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THE USE OF IGF-IR INHIBITORS IN CANCER THERAPY - A POTENTIAL APPROACH FOR SENSITIZING TUMOR CELLS TO IONIZING RADIATION

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To my parents, Ana-Maria and Marius

Doctoral thesis

The use of IGF-IR inhibitors in cancer therapy - a potential approach for sensitizing tumor cells to ionizing radiation

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ABSTRACT

This study focused on insulin-like growth factor I receptor (IGF-IR), considered as a promising therapeutic target for cancer treatment. We were in particular interested in the involvement of IGF-IR in tumor cells response to radiation and we tried to provide the rationale for a combined therapy of IGF-IR inhibitors with radiation in order to improve the therapeutic outcome.

Lung cancer is the leading cause of cancer-related mortality for both men and women. Approximately 75% to 85% of all lung neoplasms are non–small cell lung cancers (NSCLC) and thoracic radiotherapy has been a common treatment for patients with locally advanced NSCLC. Malignant gliomas represent among the most treatment-refractory tumors encountered by clinicians. For several decades, the standard of care has represented maximal total resection, followed by radiation ± nitrosurea-based chemotherapy. Central nervous system and lung cancers constitute sites that are particularly difficult to irradiate due to a large number of conceptual difficulties, allowing them to be considered as 2 particularly interesting study models.

For our *in vitro* studies we chose NSCLC and high grade glioma (HGG) cell lines.

Our research demonstrated IGF-IR expression in a panel of cell lines that represented both tumor types. IGF-IR proved to be of importance in the survival of NSCLC cell lines and the receptor inhibition decreased cell proliferation by activating different apoptotic pathways. IGF-IR inhibition combined with irradiation induced a higher degree of cell death, in association with an accumulation of cells in G2 phase and a concurrent reduction within the S phase, and thus indicated that IGF-IR was involved in the modulation of radiosensitivity in NSCLC cells. In contrast, in the HGG cell lines results showed the occurrence of a cross talk between IGF-IR and platelet-derived growth factor receptor (PDGFR). Dual targeting of IGF-IR and PDGFR caused synergy in cell death and increased radiosensitivity.

In an attempt to elucidate the IGF-IR-mediated signal transduction pathways in response to radiation we observed a complex mechanism that was not only tumor type dependent but also cell type dependent. For the NSCLC cells we were able to demonstrate for the first time that ionizing radiation activated IGF-IR. IGF-IR inhibition induced radiosensitivity via p38 kinase but not PI3 kinase and this was a novel mechanism involving nuclear Ku86. In HGG cell lines radiosensitisation was found after dual inhibition of IGF-IR and PDGFR and it was dependent on JNK activation.

Our results suggest that IGF-IR targeting might be of importance when combined with radiation. In this context it is obvious that further characterization of the complex biological processes underlying radiation response is necessary in order to allow for biologically optimized and patient individualized radiation therapy.

LIST OF PUBLICATIONS

I. **Cosaceanu D**, **Carapancea M, Alexandru O, Budiu R, Martinsson HS, Starborg M, Vrabete M, Kanter L, Lewensohn R and Dricu A.**
Comparison of three approaches for inhibiting insulin-like growth factor 1 receptor and their effects on NSCLC cell lines in vitro
Growth Factors, 2006, in press

II. **Cosaceanu D, Carapancea M, Castro J, Ekedahl J, Kanter L, Lewensohn R and Dricu A**
Modulation of response to radiation of human lung cancer cells following insulin-like growth factor 1 receptor inactivation
Cancer Letters, 2005, 222, 173-181

III. **Cosaceanu D**, **Budiu R, Carapancea M, Castro J, Lewensohn R and Dricu A**
Ionizing radiation activates IGF-1R triggering a cytoprotective signaling by interfering with Ku-DNA binding and by modulating Ku86 expression via a p38 kinase dependent mechanism
Oncogene, 2006, in press

IV. **Carapancea M, Cosaceanu D, Budiu R, Kwiecinska A, Tataranu L, Ciubotaru V, Alexandru O, Bäcklund M, Lewensohn L 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
(manuscript submitted for publication)

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LIST OF ABBREVIATIONS

A-loop	Activation loop of the receptor
AS	Antisense
ATM	Ataxia teleangiectasia mutated
ATP	Adenosine tri-phosphate
BsAb	Bispecific antibody
DSB	DNA double-strand break
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular regulated kinase
FDA	Food and Drug Administration
GH	Growth Hormone
GHRH	Growth-hormone releasing hormone
HER	Human epidermal receptor
HGG	High grade glioma
HRR	Homologous recombination repair
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IGF-IIR	Insulin-like growth factor receptor II
IGF-IR	Insulin-like growth factor receptor I
IR	Ionizing radiation
IRS	Insulin receptor substrate
JNK	c-Jun NH2-terminal protein kinase
mAb	Monoclonal antibody
MAPK	Mitogen activated protein kinase
MEK	MAP/ERK kinase
NHEJ	Non-homologous end joining repair
NRTK	Non- Receptor tyrosine kinase
NSCLC	Non-small cell lung cancer
ODN	Oligonucleotides
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor

PI3-K	Phosphatidylinositol-3´-kinase
ROS	Reactive oxygen species
RT	Radiotherapy
RTK	Receptor tyrosine kinase
SAPK	Stress activated protein kinase
SH	Src-homology
Shc	Src homology 2 domain containing transforming proteins
si-RNA	Short interfering RNA
SSB	Single-strand break
TKI	Tyrosine kinase inhibitor
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1 BACKGROUND

1.1 TARGETED THERAPIES FOR CANCER TREATMENT

1.1.1 Introduction

One of the first principles one learns in medical school is "A doctor should treat the patient not the disease". Beyond the classical understanding this is more than ever a reality in clinical practice in general and in oncology in particular. Many advances in cancer treatment have occurred lately. Treatment decisions are now influenced by the molecular profile of the tumor, which emerge as an approach of importance for the patients' response to therapy.

