

# **Strategies to increase effectiveness of growth factor receptors-targeted therapy in glioblastoma**

av

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Stockholm 2007

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Published and printed by Karolinska University Press  
Box 200, SE- 171 77 Stockholm, Sweden  
**ISBN 978-91-7357-390-0**

*"The opposite of every truth is just as true"*  
Hermann Hesse

To my family

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## ABSTRACT

Gliomas are the most common glial neoplasms, accounting for nearly 70% of all primary brain tumours. The progression of low-grade gliomas into more aggressive tumours is supported by the abnormal function of several proto-oncogenes, including growth factor receptor tyrosine kinases (GFR-TKs).

In paper I, we showed that the pharmacologic inhibition (using small molecule inhibitors) of IGF-1R, PDGFR and their intracellular signalling *via* PI3-K and ERK1/2 kinases had only modest or not antitumour activity in five glioblastoma cell lines (MO59J, MO59K, 8, 18 and 38). The ability of the ionizing radiation to improve the effect of the molecular targeted therapy was also investigated. Interaction between small inhibitors and ionizing radiation was mostly additive or subadditive, synergistic interaction was found in few analysed combinations. One explanation may be the interaction of the IGF-1R with PDGFR in maintenance the intracellular signalling activated.

Therefore, a therapeutic strategy of co-targeting both IGF-1R and PDGFR has been taken into account. In two glioblastoma cell lines (18 and 38), we found that dual targeting of IGF-1R and PDGFR increased cell death in comparison to the inhibition of either receptor alone. In addition, co-inhibition of IGF-1R and PDGFR increased radiosensitivity in 18 cells but failed to intensify the effect of radiation in 38 cells. In glioblastoma cells, radiation induced cell death has been connected to the activation of c-Jun-NH2-terminal kinase-1 (JNK1). We found that JNK1 was weakly expressed in 38 cells while it had an elevated expression in 18 cells. Exposure to ionizing radiation induced JNK1 activation in 18 cells only, suggesting that in this cell line radiation-activated JNK1 may provide an anti-proliferative signalling parallel to receptors co-targeting. To test this hypothesis, glioblastoma cells were treated with dominant negative JNK1 (dnJNK1) and the response to radiation was assayed in the presence or absence of receptors co-inhibition. Indeed, dnJNK1 protected 18 cells against  $\gamma$ -radiation induced cell death. The dnJNK1 treatment did not influence radiation response of the 38 cell line, which expressed low levels of JNK1 (paper II).

In conclusion, the combination of different targeted agents, or targeted agents and radiotherapy, seems to be a better treatment option than single agent therapy, with respect to the evident molecular heterogeneity of brain tumours. Nevertheless, a detail molecular understanding of the molecular pathogenesis of malignant gliomas is necessary to design an effective therapy against this stage of the disease.

## LIST of ARTICLES

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

- I.**     **Carapancea M., Alexandru O., Dinca I., Fetea S., Dragutescu L., Castro J., Wagner A.P., Bäcklund M., Lewensohn R. and Dricu A.**

*IGF-1R and PDGFR signaling in glioblastoma cells: therapeutic implications.*

Submitted, 2007

- II.**     **Carapancea M., Cosaceanu D., Budiu R., Kwiecinska A., Tataranu L., Ciubotaru V., Alexandru O., Bäcklund M., Lewensohn R. and Dricu A.**

*Dual targeting of IGF-1R and PDGFR inhibits proliferation in high grade gliomas cells and induces radiosensitivity in JNK-1 expressing cells.*

Published in Journal of Neuro-Oncology, 2007 june 14, DOI 10.1007/s11060-007-9417-0

## LIST of ABBREVIATIONS

BBB	Blood-brain-barrier
CML	Chronic myelogenous leukaemia
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular regulated kinase
FDA	Food and drug administration
FGF	Fibroblast growth factor
GB	Glioblastoma
GF-RTK	Growth factor receptor tyrosine kinase
GH	Growth hormone
GHRH	Growth- hormone releasing hormone
GIST	Gastrointestinal stromal tumors
HER	Human epidermal receptor
HGG	High grade glioma
IGF	Insulin-like growth factor
IGF-1R	Insulin-like growth factor type 1 receptor
IGF-2R	Insulin-like growth factor type 2 receptor
IR	Insulin receptor
JNK	c-Jun NH2-terminal protein kinase
Mab	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
NRPTK	Non-receptor protein tyrosine kinase
NSCLC	Non-small cell lung cancer
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PI3-kinase	Phosphatidylinositol-3'-kinase
PTEN	Phosphatase and tensin homolog
PTK	Protein tyrosine kinase
RPTK	Receptor protein tyrosine kinase
RTKI	Receptor tyrosine kinase inhibitor
SCCHN	Squamous cell carcinoma of the head and neck
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

# 1. BACKGROUND

## 1.1. Brain tumours

Brain tumours are various types of neoplasms that can be divided into primary and secondary (metastases) tumours (Louis, Ohgaki et al. 2007). The incidence of brain tumours has increased dramatically in the last years, particularly in the older population (Patchell, Tibbs et al. 1990; Brandes 2003; Grossman and Batara 2004). In the U.S.A., nearly 18 000 patients are diagnosed with malignant primary brain tumours each year (Patchell, Tibbs et al. 1990; Grossman and Batara 2004). Metastasis tumours account for more than 40% of brain tumours (Grossman and Batara 2004).

