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Studies on the effect of ErbB tyrosine kinase inhibitors on malignant melanoma growth and survival *in vitro*

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ABSTRACT

Malignant melanoma has one of the fastest increasing incidences among the different types of cancer in the Western world. This raise can partly be ascribed to the change in sun habits that has taken place during the last decades, since the major external risk factor for melanoma is exposure to ultraviolet radiation. Patients with early stages of melanoma can often be cured by surgery, however for patients suffering from metastatic melanoma there are only a few treatment options available. Unfortunately malignant melanoma is often resistant to radio-, bio- and chemotherapy and treatment with the currently most frequently used agent, dacarbazine, is characterized by a very low clinical response rate. Therefore, there is an urgent need for new treatment strategies which can increase the overall survival and cause less severe side effects.

The aim of this thesis was to investigate the anti-tumor effect of two different tyrosine kinase inhibitors (TKIs), gefitinib and canertinib, on two different human malignant melanoma (RaH3 and RaH5) cell lines. We investigate the effect of these two drugs on cell proliferation and survival and studied the effect of gefitinib and canertinib on ErbB1-4 receptor phosphorylation, as well as Akt, Erk1/2 and Stat3 activity.

Our results showed that phosphorylation of ErbB1, ErbB2 and ErbB3 decreased following treatment with both gefitinib and canertinib and that the subsequent downstream signaling via Akt, Erk1/2 and Stat3 was inhibited after TKI treatment. However, it was noted that the gefitinib-induced inhibition of Akt, and particularly Erk1/2, was transient and only a weak inhibition of Stat3 phosphorylation was seen. Gefitinib treatment of the RaH3 and RaH5 cells resulted in an accumulation of the cells in the G₁ phase of the cell cycle without any induction of apoptosis. Canertinib caused a more pronounced inhibition of Akt, Erk1/2, and Stat3 phosphorylation than gefitinib. This might be one explanation to why canertinib induced apoptosis in RaH3 and RaH5 cells whereas gefitinib only caused cell cycle arrest. In conclusion, gefitinib and canertinib display promising anti-tumor effects on ErbB expressing malignant melanoma and might be used in future studies in combination with conventional chemotherapy or other targeted therapies in the treatment of malignant melanoma.

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ABBREVIATIONS

AIF	apoptosis-inducing factor
Akt	a protein kinase
Bad	Bcl-2-antagonist of cell death
Bak	Bcl-2 antagonist/killer-1
Bax	Bcl-2-associated x protein
Bcl-2	B-cell CLL/lymphoma 2
Braf	v-raf murine sarcoma viral oncogene homolog B1
CDK	cyclin-dependent kinase
CDKN2A	cyclin-dependent kinase inhibitor 2A
DTIC	dacarbazine
DD	death domain
DED	death effector domain
DISC	death-inducing signaling complex
DR	death receptor
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ErbB1	erythroblastic leukemia viral (v-erb-b) oncogene homolog
ErbB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
ErbB3	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3
ErbB4	v-erb-a erythroblastic leukemia viral oncogene homolog 4
Erk	extracellular regulated kinase
FADD	Fas-associated death domain protein
FLICE	FADD-like Interleukin-1 beta-converting enzyme, known as caspase 8
FLIP _{L/S}	FLICE-inhibitory protein
Grb2	growth-factor-receptor-bound protein 2
HER1-4	human epidermal growth factor receptor 1-4
Kras	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
MDM2	murine double minute 2
MAPK	mitogen-activated protein kinase

MEK	mitogen-activated protein kinase kinase /extracellular regulated kinase kinas
Nras	Neuroblastoma RAS viral (v-ras) oncogene homolog
NRG	neuregulins
PARP	poly(ADP-ribose) polymerase
PDK1, PDK2	phosphoinositide-dependent kinase 1 and 2
PH	pleckstrin-homology
PI3K	phosphatidylinositol-3 kinase
PIP2	phosphatidylinositol (3,4)-bisphosphate
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PTB	phosphotyrosine-binding
PTEN	phosphates and tensin homolog
Raf	murine sarcoma viral oncogene homolog
Ras	rat sarcoma viral oncogene homolog
RGP	radial growth phase
RB	retinoblastoma
SH2, SH3	Src homology 2 and 3 domains
Sos	son of sevenless
Stat	signal transducer and activator of transcription
TKI	tyrosine kinase inhibitor
VGP	vertical growth phase

ORIGINAL PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals (I-II).

- I. **Djerf EA**, Trinks C, Abdiu A, Thunell LK, Hallbeck A-L and Walz TM. ErbB receptor tyrosine kinases contribute to proliferation of malignant melanoma cells: inhibition by gefitinib (ZD1839). *Melanoma Research* 2009; **19**:156-166.

- II. **Djerf EA**, Trinks C, Gréen H, Abdiu A, Hallbeck A-L, Stål O and Walz TM. The pan-ErbB receptor tyrosine kinase inhibitor Canertinib (CI-1033) promotes cell cycle arrest and apoptosis of human malignant melanoma *in vitro*. *Manuscript*.

INTRODUCTION

Malignant Melanoma

Background

The incidence of melanoma has been increasing during the last 25 years at an annual rate of 3-7 % for the Caucasian population world wide. The highest predicted lifetime risk has been reported from Australia, where the lifetime risk of melanoma is 1:25 [1]. In Europe, the highest incidence is reported in Scandinavia and the lowest in the southern part of Europe around the Mediterranean Sea. Currently, melanoma is the sixth and seventh most common cancer among Swedish women and men, respectively [2]. Seen over a 20-year period, this is one of the most rapidly increasing malignant tumors in Sweden. For men, the average increase per year is 2.3 % and for women 2.1 %. During the last decade, the increase has been even higher. Melanoma affects about 2200 individuals and leads to 450 deaths each year in Sweden [2, 3].

Risk factors

Epidemiological studies over the past several decades have identified a number of risk factors for melanoma. These include family history of melanoma, melanoma susceptibility genes, the number and type of nevi, skin type, pigmentation, childhood sunburns, and use of sunbeds [4-6]. Persons with red hair, pale skin and a tendency to freckle have a significantly higher risk to develop malignant melanoma than those with black/dark brown hair and dark skin [7]. The only known environmental risk factor in melanoma is ultraviolet radiation exposure, and the dramatic increase in melanoma incidence the last 50 years can in part be ascribed to changes in sun habits [8]. Melanoma occurs most frequently on areas of the skin exposed to the sun during sunbathing. However, the relationship between melanoma and sun exposure is complex since there is a lower incidence of melanoma among persons who work outdoors compared to persons who work indoors. One possible explanation for this paradox is that chronically tanned skin is less melanoma-prone than untanned skin exposed to bursts of high-intensity sun [9]. Blistering sunburn in childhood and adolescence is an almost universal risk factor for melanoma in white populations [10].

Treatment

The majority of cutaneous melanoma lesions are diagnosed as stage I tumors and these patients have a 90 % five-year survival rate when treated with surgery [11]. However, once malignant melanoma cells obtain the ability to metastasize they can no longer be removed by surgery and the one-year survival rate range from 40-60 % [12]. Despite attempts to treat melanoma using a wide variety of therapies, including immuno-, radio- and chemotherapy, survival for patients with metastatic melanoma is very poor. Most standard chemotherapy drugs have failed in large-scale clinical trials for melanoma. However, since its approval in 1976 the alkylating agent dacarbazine (DTIC) is still the most commonly used drug in treatment of metastatic melanoma, and when given as a single agent the clinical response rate vary in the range of 5-28 % in patients [13, 14]. Despite that multiple approaches have been taken in order to improve the response of melanoma to DTIC and other tumor specific therapies, these efforts have unfortunately failed to translate into any survival benefit for these patients. However, lately several clinical trails using targeted therapies in combination with cytotoxic chemotherapy have been initiated which show promising results [as reviewed in ref 13].