In November 1997, the Food and Drug Administration's (FDA) approval of Rituxan, an anti-CD20 monoclonal antibody (mAb), for the treatment of patients with relapsed or refractory follicular B-cell non-Hodgkin's lymphoma, represented the first targeted drug approved for the treatment of human malignancy and ushered the modern era of targeted therapeutics for cancer (http://www.fda.gov/). Decades of intensive basic research were necessary to gain a better understanding of the molecular alterations involved in carcinogenesis, fostering the emerging field of molecular therapeutics.

The basic principle of molecular therapeutics is to exploit the molecular differences between normal and cancer cells in order to allow the development of **targeted therapies**.

As most human cancers result from aberrations in cell signaling pathways, many of these aberrations represent potential pharmacological targets. Ideally, the targeted molecule should be exclusively expressed in the cancer cells, be the driving force of the proliferation of the cancer cells, and be critical to their survival. These criteria are hard to fulfill, therefore translating of a therapy from the research bench to clinical practice is not an easy task. However, a large number of molecular targets are currently being explored, both pre-clinically and in clinical trials. The major groups of **targeted therapies** include:

- inhibitors of growth factor receptors
- inhibitors of intracellular signal transduction
- cell-cycle inhibitors

- apoptosis-based therapies
- antiangiogenic compounds

The targeted therapies proved to be a very successful approach in some instances. The best example is represented by Imatinib (Gleevec) which significantly improved the outcome for patients with chronic myelogenous leukemia (Quintas-Cardama and Cortes 2006). Nevertheless it has its limitations (Azam and Daley 2006). Due to the complex molecular changes that occur during cancer progression, targeting an individual alteration is often not enough to induce a sustainable significant therapeutic response. The likely solution to this problem is to combine different targeted therapies. We have an increased understanding of the critical molecular changes driving tumor development. We have an improved ability to genotype patient's tumor, which sooner or later will be used in a large scale for tumor diagnostics. We have many potential drug targets becoming available.

Targeted therapies have proofed their tremendous potential which emphasizes that continued research in this field is imperative.

1.1.2 Protein Tyrosine Kinases, receptors and non-receptors

Protein tyrosine kinases are a large family of signaling proteins. They are involved in the regulation of normal cellular processes and required for vital functions of multicellular organisms, including cell growth, differentiation, cell-to-cell signaling, motility, and adhesion. Out of the 518 genes in the human genome encoding for kinases, 90 are identified as tyrosine kinases genes. A total of 32 encode for non-receptor tyrosine kinases (NRTKs) that can be distributed into 10 subfamilies and 58 encode for receptor tyrosine kinases (RTK), distributed into 20 subfamilies (Hubbard and Till 2000; Robinson, Wu et al. 2000).

The NRTKs are located within the cytoplasm or the nucleus and include among others Src, the Janus kinases, and Abl. The NRTKs are integral components of the signaling cascades triggered by RTKs and by other cell surface receptors such as G protein-coupled receptors and receptors of the immune system. Besides the tyrosine kinase domain they contain other domains that mediate protein-protein, protein-lipid, and protein-DNA interactions. The most commonly found protein-protein interaction domains in NRTKs are the Src homology 2 (SH2) and 3 (SH3) domains. The

activation and regulation of NRTKs vary among different members of the family (Hubbard and Till 2000; Robinson, Wu et al. 2000).

The RTKs are located within the cell membrane and function as receptors for extracellular signals. They consist of an extracellular part involved in binding polypeptide ligands. The highly conserved kinase domain is located in the intracellular part of the RTKs, flanked by a juxtamembrane domain and a carboxyterminal end (Ullrich and Schlessinger 1990). As a general mechanism, the RTKs activation is triggered by the ligand-binding-induced dimerization of the receptors, whereby several tyrosine residues in the intracellular domain are autophosphorylated. The enhancement of the tyrosine kinase activity leads to recruitment of signaling proteins, which in turn activates the RTK-dependent downstream signaling cascade (Hubbard and Till 2000).

The role of RTKs and their ligands in cancer development and progression is well established. Oncogenic RTKs are implicated in the pathogenesis of cancer by stimulating uncontrolled cell growth, inhibition of apoptosis and differentiation and imparing cellular adhesion (Zwick, Bange et al. 2002; Bennasroune, Gardin et al. 2004; Gschwind, Fischer et al. 2004). Major mechanisms underlying deregulation of RTKs activity in cancer are:

- Constitutive activation through amplification, overexpression or mutations of the RTKs (Zwick, Bange et al. 2001)
- Activation of autocrine growth factor loops (Zwick, Bange et al. 2001)

Some of the RTKs are currently used as clinical targets for the treatment of certain types of cancer. The following description includes a selection of the most studied receptors as therapeutic targets.

ErbB receptors

The human epidermal receptors (HER) or ErbB family is comprised of at least 10 different ligands and four distinct receptors (Leahy 2004). The ligands, referred to as epidermal growth factor (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 α, 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

The receptors are: Epidermal Growth Factor Receptor (EGFR) or ErbB1 or HER1; ErbB2 or HER2/neu; ErbB3 or HER3; ErbB4 or HER4. Specific for this family of receptors is the process of ligand-induced dimerization. Four domains are described in the extracelullar part of the ErbB receptors. Their conformation dictates whether or not the kinase is in an active and inactive state. HER2 has a particular conformation of the four domains that does not allow any ligand binding but induces an enhanced and permanent capacity of dimerization, thus being the preferred partner for dimerization of all the other ErbB receptors.

The ErbB family is extensively involved in tumor biology and it is one of the most studied therapeutic targets (Bianco 2004; Gross, Shazer et al. 2004; Roskoski 2004; Normanno, Bianco et al. 2005). Many drugs have been developed and they are widely used in many types of cancers.