Table 1. A simplified Classification of Brain Tumours according to the WHO classification

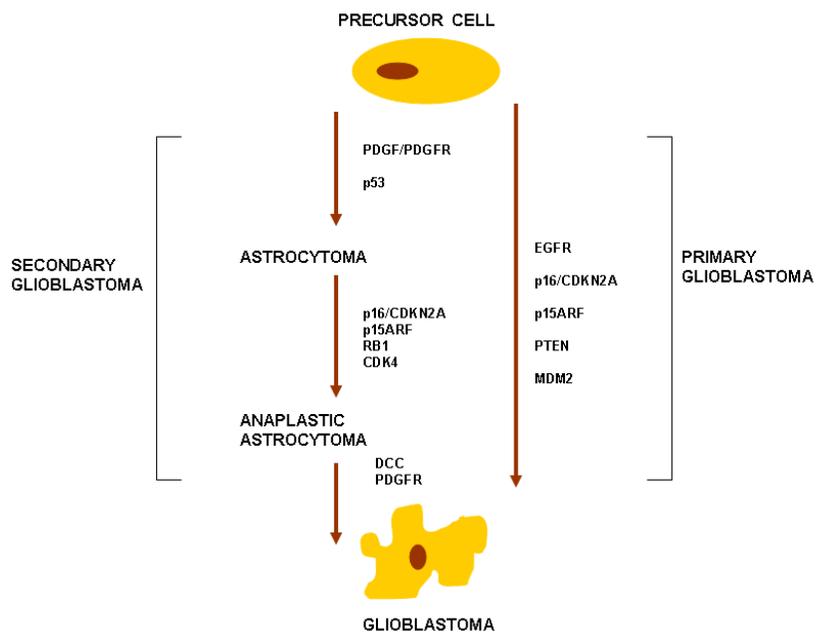
<b>A. Primary brain tumours</b>					
1. Gliomas					
	Astrocytic tumours	Pilocytic astrocytoma	Grade I	LG	Benign
		Astrocytoma	Grade II	LG	Diffuse malign
		Anaplastic astrocytoma	Grade III	HG	Diffuse malign
		Glioblastoma	Grade VI	HG	Diffuse malign
	Oligodendroglial tumours	Oligodendroglioma	Grade II	LG	Diffuse malign
		Anaplastic Oligodendroglioma	Grade III	HG	Diffuse malign
	Ependymal tumours	Ependyoma	Grade II	LG	Benign
		Anaplastic Ependyoma	Grade III	LG	Diffuse malign
	Mixed gliomas	Oligoastrocytoma	Grade II	LG	Diffuse malign
		Anaplastic Oligoastrocytoma	Grade III	HG	Diffuse malign
2. Primary CNS malignant lymphoma					
3. Meningioma					
<b>B. Secondary brain tumours</b>					
Metastases tumours					

Brain tumours classification depends on the site of the tumour, the type of tissue involved (benign or malignant) and other factors. A simplified classification of cerebral tumours is presented in Table 1. Primary brain tumours can arise from the brain cells, the meninges (membranes around the brain), the nerves or the glands, while metastatic brain tumours are tumours caused from cancer that originates in another part of the body. Glial cells give rise to numerous types of tumours, dependent on the type of cell origin. Gliomas are the most common glial neoplasms, accounting for nearly 70% of all primary brain tumours ([www.cbtrus.org](http://www.cbtrus.org); Statistical report: Primary brain tumors in the United States, 1998-2002; 2006).

Based on the degree of malignancy and histological features, gliomas are classified, according to World Health Organization (WHO), into gliomas of astrocytic origin (astrocytomas) that includes pilocytic astrocytoma (grade I), astrocytoma (grade II), anaplastic astrocytoma (grade III) and glioblastoma (GM) (grade IV); tumours derived from oligodendrocytes including grade II (oligodendrogliomas) and grade III neoplasms (oligoastrocytoma); ependymal tumours including ependyoma (grade II) and anaplastic ependyoma (grade III) and mixed gliomas including oligoastrocytoma (grade II) and anaplastic oligoastrocytoma (grade III) (Table 1).

The progression of several low-grade gliomas into more aggressive tumours is supported by the different molecular alterations involving overexpression of proto-oncogenes and loss of tumor suppressor genes (Fig. 1). Low-grade astrocytomas often have inactivating mutations of *TP53* (Okamoto, Di Patre et al. 2004), which encodes the tumour suppressor protein p53 on chromosome 17p (loss of heterozygosity (LOH)) and overexpression of platelet-derived growth factor (PDGF) and PDGF receptors (PDGFRs) (Hermanson, Funa et al. 1992). Progression to anaplastic astrocytoma is associated with inactivation of the p16-retinoblastoma (Rb) pathway and loss of 19q and cyclin-dependent kinase (CDK4) (Chakravarti, Dicker et al. 2004). Further progression to GB (so-called secondary GB) typically involves loss of chromosome 10, which includes tumor-suppressor phosphatase and tensin homolog (PTEN) (Baumann and Krause 2004). Primary GB develops *de novo* from glial cells and shows multiple genetic alterations including amplification and mutation of epidermal growth factor receptor (EGFR) and murine double minute 2 (MDM2), and deletion or mutation of the phosphatase and tensin homologue (*PTEN*) tumour suppressor gene on chromosome 10 (Baumann and Krause 2004) (Fig. 1).

Figure 1. Molecular changes associated with glioma progression.



## 1.2. Growth factor receptors tyrosine kinases -general considerations

Growth factor receptor tyrosine kinases (GFR-TKs) are closely related members of the protein tyrosine kinases (PTKs) superfamily and are among the more intensely studied proteins in cell signalling. PTKs are divided into two groups: receptor PTKs (RPTKs) and cellular, or non-receptor PTKs (non-RPTKs). Of the 91 PTKs identified so far in humans, 32 are non-RPTKs and 59 are RPTKs (Hubbard and Till 2000; Tsygankov 2003). These proteins are involved in cellular signaling pathways and regulate key cell functions such as proliferation, differentiation, motility, adhesion, and metabolic signals. Indeed, more than 70% of the known oncogenes and proto-oncogenes involved in cancer code for PTKs. Tyrosine kinases are particularly important today because of their implications in the treatment of cancer.