Development

Melanoma arises from the malignant transformation of melanocytes, which are the pigment-producing cells derived from the neural crest during embryogenic development. Melanocytes are normally confined to the basal layer of the epidermis where they produce melanin, which is transported to the surrounding keratinocytes. Once taken up by keratinocytes the pigment absorbs and dissipates ultraviolet energy and thereby protects the skin from the harmful ultraviolet radiation [15]. The Clark model describes the histological changes that take place during the progression from normal melanocytes to malignant melanoma [15]. The first stage is called nevus, a benign lesion characterized by an increased number of melanocytes in relation to keratinocytes. The growth of a nevus is limited but the growth control of the cells that constitute the nevus is however considered to be disrupted [15]. Abnormal activation of the mitogen-activated protein kinase (MAPK) signaling pathway is often an early event caused by *Nras* or *Braf* mutations, which might result in melanoma development [16]. The second stage, dysplastic nevus, is characterized by irregular nests consisting of large atypical melanocytes. At this stage of progression the molecular abnormalities affect cell growth, DNA repair, and the vulnerability to cell death [15]. In familial melanoma, a frequently occurring genetic defect inactivates *CDKN2A*, which is a single gene that encodes two tumor-

suppressor proteins, p16^{INK4A} and p19^{ARF} [17]. In non-familial melanoma, a different tumor-suppressor gene, phosphatase and tensin homologue (PTEN) is commonly inactivated by mutation [18, 19]. *CDKN2A* and *PTEN* loss both cause the cells to overcome the limited growth seen in dysplastic nevus and progress into a superficial spreading stage, known as the radial growth phase (RGP). A melanoma in RGP is confined to the epidermis and has low invasive potential. The *PTEN* loss causes an increased level of the active form of Akt in RGP cells as compared with normal melanocytes [15]. When RGP cells finally acquire the ability to penetrate the basement membrane into the underlying dermis and subcutaneous tissue, they are in the vertical growth phase (VGP). Progression from the radial-growth phase to vertical-growth phase is a crucial step in the development of melanoma which is manifested by alterations in cell adhesion molecules [15]. Normally, cell adhesion controls cell migration, tissue organization, and organogenesis, but disturbances in cell adhesion also contribute to tumor invasion. Loss of E-cadherin, increased expression of N-cadherin, α V β 3 integrin, melanoma cellular adhesion molecule (MCAM) and matrix metalloproteinase 2 (MMP-2) is evident in vertical-growth phase and metastatic melanoma [15, 20]. The expression of N-cadherin enables melanoma cells to interact with other N-cadherin-expression cells, such as dermal fibroblasts and the vascular endothelium, thereby permitting metastatic spread [21]. Metastatic melanoma develops when tumor cells dissociate from the primary lesion, migrate through the surrounding stroma and invade blood vessels and lymphatics to form tumors at distant sites.

About half of the melanomas do not arise from nevi, and the progression can occur without going through all the stages [22]. The system described by Breslow, which measures tumor thickness from the uppermost nucleated layer of the epidermis to the greatest depth of tumor invasion, is the principal prognostic factor and primary criterion in melanoma staging [23].

Genetics

A positive family history of melanoma has been reported in approximately 10 % of melanoma patients [24]. The familial cases tended to be younger, to have higher numbers of moles, and to develop multiple primary melanomas [25]. 25-40 % of the individuals with familial melanoma have mutations in the *CDKN2A* gene which encodes two proteins p16^{INK4A} and p14^{ARF}, that function in the retinoblastoma (RB) and p53 pathways, respectively [17]. p16^{INK4A} inhibits cyclin dependent

kinase 4/6 (CDK4/6)-mediated phosphorylation of RB thereby inactivating the RB protein. p14^{ARF}, which, is a product of the alternative reading frame of *CDKN2A*, inhibits murine double minute 2(MDM2)-mediated ubiquitylation and therefore cause degradation of p53.

However, the most important mutation discovered so far in melanoma is the *Braf* gene mutation, which occurs in about 66 % of malignant melanoma [26, 27]. Interestingly, the point mutations in *Braf* cluster in specific regions of biochemical importance, and 80 % of them are single nucleotide substitution resulting in a changed amino acid (V600E), conferring a constitutive activation of the Braf protein [24]. *Nras* mutations are also frequently (15-25 %) observed in both melanoma cell lines and primary tumors [as reviewed in ref 9]. *Nras* and *Braf* function in the MAPK pathway and mutations in those two genes have been found to be mutually exclusive, thus increased stimulation of this pathway is important in melanoma pathophysiology [26, 28]. Loss of PTEN protein expression occurs in 30-50 % of the melanoma cell lines and is usually the result of homozygous deletion [29]. PTEN normally regulates signaling of different growth factors by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate (PIP3) to produce phosphatidylinositol (3,4)-bisphosphate (PIP2) in the PI3K/Akt pathway. Deletion of the *PTEN* gene eliminates the inhibition of Akt resulting in an increased signaling through the phosphatidylinositol-3 kinase (PI3K)/Akt pathway [18, 19]. Interestingly, *PTEN* loss seem to be mutually exclusive with *Nras* mutations, whereas *Braf* mutations are not [30]. Therefore it has been suggested that a possible cooperation exists between *Braf* activation and *PTEN* loss in melanoma development [28].

The ErbB receptor signaling

The ErbB receptor family

The epidermal growth factor (EGF) was identified in 1962 by Dr Stanley Cohen and in 1975 its receptor, the epidermal growth factor receptor (EGFR) was identified [31, 32]. The EGFR is a member of the ErbB receptor family consisting of four receptors called ErbB1 (EGFR or HER1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4). The ErbB receptors consist of an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain (figure 1). All ErbB receptors bind ligands except ErbB2 because it has a fixed conformation where the ligand-binding site is buried and not accessible for interaction [33, 34]. Many different ligands, such as EGF, EGF-like molecules, transforming growth factor (TGF)- α and neuregulins (NRGs), activate

the ErbB receptors by binding to the extracellular ligand-binding domain and inducing the formation of hetero- or homodimers [35]. Intracellular tyrosine kinase domains are then autophosphorylated to form binding sites for intracellular signaling molecules, which activate different signaling cascades, such as the MAPK pathway, the anti-apoptotic PI3K/Akt pathway and signal transducer and activator of transcription (Stat)/Janus kinase (Jak) pathway [35, 36].

The ErbB1 receptor is expressed in the majority of nevi, as well as primary and metastatic melanoma samples [37]. The gene encoding the receptor is frequently amplified in primary lesions and this implicate an importance of the ErbB1 in malignant melanoma [37, 38]. ErbB2 is rarely expressed or amplified in this disease and the level of expression in primary and metastatic melanoma was not higher than that observed in nevi [39, 40]. However, Erbb2 is considered to be the preferred heterodimerization partner for the other ErbB receptors and to posses oncogenic potential [41]. ErbB3 is commonly expressed in melanoma lesions and has even been associated with a poor clinical outcome [39, 42]. The expression of the full-length ErbB4 receptor has not previously been shown in melanoma; however ErbB4 fragments which probably represent a cleavage product of the full-length ErbB4 has been demonstrated [43].

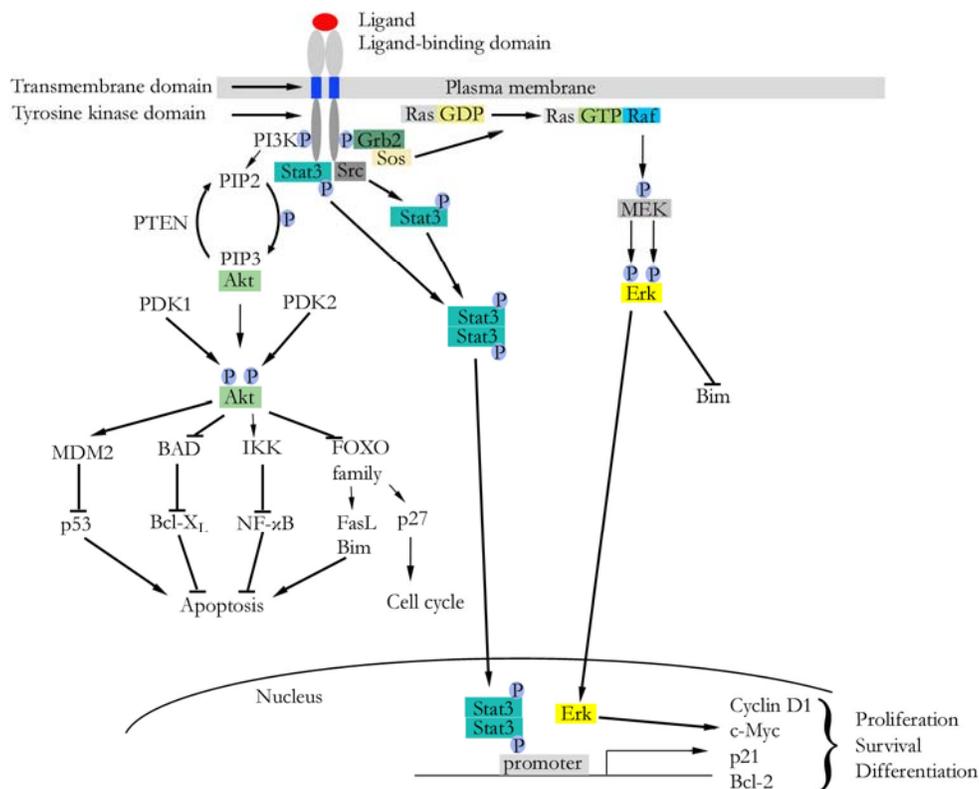


Figure 1. The ErbB receptor signaling. After ligand binding homo- or heterodimers are formed. Then the intracellular tyrosine kinase domains are cross-phosphorylated and form binding sites for intracellular signaling molecules, which activate the MAPK pathway, the PI3K/Akt pathway and the Stat pathway.