- Cetuximab (Erbitux) is a recombinant human/mouse chimeric mAb that binds specifically to the extracellular domain of EGFR. Approved in February 2004 for the treatment of EGFR-expressing metastatic colorectal carcinoma, as a single agent or in combination with irinotecan, since March 2006 it has got a second treatment indication. It is used in combination with radiation therapy for the treatment of locally or regionally advanced squamos cell carcinoma of head and neck or for the treatment of recurrent or metastatic disease as a single agent.
- Trastuzumab (Herceptin) is a recombinant DNA-derived humanized mAb that selectively binds with high affinity to the extracellular domain of HER2. Since September 1998 it has been indicated as a single agent for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein and who have received one or more chemotherapy regimens for their metastatic disease. Since february 2000 Herceptin has been used in combination with paclitaxel for treatment of patients without any previous chemotherapy and metastatic breast cancer.
- Eroltinib (Tarceva) is a tyrosine kinase inhibitor (TKI) for EGFR that belongs to the quinazolamine group. The clinical antitumor activity is not fully

characterized. Approved in November 2004 for the treatment of locally advanced or metastatic NSCLC, since November 2005 has been used too in combination with gemcitabine as a first line treatments of patients with locally advanced, unresectable or metastatic pancreatic cancer.

- Gefitinib (Iressa) is a TKI for EGFR that belongs to the quinazolamine group. As for Tarceva, the clinical antitumor activity is not fully characterized. It was approved in May 2003 as monotherapy for the treatment of patients with locally advanced or metastatic NSCLC after chemotherapy failure.

VEGFRs

The vascular endothelial growth factor (VEGF) family of angiogenic growth factors includes six secreted glycoproteins referred to as VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, placenta growth factor-1 and-2 and three receptors: VEGFR-1, VEGFR-2, and VEGFR-3. In addition, neuropilin-1 and -2 are co-receptors for specific isoforms of VEGF family members and increase the binding affinity of these ligands -to their respective receptors (Ferrara, Gerber et al. 2003).

VEGF family members are key regulators of tumor angiogenesis and they are critically involved in the pathogenesis of human cancers. Briefly, VEGFR-1 is a potent, positive regulator of physiologic and developmental angiogenesis and is thought to be important for endothelial cell migration and differentiation (Hiratsuka, Maru et al. 2001). Recently, the classical concept that VEGFRs are present only on endothelial cells has been improved. VEGFR-1 is not only present but it is also functional on human colorectal cancer cells, involved in tumor progression and metastasis (Fan, Wey et al. 2005). VEGFR-2 mediates the majority of the downstream effects of VEGF-A, including vascular permeability, endothelial cell proliferation, invasion, migration, and survival (Dvorak 2002). VEGFR-3 is involved in lymphangiogenesis, and its expression has been associated with the dissemination of tumor cells to regional lymph nodes (Stacker, Caesar et al. 2001).

Because of their well-established role many agents that selectively target this pathway are currently available and comprehensive reviews covering the subject are published (Cardones and Banez 2006; Morabito, De Maio et al. 2006).

Bevacizumab (Avastin) is a recombinant humanized monoclonal IgG1 antibody that binds and inhibits the biological activity of human VEGF. It was approved by the

FDA in February 2004, for colorectal cancer treatment in combination with chemotherapy drugs: 5-FU (5-fluorouracil), leucovorin, and oxaliplatin or irinotecan. Bevacizumab demonstrated activity in other types of cancers too, such as renal cell cancer and ovarian cancer when used as a single agent, and in lung cancer and breast cancer when combined with chemotherapy.

PDGFRs

The platelet-derived growth factor receptor (PDGF) family contains five described ligands, PDGF-AA, -BB, -AB, -CC, and-DD, which are homo- or heterodimers of four different polypeptide chains PDGF-A, -B, -C, and-D. They activate preferentially one of the three described receptors: PDGFRα by the PDGF-AA, AB, -BB, and -CC; PDGFRβ by PDGF-BB and -DD; PDGFRα/β by the PDGF-AB, -AB, and-CC (Shih and Holland 2006).

PDGFs and PDGFRs are critically involved in normal embryogenesis and organ development (Westermark, Heldin et al. 1995; Maher, Furnari et al. 2001). Various research papers suggest an important role for PDGFR overexpression and activation in the transformation process of glial tumors (Guha, Dashner et al. 1995; Westermark, 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). PDGF family involvement in tumor development is through a number of processes including autocrine stimulation of cancer cells, angiogenesis stimulation and control of tumor interstitial pressure. As for the RTKs, several types of PDGFs antagonists are described (Pietras, Sjoblom et al. 2003; Board and Jayson 2005). One example is Imatinib (Gleevec), a TKI that is clinically used for the inhibitory effects on c-kit and Abl. It also inhibits PDGFR α and β and it is under current evaluation in several clinical trials for these effects (Raymond E 2004; Wen PY 2004).

Although other RTKs are altered in human malignancies, the further discussions shall be limit to IGF-IR, the main subject of this research project.

1.2 IGF SYSTEM

IGF-IR is a member of the insulin-like growth factor (IGF) family, an important growth factor system organized in a complex regulatory network that operates throughout the organism, at cellular and subcellular levels. The IGF system is involved in development of the organism and maintenance of normal cell function. Moreover, it is implicated in diverse pathophysiological conditions with a particularly important role in cancer.

The IGF story began 50 years ago when the first report was published about a serum factor that mediated cartilage sulfation and longitudinal bone growth activity induced by somatotrophic hormone (Growth Hormone). It was named "sulfation factor" (Salmon and Daughaday 1957). Further investigations revealed more of the biological properties of that new group of substances renamed first nonsuppressible insuline-like activity (NSILA) I and II (Burgi, Muller et al. 1966; Froesch, Muller et al. 1966; Jakob, Burgi et al. 1967; Rinderknecht and Humbel 1976) and then somatomedin (Daughaday, Hall et al. 1972). In the late 70 's they received their current designation of Insulin-like growth factor I and II (Rinderknecht and Humbel 1978; Rinderknecht and Humbel 1978).

We now have a comprehensive description of the IGF system structure and function (Baserga, Hongo et al. 1997; LeRoith and Roberts 2003; Pollak, Schernhammer et al. 2004; Denley, Cosgrove et al. 2005; Russo, Gluckman et al. 2005).

A simplified representation of the IGF system includes: the IGF ligands, the IGF receptors, the IGFBPs and it is summarized in Figure 1.