Non-RPTKs can be located in the cytoplasm, nucleus or anchored to the inner leaflet of the plasma membrane. They are grouped into eight families: Src, JAK, Abl, FAK, FPS, CSK, SYK and BTK, each family consisting of several members. With the exception of homologous kinase domains (Src Homology 1, or SH1 domains), and some protein-protein interaction domains (SH2 and SH3 domains), they have little in common, structurally. Many non-RPTKs, such as Src, are involved in cell growth (Planas-Silva and Hamilton 2007). Two non-RPTKs, Abl PTKs and FPS PTKs, are involved in growth inhibition and differentiation, respectively. Some members of the cytokine receptor pathway interact with Janus kinases, which phosphorylate the transcription factors, STATs (Shudo, Yang et al. 2007). Other RPTKs activate pathways whose components and functions are unknown.

RPTKs are located in the cell membrane and consist of an extracellular ligand-binding domain, a transmembrane domain and a cytoplasmic catalytic portion. In 1994 Lemmon has established a general mechanism for ligand-induced activation of RPTKs, in which ligand binding to the extracellular domain induced dimerization of the receptors (Lemmon and Schlessinger 1994). Receptor dimerization leads to activation of the catalytic RPTK domain and to tyrosine autophosphorylation. Phosphorylation of tyrosine residues leads to generation of docking sites for adaptor proteins such as Shc, Grb2 and Crk (Pawson and Scott 1997). Signaling proteins that bind to the intracellular domain of RPTKs in a phosphotyrosine-dependent manner include RasGAP, PI3-kinase (phosphatidylinositol-3'-kinase) and phosphotyrosine phosphatase SHP.

RPTKs are divided into at least 13 different GFR-TK families such as epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) etc. (Ullrich and Schlessinger 1990). GFR-TKs are glycoproteins that are located in the cell membrane and consist of an extracellular ligand-binding domain, a transmembrane domain and a cytoplasmic catalytic domain (Ullrich and Schlessinger 1990). Most GFR-TKs possess a single polypeptide chain and are monomeric in the absence of ligand. Following ligand binding, the tyrosine kinase intracellular domain of the receptor is activated by autophosphorylation, which initiates a cascade of intracellular events (Hubbard and Till 2000).

## **IGF family**

The IGF family is an important growth factor system organized in a complex regulatory network that operates at cellular and subcellular levels. The IGF family consists of three cell-surface tyrosine kinase receptors (IGF-1R, IGF-2R and IR), three ligands (IGF-1, IGF-2 and insulin), a family of six high-affinity IGF binding proteins (IGFBP 1-6), as well as associated IGFBP proteases and all of them are involved in IGF system function.

### *The IGF ligands*

The IGF ligands are part of a complex system that cells use to communicate with their physiologic environment. IGF-1, IGF-2 and insulin are the three major ligands of IGF system. IGF-1 and IGF-2 are growth factors, while insulin regulates glucose uptake and cellular metabolism. IGFs are single polypeptides with a molecular mass of 7.5 KDa for IGF-1 and 7.4 KDa for IGF-2 and they have high sequence similarity to insulin. The IGFs are organized into four domains: A, B, C and D. Domain A and B are similar in structure with the insulin counterparts, 43% homology for IGF-1 and 41% homology for IGF-2, respectively (Daughaday and Rotwein 1989). The C domain is similar in structure to the C-peptide of proinsulin, whereas the D-domain seems to be absent in proinsulin. IGF-II is thought to be a primary growth factor required for early development while IGF-I expression is required for achieving optimal growth (Yaghmaie, Saeed et al. 2006).

Most of the circulating IGF-1 is mainly secreted by the liver as a result of stimulation by growth hormone (GH), a pituitary gland hormone, which in its turn is under the regulation of the hypothalamic factors and growth-hormone releasing hormone (GHRH) (Sara and Carlsson-Skwirut 1986; Milner and Hill 1989; Bichell, Kikuchi et al. 1992; Thissen, Ketelslegers et al. 1994; Meton, Boot et al. 1999). Almost every cell in the human body is affected by IGF-1, especially cells in muscle, cartilage, bone, liver, kidney, nerves, skin, and lungs. In addition to the insulin-like effects, IGF-1 can also regulate cell growth and development, especially in nerve cells, as well as cellular DNA synthesis. IGF-2 is secreted by the brain, kidney, pancreas and muscle and the serum concentration of the peptide is not regulated by GH. IGF-2 is more specific in action than IGF-1. In adult humans it is found at 600 times the concentration of insulin. The IGFs are known to bind the IGF-1 receptor, the insulin receptor, the IGF-2 receptor, the insulin-related receptor and possible other receptors. The IGF-1 binds IGF-1R at significantly higher affinity than it binds the insulin receptor. The IGF-2 receptor only binds IGF-2 and activates no intracellular signaling pathways, functioning only as an IGF-2 sequestering agent and preventing IGF-2 signaling.

## **IGF receptors**

Three receptors are included in the IGF family: insulin receptor (IR), insulin-like growth factor type 1 receptor (IGF-1R) and insulin-like growth factor type 2 receptor (IGF-2R). In addition, the presence of IGF-1R subtypes is described in the literature as hybrid receptors and atypical receptors (Moxham, Duronio et al. 1989; Soos, Whittaker et al. 1990; Siddle, Soos et al. 1994; Entingh-Pearsall and Kahn 2004). The hybrid receptors result from the dimerization of IGF-1R and IR hemi-receptors and have high affinity for IGF-1, while the affinity for insulin decreases dramatically. The atypical IGF receptors possess the ability to bind insulin as well as IGFs with relatively high affinity. Both IR and IGF-1R have tyrosine kinase activity and share 70% homology (Ullrich, Gray et al. 1986).