Ras/MAPK pathway

The MAPK signaling pathway is an important downstream target of activated growth factor receptors involved in mediating cellular responses to extracellular stimuli. Activated receptors autophosphorylated on tyrosine residues form binding sites for various cytoplasmic proteins containing Src homology 2 (SH2) and phosphotyrosine-binding (PTB) domains [44]. The growth-factor-receptor-bound protein 2 (Grb2) has a central SH2 domain and two flanking SH3 domains. The SH2 domain binds to the receptor and the SH3 domains binds to Sos (son of sevenless), which causes the inactive Ras-GDP to release GDP, and instead binds GTP (figure 1) [45]. This activates Ras and forms a binding site for Raf-family proteins on Ras, thereby activating Raf. Then Raf phosphorylates MEK (mitogen-activated protein kinase kinase /extracellular regulated kinase kinase) on serine residues, which in turn phosphorylates Erk on both threonine and tyrosine residues. When Erk is activated it translocates to the nucleus where it regulates the activity of various transcription factors such as Ets, Elk, Jun and Myc, resulting in the expression of genes important for cell cycle progression [as reviewed in ref 46].

PI3K/Akt pathway

In response to growth factors binding to the ErbB receptors, the PI3K/Akt pathway can be activated. The p85 regulatory unit of PI3K interacts with the intracellular part of the receptor via SH2 domains, thereby localizing PI3K to the plasma membrane [47]. The ErbB3 receptor in particular possesses several SH2 domains and it is therefore the major activator of Akt signaling [48-50]. PI3K can also be activated by other receptors such as platelet-derived growth factor receptor, fibroblast growth factor, insulin-like growth factor receptor, vascular endothelial growth factor and also by intracellular proteins such are protein kinase C, SHP1, Rac, Rho and Src [51]. When activated, PI3K phosphorylates PIP2 to produce the second messenger PIP3 (figure 1). PTEN is the antagonist of PI3K, which removes the 3' phosphate of PIP3 and inhibits signaling downstream of PI3K. The pleckstrin-homology (PH) domain of Akt binds to PIP3 thereby recruiting Akt to the plasma membrane where Akt is first phosphorylated by phosphoinositide-dependent kinase 1 (PDK1) at Thr 308 and then additionally phosphorylated in the hydrophobic C-terminal domain (Ser 473) by PDK2 to become fully activated [52, 53]. When activated, Akt moves to the cytoplasm and nucleus and phosphorylates a number of proteins important for regulating various cellular

functions such as cell proliferation, cell survival and cell migration [47, 54]. Akt is known to enhance cell survival through inhibition of pro-apoptotic proteins such as FOXO and Bcl2-antagonist of cell death (Bad). Akt also blocks apoptosis through the induction of survival proteins such as Bcl-2, Ikappa kinase (IKK), and MDM2 [55]. Furthermore, the PI3K/Akt signaling pathway is important in regulating cell cycle progression and proliferation. Akt promotes the G₁-S phase transition by blocking the FOXO-mediated transcription of the cell cycle inhibitor p27^{KIP1} or directly by phosphorylating and inactivating p27^{KIP1}. The control of cell growth by the PI3K/Akt pathway through regulating cell survival and cell cycle progression implicates the importance of this pathway in carcinogenesis and cancer development.

Stat pathway

Stat proteins are involved in both signal transduction and activation of transcription. All Stat proteins share a similar molecular structure and consist of three different domains: an oligomerization domain, a DNA binding domain and a SH2 domain. There are nine known Stat proteins including Stat1, Stat1 β , Stat2, Stat3, Stat3 β , Stat4, Stat5a, Stat5b and Stat6 [56]. Stat1 β and Stat3 β are two naturally occurring splice variants of Stat1 and Stat3, respectively. Stat1, Stat3, Stat5a and Stat5b are known to play a role in cancer. Stat3 and Stat5 stimulate cell cycle progression, angiogenesis, and inhibition of apoptosis, whereas Stat1 cause cell cycle arrest and apoptosis [57]. Stat signaling can be induced by growth factor receptors, cytokine receptors or in a non-receptor manner [58]. The EGFR can directly activate Stat via the SH2 domain and/or indirectly induce Stat phosphorylation through the activation of Src and Jak family members [58]. Activated Stat form a hetero- or homodimer which translocates to the nucleus where it binds to DNA sequences within the promoter of its target genes or to other transcription regulatory proteins such as c-fos and c-jun (figure 1) [57]. Some of the genes regulated by Stats are *Bcl-2*, *Bcl-xL*, *mcl-1*, *p21*, and *cyclin D1* [59].

Cell proliferation

Cell cycle

Throughout a person's life damaged, diseased or worn out cells are constantly being replaced. This is done through cell division. In somatic cells, the division is called mitosis and two identical daughter cells are produced. Dividing cells go through the cell cycle, which is a regulated sequence of

events where the cellular contents is duplicated and divided in two. The cell cycle is made up of two major periods: interphase, where a cell is not dividing and the mitotic (M) phase where a cell is dividing [60]. The interphase is divided into three phases: G_1 (gap), S (synthesis) and G_2 (figure 2). G_1 phase is the period between the M phase and the S phase and during this phase the cell duplicates its organelles and cytosolic components. The duration of the G_1 phase is very variable; it can last from only a few minutes up to years for different types of cells. Some cells that remain in G_1 for a very long time, perhaps never to divide again, are said to be in G_0 state. The S phase is the period between G_1 and G_2 and its duration is also variable. DNA replication occurs in S phase and a control is made, confirming that the two daughter cells have identical genetic material. The G_2 phase is the interval between the S phase and the M phase. During G_2 phase cell growth continues, enzymes and proteins are synthesized in preparation for cell division. The M phase is the interval between G_2 and G_1 and this period consists of nuclear division, and cytosolic division. After mitosis, cells enter the G_1 phase before DNA synthesis is reinitiated in the S phase.

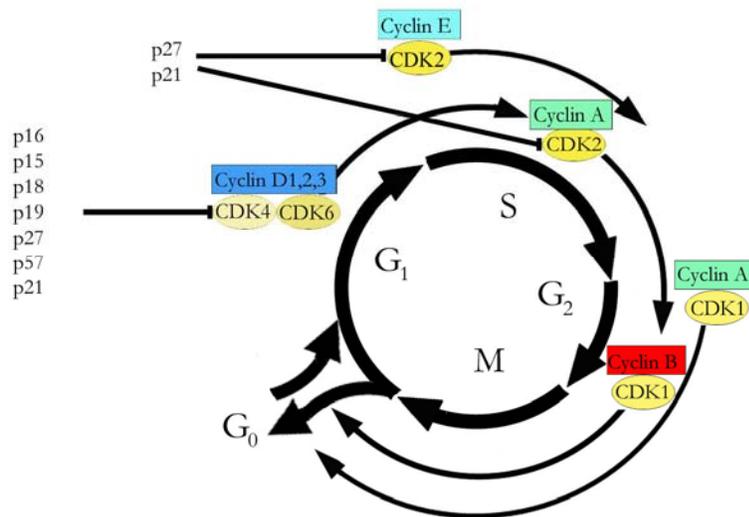


Figure 2. The cell cycle. The cell cycle is divided up into four phases, G_1 , S, G_2 and M. Cyclin-dependent kinases (CDKs) promote progression through the cell cycle and they are positively regulated by cyclins and negatively regulated by CDK inhibitors (CKIs).