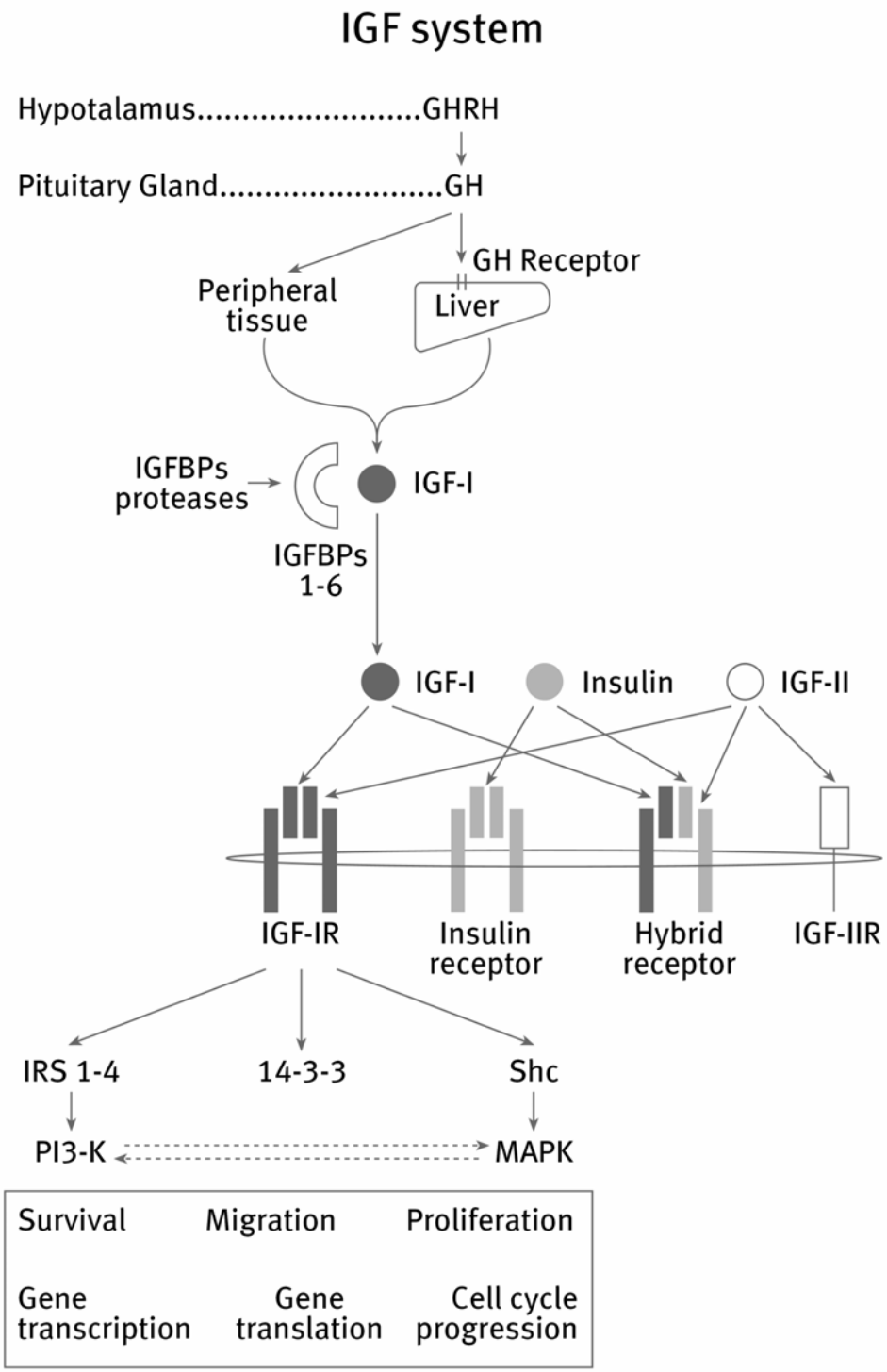

Figure 1. An overview of the IGF system

1.2.1 The IGF ligands

The three major ligands are insulin, IGF-I and IGF-II. The IGFs are major growth factors, while insulin mainly regulates glucose uptake and cellular metabolism. The IGFs consist of A, B, C and D domains and they are synthesized as prohormones. The IGFs contain a C-terminal E peptide that is cleaved in the Golgi apparatus during secretion. There is a considerable structural similarity among the ligands, with 70% sequence homology between IGF-I and IGF-II. Domains A and B of IGF-I and IGF-II have respectively 43% and 41% homology with the insulin counterparts. The C domain of IGFs shares no homology with the C peptide region of the human proinsulin and it is not removed during the prehormone processing. Therefore mature IGFs are single chain polypeptides with a molecular mass of 7.5 KDa for IGF-I and 7.4KDa for IGF-II (Zapf and Froesch 1986; Daughaday and Rotwein 1989). Variants of the IGFs are described in the literature. The "big" IGF-IIs, with molecular weights of 10-15KDa were as mitogenic as mature IGF-II (Gowan, Hampton et al. 1987). For IGF-I, a brain variant, des(1-3) or "truncated" IGF-I was described to lack the first three amino acids and to be more potent than the intact IGF-I in various cell culture system probably due to its lower affinity for IGFBPs (Giacobini, Olson et al. 1990; Oh, Muller et al. 1993; Sara, Carlsson-Skwirut et al. 1993; Russo and Werther 1994).

IGFs exert their pleiotropic function in an endocrine, autocrine and paracrine fashion. The liver produces most of the circulating IGF-I under the regulation of Growth Hormone (GH) (Bichell, Kikuchi et al. 1992; Meton, Boot et al. 1999), a pituitary gland hormone, which is in turn under the regulation of the hypothalamic factors somatostatin and growth-hormone releasing hormone (GHRH). Besides GH, also nutritional, tissue-specific and developmental factors are involved in the IGF-I expression: (Sara and Carlsson-Skwirut 1986; Milner and Hill 1989; Bichell, Kikuchi et al. 1992; Thissen, Ketelslegers et al. 1994; Meton, Boot et al. 1999). In contrast with its action on IGF-I, GH does not tightly regulate IGF-II expression.