Insulin receptor is expressed in liver, adipose tissue and muscle. Insulin binding (to both IR-A and IR-B) induces the receptor activation leading to glucose uptake and inhibition of gluconeogenesis in the liver (White and Kahn 1994). Many factors appear to contribute to differences in IGF-1R and IR signaling, including the different patterns of receptor expression, kinetics of ligand binding, recruitment of signaling intermediates and effects on gene expression (De Meyts, Christoffersen et al. 1995; Siddle, Urso et al. 2001; Mulligan, Rochford et al. 2002). Nevertheless, there is significant cross-talk between these two receptors (Nakae, Kido et al. 2001). In contrast to the well-studied IR and IGF-1R, the IGF-2R, also known as cation-dependent mannose-6-phosphate receptor, has no tyrosine kinase activity and its biological function has still to be clarified. IGF-2/M6P receptor is a single chain polypeptide with a short cytoplasmatic domain that binds two types of ligands: non-M6P-containing ligands or IGF-2 and M6P-containing ligands (lysosomal enzymes) (Morgan, Edman et al. 1987; Braulke 1999; Macdonald and Byrd 2003).

## **PDGF receptors**

The two PTKs, PDGFR and Kit, are members of the PDGFR family. Two genes encoding PDGFR- $\alpha$  and PDGFR- $\beta$  have been identified and both receptors are activated by a ligand homo and/or heterodimer consisting of four different polypeptide chains PDGF-A, -B, -C, and PDGF-D. This in turn leads to receptor dimerization with three possible configurations:  $\alpha\alpha$ ,  $\beta\beta$ ,  $\alpha\beta$  (Shih and Holland 2006).

PDGFRs and PDGFs have important roles during embryonal development (Westermarck, Heldin et al. 1995; Maher, Furnari et al. 2001) and its overexpression has been linked to different types of fibrotic disorders and malignancies. Both the PDGF receptors and its ligands are co-expressed mainly in glioblastoma and other human astrocytotic brain tumors, whereas normal brain tissue does not express these proteins (Guha, Dashner et al. 1995; Westermarck, Heldin et al. 1995; Lokker, Sullivan et al. 2002; Shih and Holland 2006). Other tumor types are cited too, such as ovarian cancer (Dabrow, Francesco et al. 1998) and prostate cancer (Fudge, Wang et al. 1994). In addition, PDGF family may function in autocrine stimulation of tumor cells, regulation of interstitial fluid pressure, and angiogenesis.

## EGF receptors

The EGFR family of PTKs comprises four members: EGFR or ErbB1 or HER-1 (human epidermal receptor); ErbB2 or HER-2/neu; ErbB3 or HER-3; ErbB4 or HER-4 (Leahy 2004). The ligands, referred to as EGF-related peptide growth factors, are divided in 3 groups based on their affinity for one or more ErbB receptors: Epidermal Growth Factor, Transforming Growth Factor  $\alpha$ , and Amphiregulin bind to EGFR, Betacellulin, Heparin-binding Growth Factor, and Epiregulin have dual specificity for EGFR and ErbB4, Tomoregulin and Neuregulins or Heregulins with two subgroups: neuregulin1 and 2 which bind to ErbB3 and ErbB4 and neuregulin 3 and 4 which bind to ErbB4.

Specific for this family of receptors is the process of ligand-induced dimerization. Four domains are described in the extracellular part of the ErbB receptors. Their conformation dictates whether or not the kinase is in an active or inactive state. HER-2 has a particular conformation of the four domains that does not allow any ligand binding but it is well known that HER-2 acts as a co-receptor for the other EGFR family members. The ErbB family is one of the most studied therapeutic targets because of its extensive involvement in tumor biology (Bianco 2004; Gross, Shazer et al. 2004; Roskoski 2004; Normanno, Bianco et al. 2005).

### 1.3. Growth factor receptors tyrosine kinases in brain tumours

It is now well established that growth factors and growth factor receptor-mediated signaling pathways are important in transformation process in many forms of cancer, including brain tumours. The combination and accumulation of the altered oncogenes (like GFR-TKs) and tumour-suppressor genes involved are characteristic for gliomas. The progression of low-grade gliomas into more aggressive tumours is supported by the abnormal function of several GFR-TKs.

IGF-1, -2 and IGF-1R are important for the normal growth and differentiation of CNS structures. IGF-1 and IGF-2 are also potent mitogens for numerous solid malignancies, including gliomas. Expression of IGF-1, IGF-2 and IGF-1R has been demonstrated in gliomas, meningiomas and medulloblastomas and may be indicative of an autocrine stimulatory mechanism in these tumours. Therapy directed against the IGF-1/IGF-1R signaling pathway in gliomas is still under development. The only therapeutic strategies that have been utilized for gliomas, involve use of antisense IGF-1 or IGF-1R (Andrew et al. 2001).