Regulation of the cell cycle

In order to prevent inappropriate cell proliferation, mechanisms exist that control the cell cycle. The main regulator proteins which allow the transition from one cell cycle phase to another are called cyclin-dependent kinases (CDKs). This is a family of serine/threonine protein kinases which are activated at specific points during the cell cycle [60, 61]. Currently nine different CDKs have been identified and five of these are active during the cell cycle; during G_1 (CDK4, CDK6 and CDK2), S (CDK2), G_2 and M (CDK1), and during all phases (CDK7) (figure 2) [60]. When the CDKs are activated they phosphorylate their downstream target proteins. The cyclin levels rise and fall during the different stages of the cell cycle and in that way periodically activate the CDKs [60]. The three D cyclins (D1, D2 and D3) bind to CDK4 and CDK6 and these complexes are essential for entry in G_1 phase [62]. The progression through G_1 is mediated by cyclin D isoforms and CDK2, -4, and -6 [63]. The D cyclins are not expressed periodically but rely on growth factor stimulation to be synthesized [64]. Cyclin E is another G_1 cyclin which binds to CDK2 to regulate the progression from G_1 to S phase [65]. Cyclin A is activated during S phase and binds to CDK2. In late G_2 and early M, cyclin A forms complexes with CDK1 to promote entry to M. Mitosis is further regulated by cyclin B in complex with CDK1 [66]. The CDK activity is also regulated by cell cycle inhibitory proteins, called CDK inhibitors (CKI), by binding to CDK or the CDK-cyclin complex. So far two different families of CDK inhibitors have been discovered, the INK4 family and Cip/Kip family [67]. The INK4 group involves p15^{INK4b}, p16^{INK4a}, p18^{INK4c}, and p19^{INK4d} [67]. These proteins are structurally related and recognize CDK4 and CDK6, but not CDK2, and cause an arrest in G_1 by competing with cyclin D for binding with CDK4. The Cip/Kip family includes p21^{WAF1}, p27^{KIP1}, and p57^{KIP2} which interact with the cyclin-CDK complexes and prevent kinase activity of cyclin A/CDK2 and cyclin E/CDK2. [68]. Overexpression of the Cip/Kip inhibitors causes G_1 cell cycle arrest, however, p21^{WAF1} and p27^{KIP1} are inhibitors of CDK2 but also activators of CDK4 [69]. Each cell cycle phase has checkpoints that allow the arrest of cell cycle progression to repair damages. When these checkpoints have been passed, the cells are irreversible committed to the next phase. When DNA is damaged or if other critical organelles or structures are malfunctioning, cell cycle arrest can be activated or even the apoptosis cascade can be initiated leading to cell death. Therefore, apoptosis is an important element of cell cycle checkpoints to protect the integrity of multicellular organisms and making sure unwanted or damaged cells are removed.

Cell death

Apoptosis compared to necrosis

There are two different types of cell death, apoptosis and necrosis, and the major differences between them is in the cause and histology [70, 71]. Apoptosis is a controlled form of cell death whereas necrosis is accidental cell death. Necrosis frequently occurs in groups of cells and is often caused by hypoxia, lack of sufficient oxygen. However, necrosis can also be caused by chemical injury from different toxins such as arsenic, cyanide, insecticides and heavy metals. During necrosis the cell membrane permeability is increased, which cause cell and organelle rupture, leading to the release of cytoplasmic and nuclear components into the interstitial fluid [70, 71]. The release of the intracellular materials into the extracellular fluid recruits neutrophils and macrophages to the necrotic area, causing inflammation. These migratory cells phagocytose the necrotic debris to prevent further damaging of surrounding cells. Therefore, inflammation is a major marker for necrosis. Apoptosis on the other hand occurs in single cells and not in groups of cells. The word apoptosis is Greek and means the dropping off or falling off as in leaves from a tree or from a flower. Apoptosis is an energy consuming process, in which an organism destroys cells that are not needed, or potentially dangerous [70]. Apoptosis is induced by a stimulus and follows a precise signal transduction pathway [70]. During apoptosis the chromatin is condensed and the cell detaches from the extracellular matrix and surrounding cells. The DNA is fragmented, condensation or shrinkage of the cytoplasm occurs and small apoptotic bodies containing cellular contents are formed. The apoptotic bodies are released and phagocytosed by surrounding cells. No inflammation is involved since no intracellular materials are released from the cell.

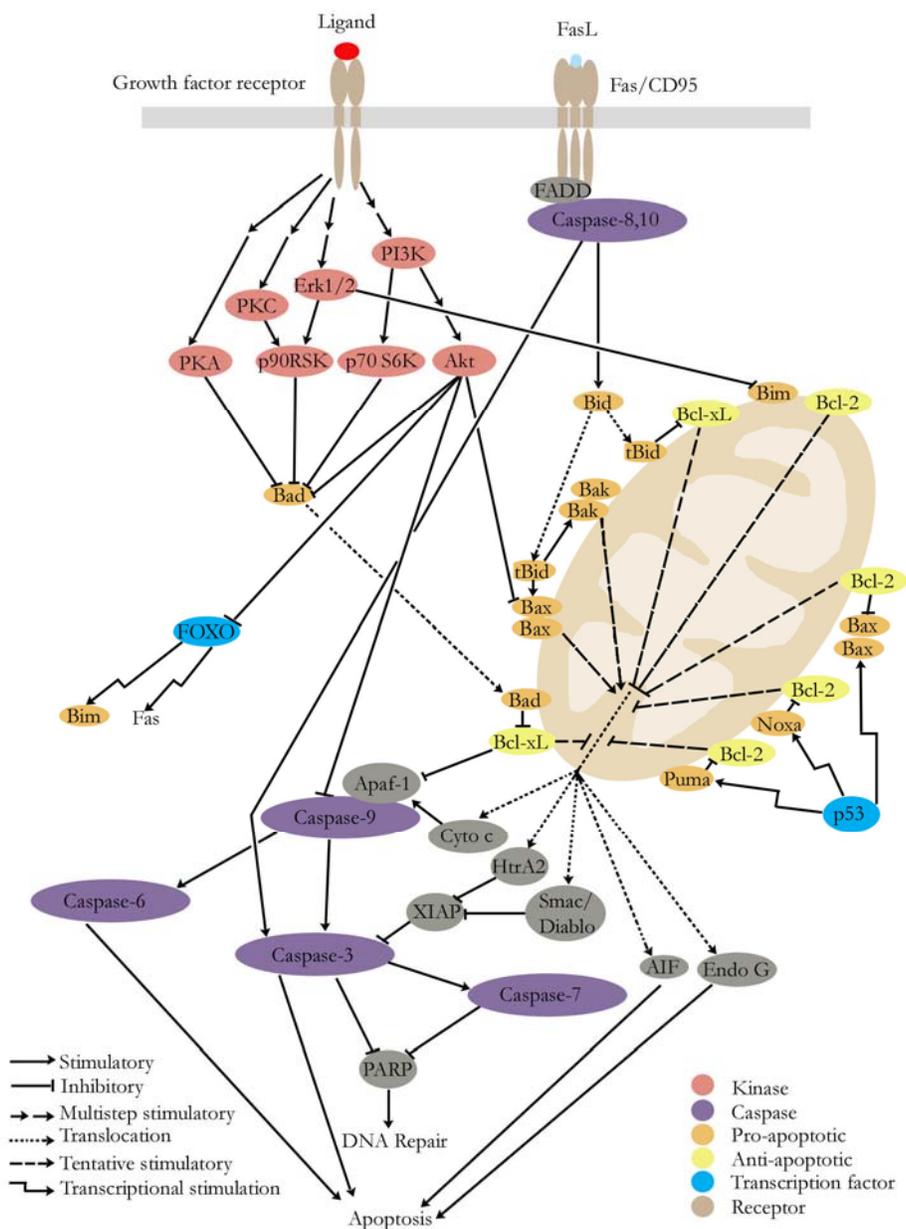


Figure 3. Induction of apoptosis can occur through two different pathways, the death receptor pathway or the mitochondrial pathway. The death receptor pathway is activated by FasL binding to its receptor Fas. This activates Caspase-8 and Caspase-10, which cleave Caspase-3 and thereby initiating the caspase cascade. The mitochondrial pathway is activated in response to different types of stress signals resulting in the formation of pores which releases, among others cytochrome c which activate the caspase cascade. The caspase cascade cause cell shrinkage, membrane blebbing, DNA fragmentation and ultimately leads to apoptosis.

Anti-apoptotic



BH domains 1-4: Bcl-2, Bcl-X_L, Bcl-w

Pro-apoptotic



BH3 only: Bim, Bik, Puma, Noxa

Pro-apoptotic



BH domains 1-3: Bax and Bak

Figure 4. Proteins of the Bcl-2 family are either pro- or anti-apoptotic and they are divided into different groups depending on their BH domains.

Regulation of apoptosis, the pathway of apoptosis

Apoptosis can be induced by a number of factors such as UV- or γ -radiation, chemotherapeutic drugs or signaling by death receptors (DR). There are two different pathways that trigger apoptosis: the death receptor pathway and the mitochondrial pathway (figure 3). The DR pathway is initiated by the binding of ligand to death receptors, such as CD95L binding to CD95 and results in the recruitment of signaling molecules to the death domain (DD), located on the intracellular part of the DR [72]. A death-inducing signaling complex (DISC) is formed comprising of the receptor, the DD-containing adaptor molecule Fas-associated death domain protein (FADD), two isoforms of procaspase-8 (also known as FLICE), procaspase-10 and the cellular FLICE-inhibitory protein (FLIP_{L/S}) [72]. The DD on the receptor interacts with the DD on FADD and the death effector domain (DED) on FADD interacts with the N-terminal DEDs on procaspase-8, procaspase-10 and FLIP_{L/S} resulting in an activation of caspase-8 and caspase-10 [72]. Caspase-8 then activates the executioner caspase-3 (figure 3).