The actions of IGF-I and IGF-II result from the activation of an array of cell surface receptors. Most, if not all, of the effects of IGF-I result from the activation of IGF-IR. IGF-I has a relative affinity almost two orders of magnitude higher for the IGF-IR than for insulin receptor. IGF-II interacts with high affinity with IGF-IIR, with insulin receptor, mainly the molecular isoform that lacks the exon 11 sequence (insulin

receptor-A) and with low affinity with IGF-IR (LeRoith, Werner et al. 1995; Frasca, Pandini et al. 1999; LeRoith and Roberts 2003).

The circulating IGF-I levels vary considerably among normal individuals and the IGF-II levels are consistently several-fold higher than that of IGF-I. This variation is thought to be due to both genetic effects and environmental or lifestyle factors. Observational epidemiologic studies suggest that circulating levels of IGF-I and IGF-II are related to the risk of developing several epithelial cancers (Pollak, Schernhammer et al. 2004; Renehan, Zwahlen et al. 2004). In particular, the results of large prospective studies consistently show higher risk of breast and prostate cancer, and possibly colon and lung cancer, in individuals with relatively high levels of IGF-I. Furthermore, the IGF system is known to interact with insulin (Giovannucci 2003) and estrogens at various levels. Animal model systems as well as analyses of human tissue show a very complex picture with normal IGF signaling being potentially disrupted during one or more stages of the carcinogenic process.

1.2.2 The IGF receptors

The IGF system includes three receptors: insulin receptor, IGF-IR and IGF-IIR. In addition, several publications describe in a variety of cell types, the existence of IGF-IR subtypes: atypical receptors and hybrid receptors (Moxham, Duronio et al. 1989; Soos, Whittaker et al. 1990; Siddle, Soos et al. 1994; Entingh-Pearsall and Kahn 2004). The atypical IGF receptors possess the ability to bind insulin as well as IGFs with relatively high affinity. The hybrid receptors result from the dimerization of IGF-IR and insulin receptor hemireceptors and have high affinity for IGF-I, while the affinity for insulin decreases dramatically. The picture is complicated by the presence of the insulin receptor isoforms A and B that have different IGF-II binding characteristics. There are conflicting reports published on this subject. Some data showed significant difference between the characteristics of the two splice variants of the hybrid receptors (Pandini, Frasca et al. 2002; Slaaby, Schaffer et al. 2006). In contrast, a recently published paper found that irrespective of the type, the hybrid receptor presented very similar binding properties to those of native IGF-IR, suggesting that insulin signaling through hybrid receptors might be physiologically irrelevant (Slaaby, Schaffer et al. 2006). It might be that these IGF-IR subtypes take

part in the complexity of the IGF signaling, however their biological role is still unclear.

Insulin receptor and the IGF-IR belong to the tyrosine kinase receptor family and share striking homology. Considered a key player in the IGF system, IGF-IR structure and functions will be detailed in a separate chapter.

Insulin receptor is expressed in liver, adipose tissue and muscle. Insulin binding 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-IR and insulin receptor 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 insulin receptor and IGF-IR, the IGF-IIR, also known as the cation-dependent mannose-6-phosphate receptor, has no tyrosine kinase activity and its biological function has still to be clarified. IGF-II/M6P receptor is a single chain polypeptide with a short cytoplasmatic domain that binds two types of ligands: non-M6P-containing ligands or IGF-II and M6P-containing ligands (lysosomal enzymes). It is thought to act as a clearance receptor for IGF-II, it functions in the mediation of lysosomal enzyme trafficking, endocytosis, lysosomal degradation of extracellular ligands and it somehow intrigues even in apoptotic/mitogenic effects and possible intracellular signal transduction (Morgan, Edman et al. 1987; Braulke 1999; Macdonald and Byrd 2003).

1.2.3 IGFBPs

The bioavailability of IGFs is influenced by the presence of insuline-like growth factor binding proteins (IGFBPs), a family of six high-affinity proteins, designated as IGFBP 1 to 6 that do not bind insulin. The IGFBPs function is very complex and it can be summarized as follows:

a. Transport of IGFs in plasma and control of its diffusion and efflux from the vascular space
b. Increase the half-life and control IGFs clearance.

c. Provide specific binding sites for IGFs in the extra- and pericellular space.
d. Inhibit or allow IGFs interactions with their cognate receptors.

IGFBPs are in turn regulated by the IGFBPs proteases through cleavage and generation of small fragments with reduced or no binding affinity for IGFs. These proteases include kallikrein-like serine proteases, cathepsin, and matrix metalloproteinases and act either within specific tissues or in the bloodstream and extracellular space.

Recent studies pointed out certain functions of IGFBPs, independent of their capacity to bind IGFs. IGFBP -2, -3 and -5 were shown to influence proliferation, migration and sensitivity to apoptosis. These independent actions of IGFBPs need be better characterized (Baxter 2000; Firth and Baxter 2002).

1.3 IGF-IR

1.3.1 IGF-IR structure. Signaling pathways. Biological properties and role in human cancer. Cross-talk with other RTKs

IGF-IR structure

IGF-IR is composed of two extracellular α-subunits that contain a ligand-binding domain and two transmembrane β-subunits that possess tyrosine kinase activity, connected by disulfide bonds (LeRoith, Werner et al. 1995). The α- and β-subunits contain 706 and 627 amino acids, respectively and their molecular weights are 135kDa for α and 90 kDa for β (Ullrich, Gray et al. 1986). The ligand pockets of IGF-IR are formed by the α-subunits and possibly by some extracellular part of the β-subunits. The β-subunits span the cell membrane and its intracellular part is essential for the receptor activity. It consists of a juxtamembranous tyrosine kinase part and a C-terminal domain. The structure of IGF-IR tyrosine kinase catalytic domain displays similarities with the insulin receptor, sharing sequence homology of 70% overall, 84% within the tyrosine catalytic domains and 100% within the ATP binding site, a highly conserved region of the IGF-IR (Ullrich, Gray et al. 1986). The structures of the IGF-IR and insulin receptor catalytic domains display the typical two-lobed protein kinase fold (Hubbard 1997; Favelyukis, Till et al. 2001) and the ATP binding and catalysis