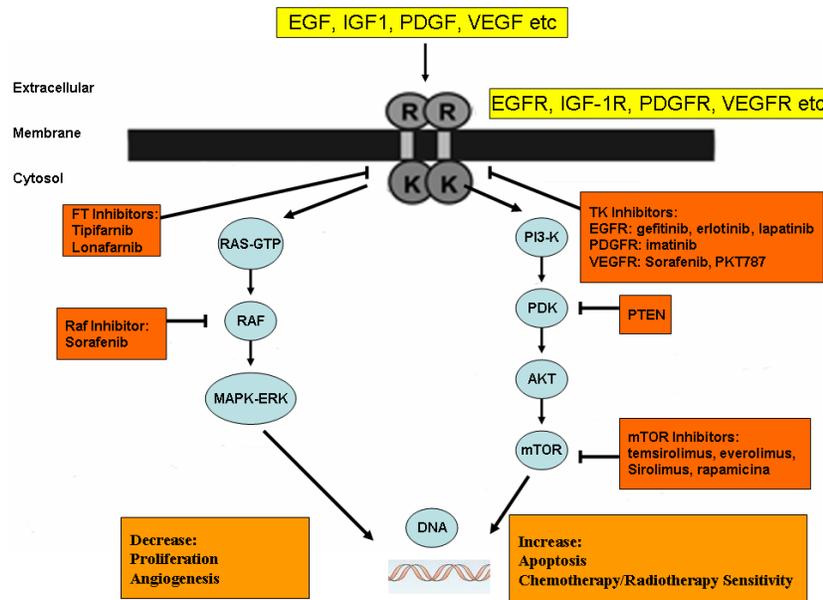
Several studies evaluating targeted molecular agents in glioblastoma are in clinical trials. Many studies were reported using Imatinib (a small-molecule inhibitor of the PDGFR) as monotherapy for treatment of glioblastoma (GB). The reasons underlying the efficacy of Imatinib as monotherapy are not fully understood. Kilic et al. demonstrated that imatinib significantly inhibited the growth of GB cell lines *in vitro* and *in vivo* (Kilic et al. 2000). The North American Brain Tumour Consortium (NABTC) and the European Organization for Research and Treatment of Cancer (EORTC) conducted clinical trials of imatinib in patients with GB. Only minimal activity was observed. Studies with more potent PDGFR inhibitors and improved CNS penetration, such as pazopanib and dasatinib, are planned.

Another potential target in GB is EGFR. Clinical studies using EGFR inhibitors (i.e. gefitinib and erlotinib) showed that these agents were well tolerated but responses were limited.

Furthermore, the responses with both agents have not been durable. The first significant advance in the treatment of the glioblastoma was the introduction of a new chemotherapeutic agent, temozolomide, highly attractive because of its low toxicity and easy application. Co-administration of erlotinib with temozolomide was shown to be safe in a phase I trial (Prados et al. 2006).

Trials with many of these agents in combination with radiotherapy are in progress. Early experiences with radiosensitizers in glioblastoma were disappointing, but there is renewed interest in this approach using targeted molecular agents. In addition to PDGFR and EGFR inhibition, now a day antiangiogenesis agents (against VEGFR) are used in clinical trials, as well as inhibitors against molecules involved in signal transduction from activated GFR-TKs, such as Ras (farnesyltransferase inhibitors), Raf and mTOR (Fig. 2). The intracellular signaling pathways are mediated by two major pathways, one is Ras/Raf/MAPK pathway and the other is PI3-K/Akt pathway.

Figure 2. Summary of agent used in targeted molecular therapy of malignant gliomas in clinical trials.



These agents used as targeted molecular therapy are generally well tolerated, but produce only modest benefits in patients with malignant gliomas. An explanation may be the complexity of the molecular abnormalities in malignant gliomas. Therefore, the improvement in our understanding of the reasons for the success or failure of clinical studies and to select the subgroups of patients who are most likely to respond to specific drugs is of major importance.

## **2. THE PRESENT STUDY**

### **2.1. Aims**

- To analyse the expression of PDGFR, IGF-1R membrane proteins and PI3-K, ERK1/2 intracellular tyrosine kinase proteins in a panel of glioma cell lines.
- To investigate the importance of PDGFR, IGF-1R and their common intracellular signalling for the proliferation and the response to ionizing radiation of glioma cell lines.

### **2.2. Materials and methods**

#### **Cells and cell culture**

The primary cell cultures 8, 18 and 38 were established from tumours at the Academic University Hospital in Uppsala according to standard procedures (Ponten and Westermarck 1978). The cell lines have been previously characterized (Hagerstrand, Hesselager et al. 2006). The cell lines MO59J and MO59K were purchased from American Type Culture Collection, USA. The cell lines were cultured in Minimum Essential Modified (MEM) medium containing 10% fetal bovine serum (FBS), 2 mM glutamine and antibiotic (100 IU/ml penicillin and 100 IU/ml streptomycin). The cells were grown in tissue culture flasks maintained in a 95% air/5% CO<sub>2</sub> atmosphere at 37°C in a humidified incubator.

#### **Materials**

Cell culture reagents were purchased from Invitrogen/Life Technologies, Inc. (Rockville, MD, USA). The antibodies against IGF-1R $\alpha$  (1H7), IGF-1R $\beta$  (C-20), PDGFR $\beta$  (958), PI3-K (D-4), ERK1/2 (C-16), JNK1 (FL), phospho-JNK1 (G-7) and actin (C-2) rabbit polyclonal IgG were purchased from Santa Cruz; anti-rabbit and anti-mouse IgG horseradish peroxidase-linked antibodies and ECL immunodetection reagents were purchased from Amersham Bioscience; normal goat serum was purchased from Dako, goat-anti-rabbit FITC was purchased from Jackson; 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) from Sigma-Aldrich; IGF-1R, PDGFR, PI-3K and ERK1/2 were inhibited by Tyrphostine AG1024, Tyrphostine AG1433, LY29004 and PD98059 respectively, and were purchased

from Calbiochem (La Jolla, CA). AG1024, AG1433, Ly294002 and PD98059 were diluted in DMSO to a stock concentration of 10mM and stored at -20°C. The DMSO concentration was below 0.1% when the inhibitors were added in the cultured medium. MTT Cell proliferation Kit was purchased from Roche Diagnostics GmbH (Mannheim, Germany) and Face JNK ELISA kit from Active Motif. Dominant negative (dnJNK1) plasmid was a kind gift from Dr Lynn Heasley (University of Colorado, Denver) and Lipofectamine™ 2000 Transfection Reagent was purchased from Gibco. Appropriate control groups with diluents only were included.