The mitochondrial pathway is regulated by members of the Bcl-2 family that are divided into pro-apoptotic, such as Bcl-2-associated x protein (Bax), Bcl-2 antagonist/killer-1 (Bak) and Bid, and anti-apoptotic, such as Bcl-2 and Bcl-X_L (figure 4) [73]. They regulate the mitochondrial pathway by controlling the permeabilization of the outer mitochondrial membrane. In response to different types of stress or damage BH3-only proteins activate the pro-apoptotic Bax or Bak. Activated Bax and Bak form homo-oligomers and participate in the formation of pores in the outer mitochondrial membrane [74]. Through these pores pro-apoptotic molecules such as second mitochondria-derived

actor of caspase (Smac) and cytochrome C escapes. In the cytosol, cytochrome C will first associate with Apaf-1 and then with procaspase-9 to form a complex called an apoptosome [75]. Apoptosis-inducing factor (AIF) and several other procaspases including procaspase-2, 3, and 9 are also released from the mitochondria after induction of apoptosis.

A crosstalk exists between the two pathways through caspase-8 activation of Bid. Cleaved Bid is transported to the mitochondria where it exerts its pro-death activity by promoting cytochrome C release. The death receptor and mitochondrial pathways converge at caspase-3 activation. Downstream of caspase-3 the apoptotic signaling branches out into a number of subprograms, which in the end results in the dismantling, and removal of the cell. The activated caspases cleave a variety of target proteins, thereby disabling important cellular processes and breaking down structural components of the cell. One target of such cleavage events is poly(ADP-ribose) polymerase (PARP) resulting in inactivation of the Poly(ADP-ribosylation) which is crucial in DNA repair and replication, transcription and cell death, and represents a cellular emergency reaction [76].

ErbB targeted therapies

The ErbB receptors are overexpressed or aberrantly activated in a wide range of human tumors, and therefore constitute attractive candidates for selective anti-cancer therapies. Currently the development of targeted therapy in oncology is principally focused on two different types of agents; monoclonal antibodies (Mabs) and tyrosine kinase inhibitors (TKIs). The treatment of tumor cells with these agents affects many signal transduction pathways that are involved in cancer development and progression. In preclinical studies, it has been observed that ErbB-targeted TKIs and antibodies may cause a rapid down-regulation of PI3K, Akt, MAPK, Src and Stat signaling and results in inhibition of proliferation of tumor cells. The first drug used as a targeted therapy was trastuzumab (Herceptin®), which was approved for the treatment of ErbB2-overexpressing metastatic breast cancer in 1998 [34]. The drug was developed after the discovery that the gene encoding the ErbB2 receptor was amplified as much as 100-fold in up to 30 % of the tumor cells from patients with invasive breast cancer [77]. It was also found that a significant clinical correlation existed between ErbB2 gene amplification and overexpression, and different parameters of malignancy such as survival and reduced time to relapse when comparing to patients with normal receptor levels [78, 79]. In recent years, several pharmaceutical companies have developed small-molecule inhibitors of ErbB tyrosine kinase activity. In contrast to antibodies these small TKIs has the ability to enter the plasma

membrane and inhibit the receptor by binding to the intracellular part of the receptor. This enables inhibition of receptors both located at the plasma membrane and intracellular receptors not expressed at the surface. One such ErbB1 TKI is gefitinib (Iressa, ZD1839) which prevent ATP binding to the receptor and thereby inhibit tyrosine kinase activity, autophosphorylation and subsequently block the ErbB1 signal transduction. Gefitinib-inhibited growth is associated with cell cycle arrest caused by the upregulation of p27 [80]. Gefitinib was the first commercially available ErbB1 TKI and is currently licensed for the treatment of advanced non-small-cell lung cancer (NSCLC) in 36 countries worldwide [81]. In December 2004, AstraZeneca announced the results of the phase III ISEL (Iressa Survival Evaluation in Lung Cancer) study which compared gefitinib with placebo in patients with advanced NSCLC who had failed one or two prior chemotherapy regimens. Even though the ISEL study showed some improvement in survival with gefitinib as compared with placebo, this failed to reach statistical significance. However, a subset of patients were found to benefit from ErbB1-targeted TKIs and those were associated with Asian ethnicity, non-smokers, adenocarcinoma histology, and female sex [82-85]. After the announcement of the ISEL data, AstraZeneca voluntarily withdrew the European submission for gefitinib and regulatory authorities in the USA and Canada limited the use of gefitinib to those patients already experiencing benefit from the drug. However, in the Asia Pacific region, due to the molecular differences in lung cancer, gefitinib has become an established therapy for pre-treated advanced NSCLC and use of the drug in the first-line advanced setting is now being studied in a large phase III pan-Asian trial known as the IPASS study.

In recent years a second-generation TKIs has been developed and these substances are able to permanently inhibit receptor activation and may also possess the ability to inhibit multiple tyrosine kinases. One such drug is canertinib (CI-1033) which is an irreversible inhibitor of ErbB1-4 receptors. When canertinib is bound to the ATP pocket of the ErbB receptors the formation of a covalent bond occurs at cysteine 773 of ErbB1, cysteine 784 of ErbB2 or cysteine 778 of ErbB4 [86, 87]. To restore ErbB signaling after inhibition with canertinib new synthesis of the receptors is required. Therefore, irreversible inhibitors have a tendency to produce a more prolonged suppression of receptor activity than other reversible inhibitors. Since canertinib blocks signaling through all members of the ErbB receptor family it is more efficient and has a broader antitumor effect than inhibitors that only prevent signaling from one of the ErbB receptor. Canertinib has been shown to inhibit growth and induce apoptosis in several cancer cell lines and xenografts [88-90]. In clinical studies canertinib has been shown to have acceptable side-effects. However, in phase II

studies canertinib has only been able to show modest effects on breast cancer and NSCLC patients [91, 92]. Therefore, it is important to identify the patients that are the most likely to respond to treatment.

AIMS OF THE THESIS

General aim

The general aim of this thesis was to investigate the effect of ErbB tyrosine kinase inhibitors on malignant melanoma cell growth and survival *in vitro*.

Specific aims:

- To investigate the effect of the ErbB1 tyrosine kinase inhibitor gefitinib on malignant melanoma cell proliferation and survival.
- To study the effect of gefitinib on ErbB receptor phosphorylation and the subsequent downstream signaling molecules Akt, Erk1/2 and Stat3.
- To investigate the effect of the pan-ErbB tyrosine kinase inhibitor canertinib on malignant melanoma cell proliferation and survival.
- To study the effect of canertinib on ErbB receptor phosphorylation and the subsequent downstream signaling molecules Akt, Erk1/2 and Stat3.

MATERIALS AND METHODS

Materials

Cell culture (Paper I-II)

Human malignant melanoma cell lines, RaH3 and RaH5, derived from two different melanoma patients with metastatic disease were kindly provided by Dr. J. Hansson (Radiumhemmet, Karolinska Hospital, Stockholm, Sweden). The cells were maintained in continuous exponentially growing cultures in 75-cm² cell culture flasks (Costar, Cambridge, MA, USA) and grown in growth medium consisting of RPMI 1640 (Gibco, Paisley, Scotland) supplemented with 10 % fetal calf serum (FCS; Gibco), 0.1 % sodium bicarbonate, 2 mM L-glutamine and penicillin (50 units/ml), and streptomycin (50 µg/ml). The cultures were incubated in humidified air containing 5 % CO₂ at 37°C and subcultivated once a week with a split ratio of 1:20, using 0.25 % trypsin as a detaching agent.

Chemicals (Paper I-II)

Gefitinib (ZD1839) used in paper I was a gift from AstraZeneca (Macclesfield, U.K.). Gefitinib was dissolved in sterile dimethyl sulfoxide (DMSO) at a 10 mM stock concentration and stored at -20°C. A working solution was prepared in growth medium and used within 30 min after preparation; the resulting concentration of DMSO was less than 0.1 % in cell medium.

Canertinib (CI-1033) used in paper II was from Pfizer Pharmaceuticals (Ann Arbor, MI, USA). Canertinib was dissolved in DMSO at a stock concentration of 10 mM and stored at -20°C. A working solution was prepared in growth medium and used within 30 min after preparation.

Recombinant Human neuregulin-1-β1 (NRG1-β1: R&D systems, Minneapolis, MN, USA) was dissolved in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 6.7 mM Na₂HPO₄ x 2H₂O and 1.5 mM KH₂PO₄, pH 7.4) containing 0.1 % Bovine Serum Albumin (BSA: Saveen Biotech AB, Limhamn, Sweden) to make a stock of 10 µg/ml and stored at -20°C.