take place in a cleft between the lobes. Within the tyrosine kinase domain there is a segment known as the activation loop (A-loop) which contains three principal autophosphorylation sites (LeRoith, Werner et al. 1995). Ligand binding to the extracellular α-subunits induces a conformational change in the preformed dimeric receptor that leads first to the autophosphorylation of Y1135, followed by Y1131 and then by Y1136 (Favelyukis, Till et al. 2001). In the unphosphorylated state, the receptor catalytic activity is very low due to the inhibitory conformation of the specific domain in the kinase region, which interferes with ATP-binding and tyrosine phosphorylation. The changes in the A-loop conformation allow substrate and ATP access. Each of the three tyrosines is important for the full activation of the IGF-IR kinase domain and has specific functions. Y1135 and Y1136 seem to destabilize the autoinhibitory conformation of the A-loop and Y1136 appears to play a key role in structural stabilization of the A-loop (Li and Miller 2006). Tyrosine phosphorylation of the triad further activates the intrinsic activity of the IGF-IR toward phosphorylation of other sites in the receptor and adaptor proteins, which in turn trigger different downstream pathways.

Signaling pathways

IGF-IR signal transduction processes possess a high degree of complexity and there is still a lot to do on characterizing them.

At least five adaptor proteins appear to bind to the cytoplasmic region of the IGF-IR. They are insulin receptor substrate (IRS) proteins family, Src homology 2 domain containing transforming proteins (Shc), the p85 subunit of the phosphatidylinositol 3 kinase (PI3-K), the SH2 domain-containing protein tyrosine phosphatase-2 (PTP1D) and growth factor receptor-bound protein 10 (GRB10). All of them have the ability to trigger distinct signaling systems (Butler, Yakar et al. 1998). As for other RTKs, the two main signaling pathways are mitogen-activated protein kinase (MAPK) and PI3-K (LeRoith and Roberts 2003). A third pathway is described as the 14-3-3 pathway that results in the mitochondrial translocation of Raf-1 kinase (Peruzzi, Prisco et al. 1999; Navarro and Baserga 2001; Peruzzi, Prisco et al. 2001).

When describing the signal transduction mechanism two aspects are of great importance: the cell type, since the expression of the adaptor proteins can markedly

differ (Petley, Graff et al. 1999) (Butler, Yakar et al. 1998) and the existence of the potential overlap among the different pathways.

Biological properties and role in human cancers

IGF-IR is essential for fetal and postnatal growth (Liu, Baker et al. 1993). It is an important factor for the development of specific organs such as the nervous system. IGF signaling regulates neuronal proliferation, apoptosis and cell survival (Russo, Gluckman et al. 2005). It is also involved in the regulation of the metabolism (Kulkarni, Holzenberger et al. 2002).

Several model systems provide evidence for IGF-IR involvement in proliferation and metastasis of cancer. IGF-IR activation is a key event of the receptor involvement in cancer biology and it is triggered either by higher levels of circulating IGF ligands in the host or by autocrine production of ligands by the neoplastic cells (Khandwala, McCutcheon et al. 2000). Upregulation of IGF-IR is shown in a variety of tumors; however the molecular mechanism by which IGF-IR expression is increased is unidentified. Neither amplifications nor mutations of the IGF-IR are described, in contrast to the ErbB receptors family. Thus it is generally accepted that activation of IGF-IR requires ligand binding and IGF-IR seems to have important roles even if the expression levels are low (Tennant, Thrasher et al. 1996).

A broad range of human cancer overexpressed the receptor or had an increased IGF-IR kinase activity. IGF-IR mRNA was detected in a majority of primary breast tumor samples, with IGF-IR overexpressed in 30% to 40% of breast cancers (Cullen, Yee et al. 1990). The IGF-IR was overexpressed (Milazzo, Giorgino et al. 1992; Pezzino, Papa et al. 1996) and hyperphosphorylated (Resnik, Reichart et al. 1998; Surmacz 2000) in primary tumors compared to benign tumors or normal breast epithelium. In primary prostate cancer cells a significant upregulation of IGF-IRmRNA and protein level was found in comparison to benign prostatic epithelium (Kurek, Tunn et al. 2000; Hellawell, Turner et al. 2002). During progression from colorectal adenoma to carcinoma an increase of IGF-IR expression was detected in human tissue samples. Strong IGF-IR staining correlated with a higher grade and stage of tumors (Hakam, Yeatman et al. 1999; Hassan and Macaulay 2002). Similarly, progression from benign nevi to malignant melanoma was associated with increase in IGF-IR expression (Kanter-Lewensohn, Dricu et al. 2000).

IGF-IR, activated by its ligands, has a variety of functions that help controlling cells proliferation, namely:

- It sends a mitogenic signal
- It protects cells from a variety of apoptotic injuries
- It promotes growth in cell size
- It plays a crucial role in the establishment and maintenance of the transformed phenotype
- It regulates cell adhesion and cell motility
- It can induce terminal differentiation.
- It can offer protection against therapy

These various functions of the IGF-IR are discussed in many reviews, to which the reader is referred for detailed references (Baserga, Hongo et al. 1997; Baserga 2000; Valentinis and Baserga 2001; Baserga, Peruzzi et al. 2003).

Cross-talk with other RTKs

Off many studies on receptor-mediated signal transduction a recurring subject has emerged in the last years, namely the interplay or the cross-talk that occurs between structurally distinct families of receptors in response to ligand activation. It is an important subject with crucial implications on the therapeutic response. This holds up for IGF-IR and its cross-talk was described in different biological systems and may take place at a variety of levels.

Interconnections were identified with nuclear steroid receptors (Dupont, Karas et al. 2000), G protein-coupled receptors (Dalle, Ricketts et al. 2001) or the two transmembrane serine/threonine kinase receptors of TGFβ ligands family (Danielpour and Song 2006). Most studies reported the IGF-IR cross-talk occurring within the RTK family.