### **Cell treatment**

Cells at the same passages were used for experimental propose. The cell lines were cultured in MEM containing 10% fetal bovine serum (FBS), 2 mM glutamine and antibiotic (100 IU/ml penicillin and 100 IU/ml streptomycin). The cells were grown in 35-, 60-, 150-mm dishes or in 96-well culture plates, maintained in a 95% air/5% CO<sub>2</sub> atmosphere at 37°C in a humidified incubator. IGF-1R and PDGFR were inhibited by 10 μM AG1024 and AG1433 respectively, for different periods of times. The drugs (AG1024 and AG1433) were renewed after 48 h. The ionizing radiation was given in a 2, 4, 6, 8 or 10-Gy single-dose. To study the effects of IGF-1R activation on radioresistance, standard medium was replaced with serum-free medium containing 50 ng/ml IGF-1. For the analysis of JNK activation, standard medium was replaced with serum-free medium, serum-free medium containing 50 ng/ml IGF-1 or 50 ng/ml PDGF for different periods of times. Appropriate control groups with diluents only were included.

### **Western blotting**

Cells were lysed in lysis buffer [150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1% NP40, 1 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, and protease inhibitor mixture (Boehringer Mannheim Co.)] at 4°C. The lysate was centrifuged (12,000 g) for 10 min at 4°C to remove insoluble components. Protein was quantized by the Bio-Rad Dc protein assay. Equal amounts of protein were separated on SDS-PAGE 10% gels and transferred to an Immobilon-P PVDF membrane (Millipore). The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST), then incubated with primary antibody in 1% non-fat dry milk in TBST, followed by secondary antibody linked to horseradish peroxidase diluted in 1% non-fat dry milk in TBST. The ECL detection system for western blot analysis was followed according to the manufacturer's instructions for antibody detection.

## **Irradiation**

Cells were incubated in a 95% air/5% CO<sub>2</sub> atmosphere at 37°C in a humidified incubator in culture medium until 70% to 80% confluence. Cells were then irradiated in a culture medium at room temperature with 2, 4, 6, 8 or 10 Gy single-dose, using a <sup>137</sup>Cs radiation source.

## **Growth inhibitory assay**

To investigate the effect of IGF-1R, PDGFR, PI-3K and ERK1/2 inhibitors, GB cells were treated with the following drugs: 20µM AG1024, 20µM AG1433, 10µM LY294002 and 10µM PD98059 with or without concomitant exposure to a radiation dose of 2 or 8 Gy. After 72h the cell growth was evaluated by using MTT assay.

MTT assay is based upon the cleavage of the yellow tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to purple formazan crystals by metabolically active cells. 1x10<sup>3</sup> cells/w/200µl medium were seeded in 96-well culture plates, incubated for 8 h and exposed to different concentrations of tyrosine kinase inhibitors. After 72h of incubation 10 µl of MTT labelling reagent were added to each well and plates were incubated at 37°C for 4 h. Formazan products were solubilised and the optical density (OD) was measured at 595nm. Results were expressed as a percentage of that in untreated control cultures.

For cell growth assessment we used the MTT and the colony-forming assay. The growing inhibition effect of the drugs and the cellular sensitivity to radiation treatment was also determined from the loss of colony-forming ability. Cells were trypsinized, re-suspended in complete medium and counted. Known numbers of cells were cultured on 60-mm agar coated tissue culture dishes and subjected to treatment. The number of cells per dish initially inoculated, varied with the irradiation dose so that the number of colonies surviving was in a range that could be counted conveniently. Cells were allowed to grow until the surviving cells produced macroscopic colonies (of more than 50 cells) and after staining with methylene blue the colonies were counted under magnification.

## **Transfection**

18 and 38 HGG were plated at subconfluent density, transiently transfected with 2mg/ml DNA using Lipofectamine 2000 as described by the manufacturer. Five hours after transfection, the media were replaced with MEM containing 10% FBS and the cells were subjected to different experiments.

## **Flow Cytometry for protein detection**

The human glioblastoma cells were trypsinized, washed with FACS buffer (PBS containing 3% FBS and 0.02% NaN<sub>3</sub>), blocked in 10% FBS and stained with anti-IGF-1R Ab or anti-PDGFR Ab, working dilution 1:10 for 40–45 min at 4°C. The cells were then stained with FITC-conjugated second antibody for 30 minutes on ice. Relevant isotype controls were used. Cells were analysed using a FACS Calibur flow cytometer (BD Biosciences, Heidelberg, Germany) and the CellQuest™ software. For each measurement 100,000 events were acquired.

## **Immunofluorescence**

Double staining for nuclei (DAPI) and JNK1 or IGF-1R $\beta$  or PDGFR- $\beta$  (FITC) was performed on 18 and 38 paraffin embedded cells. Deparaffinized and rehydrated 4- $\mu$ m sections were microwave heated 6 min in 0.1 M citrate buffer (pH 6.0) for antigen retrieval. Antibodies were diluted in TBS containing 1% bovine serum albumin and 0.05% sodium azide. Sections were blocked for nonspecific antibody binding with normal goat serum (30 min). After the incubation with the primary antibody, rabbit-anti-JNK1 at 4°C, overnight, cells were incubated with 1:100 goat anti-rabbit FITC for 40 min. Nuclei were co-stained with 1  $\mu$ g/ml DAPI for 10 min. Between all incubations the sections were thoroughly rinsed in PBS + 0.01% Tween. Incubation with PBS instead of primary antibody was used as a negative control for the detection system. The slides were evaluated using a fluorescence microscope (Olympus BX60, Tokyo, Japan) equipped with a digital camera (Sony DKC-5000, Tokyo, Japan) and filter cubes was used to document specific FITC, and DAPI fluorescence images which were edited and overlaid by Adobe Photoshop 6.0.