Methods

The methods have been described in detail in each paper, and therefore the principle of methods will be discussed.

Cell counting experiments (paper I-II)

Cell counting was performed using a Coulter Counter Z2 (Beckman Coulter, Krefeld, Germany). The counter can be used to measure any particulate material suspended in an electrolyte. Particles as small as 1 μm and as large as 120 μm in diameter can routinely be measured using this counter. In a Coulter counter, a tube with a small aperture on the wall is immersed into a beaker that contains particles suspended in a low concentration electrolyte. Two electrodes are placed in the beaker, one inside the aperture tube and one outside the aperture tube, and a current path is provided by the electrolyte when an electric field is applied. The impedance between the electrodes is then measured. The aperture creates what is called a “sensing zone”. Particles in low concentration, suspended in the electrolyte, can be counted by passing them through the aperture. As a particle passes through the aperture, a volume of electrolyte equivalent to the immersed volume of the particle is displaced from the sensing zone. This causes a short-term change in the impedance across the aperture, which can be measured as a voltage pulse or a current pulse. The height of the pulse is proportional to the volume of the sensed particle. If a constant particle density is assumed, then the height of the pulse is also proportional to the particle mass. This technology is also called aperture technology. Using count and pulse height analyzer circuits, the number of particle and volume of each particle passing through the sensing zone can be measured. If the volume of liquid passing through the aperture can be precisely controlled and measured, the concentration of the sample can be determined.

Determination of the protein concentration (paper I-II)

Protein lysates were prepared from RaH3, RaH5 and MCF7 cells as described in paper I and paper II. Total protein concentration was determined spectrophotometrically using the Bradford method [93]. The Bradford assay has become the preferred method for quantifying protein in many laboratories. This technique is simpler, faster, and more sensitive than the Lowry method.

Furthermore, when compared with the Lowry method, it is subject to less interference by common reagents and non-protein components of biological samples. The assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when being bound to protein. Thus, the quantity of protein can be estimated by determining the amount of dye in the blue ionic form. The dye appears to bind most readily to arginyl and lysyl residues of proteins. This specificity can lead to variation in the response of the assay to different proteins, which is the main drawback of the method.

Immunoprecipitation (paper II)

Immunoprecipitation is a method that precipitates an antigen out of a solution by using an antibody. This method can be used to increase the concentration and purity of an antigen in a protein lysate. A so-called co-immunoprecipitation can be used to identify interactions between different proteins and peptides. A protein lysate is taken and pre-cleared with rabbit serum and protein G Plus/ protein A agarose beads. Any antibody that does not recognize the antigen can be used for pre-clearing but rabbit serum is often the best choice since it is commercially available in large amounts and binds with its Fc domain tightly to protein A. Since protein A has different affinities for antibodies from different species, protein G can be used to increase the concentration of bound antibody. After the pre-clearing, lysates are centrifuged to remove the rabbit serum and the agarose beads. Then the antibody towards the protein of interest and protein G Plus/ protein A Agarose beads are added to the protein lysates and time is allowed for the antibody to bind to the antigen and to the beads. The lysate is centrifuged and the pellet is kept and washed with PBS thereby discarding non-specific proteins from the lysate. Laemmli buffer is added and lysates are boiled for 4 min under which the protein is released from the antibody and the proteins are denatured. The lysate is then centrifuged to remove the beads and the lysate is added on to a sodium dodecyl (lauryl) sulfate (SDS) gel and western blot is performed.

Western blot analysis (paper I-II)

Western blot is a method where proteins are transferred from a gel to a membrane using electrophoresis [94]. This method is used to detect and quantify a specific protein in a sample.

In western blot, proteins are separated by polyacrylamide gel electrophoresis (PAGE) and the gels can be prepared for a wide range of size resolution. We used the Laemmli buffer system (Tris-Glycine) where a large-pore gel is casted on top of a small-pore gel. The polyacrylamide gels are formed by the co-polymerization of acrylamide and a crosslinking agent such as bis-acrylamid. Free radicals are required to initiate the polymerization reactions which are formed from ammonium persulfate (APS). To accelerate the formation of free radicals from APS tetramethylethylenediamine (TEMED) is added. This produces a chain reaction where polymers are elongated and randomly crosslinked to produce a polymer network with a defined pore size. The size of the pores is determined by the concentration of acrylamide, the amount of crosslinker and the concentration of APS and TEMED. The buffer used to prepare the gels is Tris-HCl whereas the buffer in the tank is Tris-glycine. The two different buffers create two ion fronts making the protein packed in a very tight zone. Laemmli buffer containing β -mercaptoethanol is added to the protein lysates, which are then boiled for 4 min. During the boiling, the proteins are denatured thereby removing the proteins' 3D-structures, which enables the proteins to unfold completely. The Laemmli buffer, gels and the buffer in the tank all contain the strongly anionic detergent SDS that binds to all proteins making them all negatively charged. SDS also strengthens the denaturation performed by β -mercaptoethanol. When the lysates have been added to the gel an electric current is applied, making the negatively charged proteins move forward in the gel towards the positive anode. The smaller proteins will encounter less resistance than the longer proteins, making them migrate faster than the longer ones. In this way many proteins of different length can be separated from each other on a single gel. The proteins then are transferred to a membrane [nitrocellulose or polyvinylidene fluoride (PVDF)] by using an electric current. Nitrocellulose membranes generally have a low background but they are more fragile and tear easily. PVDF on the other hand have a higher background but they are easier to handle and rarely tear. In this thesis PVDF membranes has been more frequently used but occasionally we have used nitrocellulose if the antibodies did not bind well to PVDF (as in the case of the antibody phosphotyrosine 4G10). After transfer membranes are incubated with a protein solution, such as milk or bovine serum albumin, to block all free binding sites on the membrane. This is done to prevent the antibody from binding non-specifically. The protein solution also contains Tween-20, which is a detergent that improves the antigen-antibody binding. The membranes are incubated with a primary antibody directed towards the protein of interest. Membranes are then washed thoroughly so that all non-bound antibodies are removed and thus minimizing the background. Subsequently, the membranes are incubated with a secondary antibody

which binds to the primary antibody. The secondary antibodies we used are conjugated with horseradish peroxidase (HRP). The membranes are washed which removes excess antibodies. Then enhanced chemoluminescence (ECL) is used which produce a signal with HRP that can be detected using an ECL film.

Enzyme-linked immunosorbent assay (paper I)

Enzyme-linked immunosorbent assay (ELISA), is a method used to detect an antibody or an antigen in a sample. This method was originally developed in 1971 for the quantification of IgG [95].

In paper I a sandwich ELISA was used to detect the differences in expression of phosphorylated ErbB4 when comparing serum-starved to NRG1- β 1 stimulated and gefitinib treated to exponentially growing RaH3 and RaH5 cells. A 96 well plate was coated with a phospho-ErbB4 capture antibody and then the plate was blocked to prevent non-specific binding. The same concentration of protein lysate was added to each well on the plate and time was allowed for the protein to bind to the antibodies. Excess protein was washed away and a HRP-conjugated anti-phospho-tyrosine detection antibody was added. The detection antibodies that did not bind were washed away and K-Blue substrate solution was added to each well. Enhanced K-Blue develops a deep blue color in the presence of peroxidase labeled conjugate. 1 M HCl was used to stop the reaction and the absorbance was measured at 450 nm.

Flow cytometry (paper I-II)

Flow cytometry is a well-known method that is frequently used to measure multiple parameters on a single cell. A carrier liquid is used to move cells or particles passed a measuring point. The measurement takes place in a cuvette where a light sensitive detector measures light that is spread or fluoresce from each cell. The light source in a flow cytometer can be a laser or a mercury lamp which makes the light that hits the cells as they pass through the measuring point very intense and focused. The light hits the cells is scattered in all directions and it has been shown that the amount of light that is spread in the same direction as the light source correspond to the size of the cell. This is known as Forward Scatter (FSC). The cells granularity is equivalent to the amount of light that is scattered at a 90 degree angles of the direction of the light source, also known as Side Scatter (SSC). Different antigens can be investigated on the surface of cells by using specific antibodies with a

fluorochrome bound to it. When light from the laser hits the fluorochrome, the energy is absorbed and photons of a shorter wavelength are emitted. These emitted photons are detected and measured by the flow cytometer.