IGF-IR upon ligand stimulation shares the RAS pathway with other RTKs, especially with EGFR and PDGFR, as suggested by the studies on wild type mouse embryo cells growth (Sell, Rubini et al. 1993; Coppola, Ferber et al. 1994; DeAngelis, Ferber et al. 1995).

Stimulation of IGF-IR signaling interfered with antitumoral activity of EGFR inhibitors. In glioblastoma cells investigators saw a compensatory upregulation of IGF-IR level in response to EGFR inhibitory treatment (Chakravarti, Loeffler et al.

2002) and the impairment of IGF-IR function increased the apoptotic effects of EGFR inhibition (Steinbach, Eisenmann et al. 2004). In several human breast cancer cell lines that expressed IGF-IR similarly but EGFR differently, co-targeting IGF-IR and EGFR activity caused additivity or synergy in growth inhibition and apoptosis induction (Camirand, Zakikhani et al. 2005). The two receptors showed reciprocal transactivation. The IGFI/IGF-IR pathway transactivated the EGFR via an autocrine release of EGF-like growth factors (Vardy, Kari et al. 1995; Roudabush, Pierce et al. 2000). In mammary epithelial cells, IGF-IR protected against apoptosis via EGFR transactivation (Gilmore, Valentijn et al. 2002). In turn, in NSCLC cells amphiregulin transactivated IGF-IR independent of binding to its specific receptor EGFR (Hurbin, Dubrez et al. 2002). For more details, the reader is referred to a review which focuses on the studies upon IGF-IR/EGFR cross-talk (Adams, McKern et al. 2004). The mechanism of cooperation between IGF-IR and EGFR is not clearly elucidated, but further insights were gained for HER2 and IGF-IR interaction.

HER2 is another ErbB receptor in close connection with IGF-IR. In breast cancer cell models that overexpressed HER2, an increased level of IGF-IR signaling interfered with Trastuzumab-induced HER2 inhibition (Lu, Zi et al. 2001). Other experiments performed on mammary tumors demonstrated the existence of a hierarchical interaction between IGF-IR and HER-2, in which IGF-IR directed HER-2 phosphorylation. The physical association of both receptors, resulting in the formation of a heteromeric complex was the underlying reason for this interaction (Balana, Labriola et al. 2001). More recently, the heterodimer was again reported but this time in trastuzumab resistance breast cancer cell only (Nahta, Yuan et al. 2005). The authors suggested that the IGF-IR/HER-2 heterodimer contributed to trastuzumab resistance but in contrast to Lu et al, independently of total IGF-IR levels (Camirand, Lu et al. 2002).

Cross-talk between IGF-IR and PDGFR was described in different cellular models (Bridle, Li et al. 2006; Novosyadlyy, Dudas et al. 2006) and the dual blockade of the IGF-IR and PDGFR was suggested to be a valuable strategy for the rabdomyosarcomas treatment (Blandford, Barr et al. 2006).

Stem cell factor (SCF)/Kit and IGF-I/IGF-IR autocrine loops play a prominent role in the growth of small cell lung cancer. One study showed that cell growth was efficiently inhibited in cells that are highly dependent on IGF-I signaling by using a potent and selective IGF-IR kinase inhibitor only. However for optimal growth

inhibition of small cell lung cancer cells with an active SCF/Kit autocrine loop, a combination of a Kit inhibitor (STI571) and an IGF-IR inhibitor (NVP-ADW742) appeared to be necessary (Warshamana-Greene, Litz et al. 2004). In addition, targeting of both IGF-IR and c-kit synergistically increased the antiapoptotic effect in comparison to either of the receptor inhibition alone using a downstream pathways ERK1/2 dependent (Camirand and Pollak 2004).

1.3.2 IGF-IR blockade therapy

IGF-IR was one of the very first RTKs to be cloned. In the last 20 years several lines of epidemiological and mechanistic evidence linked IGF-IR activation and signaling to tumor biology and a lot of preclinical data encouraged the use of receptor blocking as a targeted therapy. However, long after the success of other targeted drugs like trastuzumab (Herceptin) and imatinib (Gleevec), the first two phase I clinical trials testing IGF-IR inhibitors were launched in 2004:

- Phase I clinical trial of CP-751,871 (a fully human IgG2 anti-IGF-IR antibody) for patients with multiple myeloma.

(http://www.moffitt.usf.edu/about_moffitt/publications/clinical_trials_update/nbcmonths/2005s2.pdf)

- Phase I clinical trial of INSM 18 (a small molecule with structure and biological activity not disclosed) for patients with relapsed prostate cancer.

(http://www.drugresearcher.com/news/ng.asp?id=55546-insmed-trials-encourage)

Two main reasons caused this delayed introduction of the IGF-IR blockade therapy. Firstly, IGF-IR shares 70% homology with the insulin receptor, therefore one was concerned about accidentally hitting the insulin receptor with IGF-IR inhibitors and secondly, IGF-IR is ubiquitously expressed, in normal tissue throughout the body with unpredictable side-effects after IGF-IR inhibition.

Fortunately, in the past few years drug discovery evolved enormously and IGF-IR, being involved in so many malignant processes and sharing a complex cross-talk with other RTKs became a subject of intensive drug targeted therapy research. In addition, highly specific IGF-IR selectivity has become an open question. The dilemma was raised by published data suggesting that the insulin receptor played an important role in regulating insulin or IGF-II action in cancer cells, thus inhibition of insulin receptor

or hybrid receptor might be a requirement for an effective antitumor therapy (Sciacca, Costantino et al. 1999).

Anyhow, many big pharmaceutical and biotech companies have set all their reservations aside and are intensively pursuing drugs and small molecules that target IGF-IR.

Researchers have developed and explored several therapeutic strategies to disrupt the IGF-IR-mediated signaling at many levels. They can be roughly divided into two categories: strategies that alter the IGF-IR ligands bioavailability and strategies directed against the IGF-IR. The latter accounts for most of the approaches studied. Although not all of them will probably have clinical applicability, many of the inhibitory strategies can be summarized as follows:

Antagonists of GHRH (Braczkowski, Schally et al.2002; Letsch, Schally et al.2003; Szereday, Schally et al 2003) **or GH** (McCutcheon, Flyvbjerg et al. 2001).