## **Elisa**

Face Elisa for JNK phosphorylation was done according to the manufacture. Cells were cultured in a 96-well plate, treated and then assayed with the anti-phospho JNK kinase antibody, using the following procedure: at the desired time, cells were fixated in formaldehyde 4% for 20 min at room temperature. A buffer containing 1% H<sub>2</sub>O<sub>2</sub> and 0.1% sodium azide was added for another 20 min, followed by 1 h incubation with antibody blocking buffer, both steps at room temperature and with mild agitation. The primary antibody was added in 1:200 dilutions, overnight, at 4°C. The 1:1,000 diluted HRP conjugated secondary antibody was incubated for 1 h at room temperature. Each step described above was followed by a 15 min of washing. The colorimetric reaction was started by adding 100  $\mu$ l of developing solution, protected from direct light. After 10–15 min, when the darkest stained wells were medium to dark blue, the reaction is stopped with 100  $\mu$ l stop solution. The absorbance was read within 5 min at 450 nm wavelength. In the same experiment, the cell number was quantified by using crystal violet staining. After 30 min incubation at room temperature with 100  $\mu$ l of crystal violet stain, 100  $\mu$ l of 1% SDS were added per well and incubated on an orbital shaker. The absorbance was read 1 h later at 595 nm wavelengths. The JNK1 phosphorylation was evaluated relative to cell number.

### 2.3. Summary of the papers

In paper I, we evaluated the cell surface expression of IGF-1R, PDGFR membrane receptors and PI3-K and ERK1/2 that are the key proteins in the receptors common intracellular pathway, in five primary glioblastoma cell lines (MO59J, MO59K, 8, 18 and 38).

Our results indicated that the IGF-1R and PDGFR membrane tyrosine kinase proteins and PI3-K and ERK1/2 intracellular tyrosine kinase proteins could be detected in the GB tumour cell lines, excepting a low level of PDGFR and ERK1/2 in MO59J cell line. We also investigated the importance of these proteins, with respect to glioblastoma cells proliferation and the response to radiation. To this end, we tested the effects of pharmacologic inhibition of IGF-1R, PDGFR, PI3-K and ERK1/2 using small molecule inhibitors alone or in combination with ionizing radiation on human GB cell lines.

In general, the small molecule inhibitors used in this study have only modest or not antitumour activity on glioblastoma cells and therefore their combination with other therapy modalities was evident. Interaction between small inhibitors and radiation was mostly additive or subadditive, synergistic interaction was found in few analysed combinations. One explanation may be the interaction of the IGF-1R with PDGFR in maintenance the intracellular signalling activated (paper I). Therefore, a therapeutic strategy of co-targeting both IGF-1R and PDGFR has been taken into account.

In two glioblastoma cell lines (18 and 38), we found that dual targeting of IGF-1R and PDGFR increased cell death in comparison to the inhibition of either receptor alone. In addition, co-inhibition of IGF-1R and PDGFR increased radiosensitivity in 18 cells but failed to intensify the effect of radiation in 38 cells.

In glioblastoma cells, radiation induced cell death has been connected to the activation of c-Jun-NH2-terminal kinase-1 (JNK1). We found that JNK1 was weakly expressed in 38 cells while it had an elevated expression in 18 cells. Exposure to ionizing radiation induced JNK1 activation in 18 cells only, suggesting that in this cell line radiation-activated JNK1 may provide an anti-proliferative signaling parallel to receptors co-targeting. To test this hypothesis, glioblastoma cells were treated with dominant negative JNK1 (dnJNK1) and the response to radiation was assayed in the presence or absence of receptors co-inhibition. Indeed, dnJNK1 protected 18 cells against  $\gamma$ -radiation induced cell death. The dnJNK1 treatment did not influence radiation response of the 38 cell line, which expressed low levels of JNK1 (paper II).

## 2.4. Discussion

The tyrosine kinase inhibitory activities of anilinoquinazolines were first described in 1994 by three separate groups (Fry et al. 1994; Ward et al. 1994; Osherov et al. 1994). Later, several ATP analogues of the quinazoline and pyridopyrimidine family have been described to interact with the conserved ATP-binding site of the kinase domain of the growth factor receptors (Palmer et al. 1997; Smaill et al. 1999). This theoretical stage was changed in convincing fashion in 1998, when the first pyridopyrimidine small molecule targeted, Gleevec, has demonstrated clinical efficacy in chronic myelogenous leukemia and gastrointestinal stromal tumor (Carroll et al. 1997; Dan et al. 1998; Joensuu et al. 2001; Deiningner et al. 2003; Nashed et al. 2003). The Phase 1 clinical trials in CML patients showed good response and well tolerance of the drug and phase 2 and 3 trials confirmed the phase 1 results (Druker et al. 2001; Talpaz et al. 2002; Morishima et al. 2004; Schmidli et al. 2005).

Now days, molecular targeted therapy holds the promise of providing more effective treatment modalities with minimal toxicity, but the development of targeted therapy for gliomas has been particularly challenging. Gliomagenesis is driven by several signaling pathways that are differentially activated or silenced in complicated signal interconnections.