In paper I and II we used flow cytometry to quantify the amount of DNA in each cell and determine how many cells were present in the respective cell cycle phase. The staining of DNA can be done in many different ways but we used propidium iodide (PI) that is excited at 488 nm. PI is not able to penetrate intact cell membrane therefore a detergent such as trypsin is used to make the membrane more permeable. The manufacturer of the flow cytometer (FACSCalibur: BD Biosciences, San Jose, CA, USA) used, recommends using a particular protocol from Vindeløv which uses three different solutions A, B, and C [96]. The first solution A contains trypsin which make the membrane more permeable, the second solution B contains a trypsin inhibitor that prevents trypsin from breaking down the cells, and the third solution C contains PI that stains DNA. In the protocol, it is also recommended that two reference populations, chicken and trout, of known DNA contents are used. The amount of fluorescence in the two different populations is used to decide a DNA-index of the cells in the sample. Before analyzing the samples the FACSCalibur is normalized so that the known DNA-peaks appear at the right location in the FL2-A histogram. The cell samples are then run and the data from each cell is analyzed to determine their location in the cell cycle.

In paper I we also used flow cytometry to demonstrate the presence of ErbB1-4 proteins in RaH3 and RaH5 cells before and after treatment with gefitinib. Indirect immunofluorescent labeling was performed, first an antibody directed towards the receptor of interest was used then a secondary fluorochrome-conjugated antibody that recognizes the Fc-part of the primary antibody was utilized. The antibodies for ErbB1, ErbB3 and ErbB4 all recognized intracellular parts of the receptors. Therefore, an IntraStain and Permeabilisation kit (K2311, DakoCytomation) was used. First reagent A was used to fix the cells and then reagent B was used to permeabilize the membrane, allowing the antibodies to enter the cells. The ErbB2 antibody recognizes the extracellular part of the receptor. Then a fluorescein-isothiocyanate (FITC)-conjugated Goat-anti-Mouse F(ab')₂ (F0479, DakoCytomation) was added, the cells were fixed with paraformaldehyde and the samples were analyzed on a FACSCalibur (BD Biosciences).

In paper II induction of apoptosis was investigated in melanoma cells after canertinib treatment and this was done by using the Annexin V-PE apoptosis detection kit for flow cytometry. This kit is based on the fact that the membrane phospholipid phosphatidylserine (PS) is translocated to the outside of the cell early in apoptosis. Annexin V is conjugated with a fluorochrome called Phycoerythrin (PE) that has a high affinity for PS, and this is used to detect cells that expose PS. 7-amino-actinomycin D (7-AAD) is a substance that enters the cells after they have lost membrane integrity. By using both Annexin V-PE and 7-AAD cells that are viable (Annexin V-PE negative and 7-AAD negative), early apoptotic (Annexin V-PE positive and 7-AAD negative), late apoptotic or dead (Annexin V-PE positive and 7-AAD positive) can be distinguished from each other.

DNA preparation (paper I)

DNA was isolated from RaH3, RaH5 and SK-MEL according to the Wizard[®] Genomic DNA Purification Kit (Promega Madison, WI, USA). The Wizard[®] Genomic DNA Purification Kit is based on a step by step process [97]. The first step in the purification procedure lysed the cells and the nuclei. The cellular proteins were then removed by a salt precipitation step, which precipitated the proteins and left the high molecular weight genomic DNA in solution. Finally, the genomic DNA was concentrated and desalted by isopropanol precipitation. The DNA pellet was then washed with 70 % ethanol, centrifuged and excess ethanol was removed. The DNA was air-dried and then rehydrated using a Rehydration Solution over night in 4°C. The DNA concentration was measured using a Nanodrop. The 260/280 ratio were 1.8-2.0, which reveals a satisfactory removal of proteins.

Polymerase chain reaction (PCR) (paper I)

The polymerase chain reaction (PCR) is a method used to amplify specific DNA regions, which was developed in by Kary Mullis [98].

A basic PCR requires several components and reagents, which is a DNA template, two primers, DNA polymerase, deoxynucleosides triphosphates (dNTP), buffer solution, divalent cations such as Ca²⁺ and monovalent cations. DNA template containing up to 10 k base pairs (bp) can usually be amplified by most PCR methods. However, some methods can even amplify DNA targets as big as 40 k bp in size. The two primers should be complementary to the 5' or the 3' end of the DNA

region. The Taq polymerase is the most frequently used DNA polymerase that enzymatically assembles the new DNA strand from the dNTPs using the single DNA strand as a template. The buffer solution provides a chemical environment that is optimal for the activity and stability of the DNA polymerase. The mixture was placed in a PCR machine and the temperature was quickly raised to 95°C for 30 s. This broke the hydrogen bonds between the two DNA strands creating two single strands, called denaturing. Then the temperature was lowered to 55°C for 30 s and this allowed the primers to bind in to their complementary sequences, also known as annealing. Then the temperature was raised to 72°C for 30 s and thereby making DNA polymerase start to extend the primer with soluble dNTPs creating a complementary strand. In this way two double stranded DNA molecules are created from each double stranded DNA molecule in the sample. The cycle was repeated 35 times and the amount of DNA was doubled further with every cycle. The samples from each PCR were loaded on a 1.5% agarose gel (Invitrogen, Paisley, Scotland UK) and stained with ethidium bromide for detection of PCR products on a UV-table.

In Paper I we performed PCR on exons 11 and 15 of the *Braf* gene and exons 1 and 2 of the *Kras* and *Nras* genes in RaH3, RaH5 and SK-MEL cells to investigate for mutations in those regions. At the end of the PCR reaction the samples are run on an agarose gel to control the PCR products.

Single strand confirmation analysis (SSCA) (paper I)

Single strand confirmation analysis is a method used to detect DNA polymorphisms and point mutations in a DNA sample. SSCA is a method based on the fact that two DNA strands that only differ at a single base will produce two different secondary structures that will display a different migratory pattern in the gel.

In paper I we used SSCA to investigate the *Braf*, *Nras* and *Kras* genes for mutations. PCR products from exons 11 and 15 of the *Braf* gene and exons 1 and 2 of the *Kras* and *Nras* genes were marked by performing an additional PCR amplification for 8-10 cycles with the incorporation of radioactive labeled nucleotides and then denatured by heating before being separated in polyacrylamide gel. The pattern of separated bands was visualized using an x-ray film. PCR products with altered mobility compared to normal sample were excised and re-amplified for DNA-sequencing.

DNA sequencing (paper I)

During the 1970's the first attempts at sequencing DNA were made, and since its discovery the method developed by Sanger is the most widely used [99]. The Sanger sequencing method is based on the fact that dideoxynucleotides (ddNTP) lack the free 3' hydroxyl group which is crucial for the incorporation of nucleotide in PCR reactions and thereby preventing further extension. In a PCR reaction containing all dNTPs and one labeled ddNTP (ddATP, ddGTP, ddCTP or ddTTP) the reaction will stop at random points when the ddNTP is incorporated and this will create DNA stands of different length but all starting at the same nucleotide. At the end of the PCR reaction the mixture containing DNA strands of different length were then loaded into the capillary electrophoresis instrument MegaBACE 1030 (GE Healthcare). The fluorophore labeled ddNTPs can be excited by a laser to emit fluorescence of a specific wavelength that can be detected and thereby determining different mutations.

In paper I we used the Sanger method to sequence the PCR products from exons 11 and 15 of the *Braf* gene and exons 1 and 2 of the *Kras* and *Nras* genes to confirm the results obtained from the SSCA and to determine the point mutations.

RESULTS AND DISCUSSION

Paper I

There is an increasing demand for the development of new treatment options for patients with metastatic melanoma. The treatments available today are characterized by a low percentage of responders and severe side effects. However, lately new types of pharmaceuticals have been developed that acts by targeting specific growth factor receptors and their intracellular signaling pathways. One such new drug is the ErbB1 tyrosine kinase inhibitor gefitinib which was used in Paper I in the treatment of two malignant melanoma cell lines.

The aim of this study was to investigate the effect of the ErbB1 tyrosine kinase inhibitor gefitinib on cell growth and survival on two malignant melanoma (RaH3 and RaH5) cell lines. Gefitinib has previously been shown to inhibit growth and induce apoptosis in several different cancer cell lines and solid tumors [80, 100-102]. In our experiments gefitinib inhibited proliferation of RaH3 and RaH5 cells in a dose-dependent manner, however, the growth inhibition appeared not to be complete (figure 1). Gefitinib-induced growth inhibition was accompanied by the upregulation of the cyclin dependent kinase inhibitor p27 and subsequently G₁ cell cycle arrest. Analogous results have been obtained in other cancer cell types [80].

The question arose if the continued proliferation seen despite gefitinib treatment might be caused by selection of an ErbB-negative population of melanoma cells. Therefore, the ErbB receptor expression was investigated by flow cytometry following gefitinib treatment and compared to untreated cells. No selection of any cell population devoid of ErbB receptors could be detected explaining the low but continuous proliferation noted during gefitinib treatment (figure 3).

