed here did not share the same activity as Gö6976, and therefore the mutant EGFR inhibitory function of Gö6976 is likely to be independent of its PKC inhibitory effect. These findings were controversial to previous studies since it has been reported earlier that PKCs can transactivate EGFR and its downstream signaling in prostate cancer cell lines with wild-type EGFR (Kancha *et al.* 2009).

A previous study screened a chemical library of irreversible inhibitors against mutant EGFR and characterized a new class of inhibitors (WZ3146, WZ4002, and WZ8040), with activity against mutant EGFR with or without T790M more than that against wild-type EGFR (Zhou *et al.* 2009). Some structurally related inhibitors have recently entered early phase clinical trials. Gö6976, structurally unrelated therapeutic agent to WZ4002, investigated here was shown to have similar activity, with preferential inhibition of the mutant EGFR relative to the wild-type receptor, and similar inhibition of the mutant EGFR in the with or without T790M. The results of this study also showed that when Gö6976 was used instead of erlotinib, an *EGFR* mutant cell line known to develop the T790M-mediated acquired resistance showed a marked temporal delay in the development of acquired resistance and an absence of the T790M mutation. It can be speculated that Gö6976 could also delay the development of acquired resistance in a clinical setting. This study showed that beside activity *in vitro*, Gö6976 also inhibits tumor growth of *EGFR* mutant xenografts with the T790M mutation. Similar results with another PKC inhibitor, FLT3, and mutant *EGFR* have later been published by another research group, confirming the findings of this study (Lee *et al.* 2013). Even though targeted cancer therapeutics are thought to be highly specific for their targets, off-target functions can occur. Like in the case of some PKC inhibitors, off-target effects can be positive and lead to other therapeutic strategies, which should be investigated.

Development of acquired resistance has been explained by Darwinian selection among the drug-resistant clones that are initially present in the tumor. However, a recent study presented a model with drug-dependent clones that can mediate resistance and this resistance was forestalled by discontinuous dosing (Das Thakur *et al.* 2013). The current work provides evidence for a more complex development of acquired resistance. It was shown here that concurrent administration of stem cell targeting drugs and ALK TKIs blocked the development of EGFR and/or HER2 mediated acquired resistance in the H3122 line, as has been described also previously (Sasaki *et al.* 2011, Yamaguchi *et al.* 2014). Since combining salinomycin and ALK TKI blocked the formation of acquired resistance, it can be hypothesized that acquired resistance is mediated by CSCs in this cell line.

6.4 Alternative dosing schedules

Beside therapy resistance, therapy tolerability is another challenge for targeted cancer therapies. This question rises up especially with treatments with combinations of oncogene based targeted therapies such as the dual PI3K and MEK inhibition. In general, clinical trials are testing the targeted cancer therapies in dosing regimens with longer drug exposures and subtotal suppression of the targets. It is possible that higher dosing of the drugs with robust target inhibition for shorter periods of time would be clinically more effective and tolerable. There is already some preclinical evidence supporting this suggestion (Hoeflich *et al.* 2012).

Clinical studies have shown that simultaneous inhibition of multiple pathways is likely to be more toxic than inhibition of a single pathway. To overcome this challenge, the current work investigated whether maximal effect of the dual PI3K and MEK inhibition is possible to achieve by alternative dosing schedules, which may be potentially clinically more tolerable. Even though some works have tried to clarify if both PI3K and MEK inhibitors need to be administered concurrently or if either of the drugs could be used intermittently, the optimal dosing regimen have not yet been clarified (Hoeflich *et al.* 2012, LoRusso *et al.* 2012).

The data presented here suggest that even short courses of concurrent administration of PI3K and MEK blockage can cause marked cytotoxicity and/or apoptosis. Two out of the four dual inhibition-sensitive cell lines showed comparable cytotoxicity to that achieved with continuous administration of dual

inhibition when the MEK inhibitor was administered for short periods (15 min) in combination with continuous PI3K inhibitor treatment. Based on this *in vitro* data, it can be hypothesized that even short courses of dual inhibitor administration could have similar clinical effects with possibly better tolerability in the clinic. Analogous results were published in a previous work where it was shown that intermittent administration of concurrent PI3K and MEK inhibition can induce robust growth inhibition in cancer cell lines (She *et al.* 2010).

Better alternative dosing schedules could also enable the use of higher drug doses and lead to strengthened target inhibition, as already has been suggested by some scholars (LoRusso *et al.* 2012). Short but more significant target inhibition is likely to be more efficient than longer periods of sub-maximal inhibition. The data of this study underlines the significance of maximal target inhibition and a preferential role for longer inhibition of the PI3K-Akt-mTOR pathway.

7 Conclusions

The current work investigated resistance of targeted therapy in solid tumor cell lines. The aim of this study was to investigate three different types of therapy resistance: primary, adaptive and acquired resistance in NSCLC, breast cancer, colorectal cancer and melanoma cell lines.

The main conclusions of this study are:

1. In some NSCLC cell lines, dual PI3K and MEK inhibitor therapy is more efficient than either inhibitor alone. Responses to dual inhibition are not associated with any specific oncogenic genotype.
2. The maximal effect of the dual inhibition can be achieved with alternative dosing schedules.
3. Combining a third agent, such as ABT-263, entinostat or dasatinib, to dual PI3K and MEK inhibition may increase the efficiency of the therapy. Bcl-xl downregulation relates to the apoptotic response of the triple inhibitor treatment.
4. Cancer stem cells can mediate adaptive and acquired resistance to targeted therapies. Since these cells follow the stochastic model, concurrent therapy with a targeted agent and a stem cell targeting drug is needed for maximal therapeutic efficiency.
5. This study showed that Gö6976 acts as a potent therapeutic agent for *EGFR* mutant NSCLC cells both *in vitro* and *in vivo*. In addition, Gö6976 inhibits mutant EGFR despite the presence of the T790M mutation, which is the most frequent mechanism of acquired resistance to the EGFR TKIs.

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- I Jokinen E, Laurila N & Koivunen JP (2012) Alternative dosing of dual PI3K and MEK inhibition in cancer therapy. *BMC Cancer* 12(612).
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- IV Taube E, Jokinen E, Koivunen P & Koivunen JP (2012) A novel treatment strategy for EGFR mutant NSCLC with T790M-mediated acquired resistance. *Int J Cancer* 131(4): 970-979.

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