The present study demonstrates the heterogeneous expression of two tyrosine kinase growth factor receptors (IGF-1R and PDGFR) in a panel of five glioblastoma cell lines (MO59J, MO59K, 8, 18 and 38). The level of IGF-1R was moderate in four cell lines (MO59J, MO59K, 18 and 38) and high in one cell line (8). PDGFR was elevated expressed in 8 cell line, moderate expressed in 18, 38 and MO59K and very low expressed in MO59J cells. The level of PDGFR was lower than the level of IGF-1R in all cell lines, excepting 8 cell line that expressed equal amount of both receptors. The importance of IGF-1R and PDGFR in glioblastoma cell proliferation was investigated using AG1024 and AG1433, specific drug inhibitors of these signaling molecules. Inhibition of IGF-1R by AG1024 reduced glioblastoma survival to a low extent. Even if the level of PDGFR was lower than the level of IGF-1R, treatment with AG1433 was more effective in killing the GB cells but, in general, the responses with both agents were modest. For this reason we used several strategies to improve the effect of tyrosine kinase receptor-targeted therapy: (I) inactivation of PI3-K and ERK1/2, the key proteins in the common intracellular IGF-1R/PDGFR signal transduction, (II) IGF-1R-PDGFR co-targeting, (III)

inactivation of IGF-1R, PDGFR receptors and their intracellular signalling in combination with  $\gamma$ -radiation.

Inhibition of PI3-K or ERK1/2 by LY294002 and PD98059, respectively, provoked very weak or not cytotoxic effect on glioblastoma cells. Studies from many research groups that analyzed the importance of the PI3-K and ERK1/2 inactivation for growth and survival of various cancer types (Chakravarti et al. 2002; Sanson et al. 2006; Sourbier et al. 2006; Ozaki 2007; Tai et al. 2007; Wong et al. 2007) provided contradictory conclusions suggesting differences in cancer cells phenotype. Since the glioblastoma cell lines used in this study expressed both PI3-K and ERK1/2 but were refractory to their inhibitors, we interpreted this observation to mean that one or more unknown signal cascade, other than PI3-K/AKT and MEK/ERK pathways, could be induced by receptors activation.

We also tested the ability of the small molecule drug inhibitors as radiosensitizers. Of the glioblastoma cell lines analysed in this study, MO59J was previously shown to be radiosensitive and found that the most combined variants failed to induce synergy in killing glioblastoma cells.

IGF-1R and PDGFR co-inhibition were analysed in two cell lines (18 and 38). Interestingly, receptors co-targeting provide to be effective in inducing cytotoxicity in both cell lines. However, the IGF-1R and PDGFR concomitant inhibition increased radiation induced cell death in 18 cells but failed to intensify the radiation effect in 38 cells. In addition, in 18 cells, radiation induced cell death has been connected to the activation of c-Jun-NH2-terminal kinase-1 (JNK1).

In conclusion, the combination of different targeted agents, or targeted agents and radiotherapy, seems to be a better treatment option than single agent therapy, with respect to the evident molecular heterogeneity of brain tumours. Nevertheless, a detail molecular understanding of the molecular pathogenesis of malignant gliomas is necessary to design an effective therapy against this stage of the disease.

### 3. ACKNOWLEDGEMENTS

This work was founded by grants from: the Swedish and Stockholm Cancer Societies, the Swedish Heart and Lung Foundation and Swedish Society of Medicine.

I would like to express my sincere gratitude to everyone who have contributed to my research and made it an enjoyable time. Especially I would like to thank to:

**Anica Dricu**, my head supervisor, who guided my first steps in the world of research and accepted me in her research group. I do thank you for the continuous support, for being a great supervisor and a friend too.

**Rolf Lewensohn**, my co-supervisor, who welcomed me in his lab, for his constructive criticism and advices regarding my projects.

**Janne Lehtiö**, co-supervisor, for general support.

All my collaborators and co-authors:

**Daria**, my closest collaborator and dearest friend in Anica's group. All my colleagues in the group for their help, friendship and very good and fruitful collaboration in the lab: Simona, Anna, Laura, Oana, Roxana, and especially **Raluca**.

Former and present members in Rolf's group for a very pleasant and enjoyable atmosphere: Irina, Josefin, Dali, Betszy, Reidoun, Luigi, Sara, Lukas, Maria, Kristina, Petra, Brigitta S, Annica S, Meeri, Barbro, Owe, Leif. My special thoughts to our assistants, Birgitta Mörk and Lillemor Laurén, for their technical support and great company in the lab.

Evi Gustavson Kadaka, always of great assistance with all administrative work and Juan Castro for introducing me into the knowledge about FACS techniques.

My colleagues at the CCK ground: Allan, Sissi, German and Pega from Freidoun Albertioni's group, Bim, Annica J, Jesper and Annelie from Margareta Edgren's group, Baldour, Lotta, John, Helena, Ninib and Agnes from Per Kogner's group, Amos, Pawan and Parisa from Peter Biberfeld's group, Maria H., Maria B., Alexandra, Walid and Richard from Stig Linder's group, Malin, Ali and Torsten.

The chairman of the Department of Oncology-Pathology, Karolinska Institute, **Tina Dalianis**, for providing an excellent work environment.

All my friends at KI: Daiana and Radu, Anca, Anki Popescu-Greaca, Gazal, Eli and Amir. Thank you for all the good moments. My dear friends Nader, Monica and Alex, Doru, Gabi Dosoftei for your stable friendship through the years.

My family has all my gratitude and love. I thank you all for your unconditioned love and help when I was in need and for being always by my side: my mother Matilda (toata dragostea mea, mami scumpa), my father Filip who no longer is among us (Dumnezeu sa te odihneasca, tati drag), my parents-in-law

Ludmila (maman) and Matei for embracing me so heartily as their dotter, my dear sister Lena, my two beloved nephews Andreea and Aditzu, and my dear little brother Sorin, always close to my heart.

My dear husband **Andrei** for your love and support, always believing in me, for making my life complete.

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