Malignant melanoma lesions are known to frequently express ErbB1 and to harbor mutations in its gene [37, 38]. The presence of ErbB2 has been demonstrated in a low percentage of melanoma specimens [39]. However, ErbB3 expression occurs frequently in melanoma and has been correlated with increased tumor cell proliferation and poor prognosis for patients with primary melanoma [42]. The expression of full-length ErbB4 has not previously been demonstrated in malignant melanoma,

although ErbB4 fragments have been detected [43]. In our experiments we were able to demonstrate the expression and phosphorylation of all four ErbB receptors (figure 3 and 4). However, when investigating the ErbB4 receptor the presence of two bands of different length was revealed. In previous studies shorter ErbB4 bands have been demonstrated, which might represent a less glycosylated form or a cleavage product of the full-length ErbB4 [43, 103]. We confirmed that gefitinib-induced inhibited ErbB1, ErbB2 and ErbB3 receptor phosphorylation [100, 104, 105]. Interestingly, ErbB4 phosphorylation was however unaffected by gefitinib treatment. The ErbB3 and ErbB4 ligand NRG1- β 1 was unable to induce ErbB4 phosphorylation whereas all the other receptors were clearly activated suggesting that the ErbB4 receptor is non-functioning in RaH3 and RaH5 cells (figure 5). Additional studies are required in order to elucidate the function and structure of the ErbB4 receptor in RaH3 and RaH5 melanoma cells.

To further elucidate the mechanisms involved in gefitinib-induced growth inhibition, the ErbB downstream signaling was studied. The ErbB3 receptor is the major activator of the PI3K pathway and the inability to block this receptor has been particularly associated with the resistance to tyrosine kinase inhibitors [49, 100]. In the PI3K pathway, Akt displayed a decrease in activation within 30 min of gefitinib treatment (figure 6). However, after 6 h, Akt phosphorylation was partially restored. In the MAPK pathway, Erk1/2 activation was similarly inhibited within 30 min, however, Erk1/2 phosphorylation appeared to be restored within 6 h of gefitinib treatment (figure 6). Earlier studies have implied that sensitivity to gefitinib is dependent on its ability to prevent Akt and Erk1/2 signaling [104-107]. However, there are contradictory results concerning this since it has also been discovered that gefitinib may inhibit Akt and Erk1/2 without inducing apoptosis or have any anti-proliferative effects [108]. Therefore, it is still unclear if the lack of induction of apoptosis in our experimental system is due to gefitinib's inability to persistently inhibit Akt and Erk1/2 signaling.

We also investigated the effect of gefitinib on Stat3 activation. Unlike Akt and Erk1/2, Stat3 phosphorylation was not inhibited after 30 min of treatment. However, within 6 h of treatment a partial reduction in Stat3 phosphorylation was observed (figure 6). It has previously been demonstrated that Stat3 signaling is important in regulating cell growth and survival [109]. Other malignant melanoma cells have been shown to undergo apoptosis due to inhibition of Stat3 signaling and the number of apoptotic cells could be related to the degree of Stat3 inhibition [110]. In our experimental system the inhibition of Stat3 was not sufficient to induce apoptosis.

This study suggests that targeting the ErbB receptors with specific tyrosine kinase inhibitors such as gefitinib might be a new treatment strategy for malignant melanoma. However, further studies are required to fully investigate the applicability of these types of drugs in the treatment of malignant melanoma.

Paper II

In paper I we showed that the ErbB1 tyrosine kinase inhibitor gefitinib inhibited proliferation of RaH3 and RaH5 malignant melanoma cells. This drug was however not able to induce apoptosis probably due its inability to sufficiently prevent downstream signaling from the ErbB receptors. In order to investigate if a broader ErbB tyrosine kinase inhibition could display a more potent antitumor effect the pan-ErbB tyrosine kinase inhibitor, canertinib, was utilized. The aim of this study was to examine the effect of the pan-ErbB tyrosine kinase inhibitor canertinib on malignant melanoma cell growth and survival and on Akt, Erk1/2 and Stat3 signaling.

Canertinib has been shown to induce apoptosis in breast cancer cells, but its effect on melanoma cells has not previously been investigated [111]. We observed that treatment of RaH3 and RaH5 cells with canertinib significantly inhibited growth in a dose-dependent manner, with an IC_{50} of approximately 0.8 μ M in both cell lines, and at the highest concentration (10 μ M) cell death was observed (figure 1). We also investigated the effect on cell cycle distribution after treatment with a low-dose (1 μ M) and a high-dose (10 μ M) of canertinib. A low-dose treatment of RaH3 and RaH5 cells resulted in a G_1 phase accumulation, a canertinib effect that has previously also been reported in colon cancer cell lines (table 1, figure 2) [89]. However, high-dose treatment resulted in a decrease in number of cells in all cell cycle phases and after 12 h of canertinib treatment a sub-fraction of G_1 -cells appeared (figure 3). This fraction is regarded to represent apoptotic cells [112]. Western blot analysis of apoptotic PARP fragment and flow cytometry on Annexin V staining confirmed that canertinib induced apoptosis (figure 4). Our results are in line with those obtained by others who have shown that canertinib inhibits cell proliferation and induces apoptosis in a dose-dependent manner in other cancer types [88-90].

Canertinib clearly inhibited ErbB1 and ErbB3 phosphorylation in RaH3 and RaH5 cells as determined by western blot. Also, ErbB2 phosphorylation was inhibited but to a lesser extent (figure 5). Similar results have been obtained by others showing that canertinib inhibits of ErbB1-4 phosphorylation in several different cancer cells [89, 90, 113-115]. In our experiments, it is particularly interesting that canertinib potently inhibited ErbB3 activation in view of evidence that the expression of the ErbB3 receptor in malignant melanoma correlates with poor survival [42]. ErbB4 receptor phosphorylation, was as expected, unaffected by canertinib [116].

Canertinib also significantly inhibited both Akt and Erk1/2 phosphorylation within 30 min of treatment (figure 6). The inhibition of phosphorylated Akt was sustained for at least 6 h, whereas Erk1/2 phosphorylation partially reappeared within the same time period. Stat3 phosphorylation on the other hand was not affected after 30 min of canertinib treatment but was, however, markedly reduced within 6 h. These results are in line with those reported earlier on esophageal, colon and breast cancer cell lines and colon cancer xenografts [88, 89, 111, 114]. The more effective inhibition of Akt, Erk1/2 and Stat3 signaling by canertinib as compared to gefitinib, might be one explanation why canertinib induces apoptosis of RaH3 and RaH5 cells whereas gefitinib only inhibits proliferation.

CONCLUSIONS

Paper I

We report that the tyrosine kinase inhibitor, gefitinib, directed towards ErbB1 displays a potent inhibitory effect on malignant melanoma cell growth. This effect appears to be mediated through inhibition of ErbB1, ErbB2 and ErbB3 signaling with subsequent inhibition of the downstream signaling molecules Akt, Erk1/2 and Stat3. The anti-proliferative effect of gefitinib coincides with the upregulation of the cyclin-dependent kinase inhibitor p27^{KIP1} followed by cell cycle arrest in G₀/G₁ phase. We conclude that blockade of ErbB signaling might be an interesting complementary treatment strategy of malignant melanoma.

Paper II

This study clearly shows that the pan-ErbB tyrosine kinase inhibitor canertinib induces apoptosis in malignant melanoma RaH3 and RaH5 cells. We also show that treatment with low-dose of canertinib (1 μ M) resulted in accumulation of melanoma cells in the G₁ cell cycle phase, whereas higher concentrations (≥ 7 μ M) induced a non-cell cycle specific apoptosis. In addition, canertinib clearly inhibited phosphorylation of the ErbB1, ErbB2, and ErbB3 receptors in RaH3 and RaH5 cells, whereas ErbB4 phosphorylation was left unaffected. The downstream signaling molecules Akt, Erk1/2, and Stat3 were also potently dephosphorylated by canertinib. In summary, canertinib inhibits proliferation and induces apoptosis in a dose-dependent manner in malignant melanoma cells *in vitro*. Further *in vitro* and *in vivo* studies are required to establish if canertinib will prove to be effective in the treatment of malignant melanoma either as a single agent or in combination with conventional cancer therapy.

FUTURE PERSPECTIVES

We have developed cell lines resistant to gefitinib and canertinib. The mechanisms of resistance will be studied. We will also investigate if any cross-resistance between different tyrosine kinase inhibitors prevail and other tumor specific therapeutics.

We have in a pilot experiment, demonstrated that canertinib potently inhibits growth in melanoma xenografts in nude mice. These findings will be confirmed and the mechanisms of canertinib induced anti-tumor effects will be investigated.

In addition to the already studied mutations in *Baf*, *Kras* and *Nras* it would also be of importance to investigate any molecular alterations in PTEN, p53 and PI3K in order to elucidate the biology of RaH3 and RaH5 cells to ultimately find more effective treatment strategies of melanoma.

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