Considering that elevated levels of IGF-I are associated with an increased risk of developing prostate (Chan, Stampfer et al. 1998), breast (Hankinson, Willett et al. 1998) or colon cancer (Giovannucci, Pollak et al. 2000), removing the ligand might be one alternative to block IGF-IR. However this approach would affect neither the autocrine/paracrine secreted IGF-I, nor the IGF-II.

IGF-mimetic peptide

They inhibit ligand-receptor interaction by using a ligand analog that bind the receptor but avoid its activation. Synthetic IGF-I-mimetic peptides were designed and tested with promising results, but their efficacy has never been assessed in vivo and this approach will not account potential IGF-II stimulation (Pietrzkowski, Wernicke et al. 1992; Pietrzkowski, Mulholland et al. 1993).

IGFBPs

The IGF system controls the bioavailability of its ligands through the interaction with IGFBPs. IGF-I and IGF-II have a higher affinity for IGFBPs than for IGF-IR, therefore these naturally occurring proteins, could be used to neutralize IGFs actions (Firth and Baxter 2002).

Several papers investigated IGFBP 1 and 3 and they could block the growth of tumor cells *in vitro* and *in vivo* (Figueroa, Sharma et al. 1993; Oh, Muller et al. 1993; Yee,

Jackson et al. 1994; Nickerson, Huynh et al. 1997). rhIGFBP3 was demonstrated to enhance sensitivity to radiation therapy (Blouin MJ 2003; Blouin MJ 2003) and chemotherapy-induced apoptosis in models of human breast cancer (Perks C 2003). The pharmaceutical company Insmed (http://www.insmed.com/) is currently conducting a Phase I study (INSM-120-101) of rhIGFBP-3 to evaluate for the first time in humans the safety, tolerability and pharmacokinetics of single intravenous infusions of rhIGFBP-3 for different doses.

Of importance here might be the described IGFs independent effects of IGFBPs on cell survival and migration (Oh, Muller et al. 1993; Rajah, Valentinis et al. 1997).

Future research will tell about long term effects of IGFBPs administration *in vivo*.

Antibodies against the IGF-IR

They interfere with ligand binding and trigger the internalization and the degradation of IGF-IR. Inhibition of IGF-IR function by a specific antibody was first demonstrated using the mouse monoclonal antibody αIR-3 directed against the α-subunit of the receptor (Kull, Jacobs et al. 1983). Historically, its use preceded the description of the trastuzumab precursor, 4D5 and it was nearly simultaneously reported with cetuximab. Other mouse monoclonal antibodies described in the literature are MAB 391 (Hailey, Maxwell et al. 2002), 1H7 (Li, Kato et al. 1993) and 4G11 (Jackson-Booth, Terry et al. 2003). Several results raised the concern that αIR-3 was not a complete inert agent acting as an IGF-I agonist in stable transfected NIH-3T3 cells overexpressing human IGF-IR (Kato, Faria et al. 1993). On the other hand murine antibodies were not ideal therapeutics for human patients because of their immunogenic properties. In order to introduce antibodies into the clinic, numerous research groups engineered and characterized antagonistic and/or neutralizing humanized antibodies that targeted the extracellular domain of IGF-IR. They can provide a greater potential for success as therapeutics because they are less likely to induce an immune response and posses a longer half-life *in vivo* (Park and Smolen 2001). The available humanized antibodies are described in Table nr 1.

Table nr 1. Antibodies that target the extracellular domain of IGF-IR

Antibody	Phase of development	Description	References
CP-751,871	Phase I in patients with multiple myeloma	A fully human IgG2 antibody, generated by the application of Xenomouse technology. Dissociation constant of 01.5 nM. It inhibits IGF-I binding to the receptor with IC_{50} values of 1.8 and IGF-I induced receptor autophosphorilation with IC_{50} of 0.42 nM. In vivo antitumor efficacy as a single agent or in combination with Doxorubicin, 5-Fluorouracil or Tamoxifen in several tumor xenografts.	(Cohen, Baker et al. 2005)
EM164 (AVE1642)	Preclinical	A humanized IgG version of an antagonistic antibody generated by immunizing mice with murine cells overexpressing human IGF-IR. Dissociation constant of 0.1 nM. In vitro and in vivo (intravenous administration) effects as a single agent or in combination with Gemcitabine at concentration of 60 nM-120 nM.	(Maloney, McLaughlin et al. 2003; Bladt 2006; Geoerger B 2006)
IMC-A14	Preclinical	A fully human antagonistic antibody obtained after a Fab phage-display library screening. Dissociation constant of 0.04 nM. In vivo inhibition of tumor growth	(Burtrum, Zhu et al. 2003; Lu, Zhang et al. 2004; Wu, Odman et al.

		following intraperitoneally administration in many types of cancer xenograft. Antitumor activity in combination with Melphalanor Bortezomib in a multiple myeloma model.	2005)
h7C10(F50035)	Preclinical	A recombinant humanized IgG1 antibody. It inhibits IGF-I and IGF-II binding to the receptor with IC_{50} values of 4.2nM and 3.1nM, respectively. *In vivo* antitumor effects against breast and NSCLC xenografts following intraperitoneally administration. Enhanced effects after combination with Vinorelbine (tubulin depolymerizing agents) or 225Mab (EGFR antibody).	(Goetsch, Gonzalez et al. 2005)
19D12	Preclinical	A fully humanized neutralizing antibody obtained using the Medarex´s HuMab transgenic mouse technology. Dissociation constant of 3.8 pM. *In vivo* antitumor effects against ovarian, NSCLC, breasy and colon cancer xenografts.	(Wang, Hailey et al. 2005)
scFv-Fc-IGF-IR	Preclinical	A chimeric single chain antibody, generated by fusing the Fc domain of human IgG1 with the Fv region of 1H7, a mouse monoclonal antibody. In vivo antitumor effects when used as single agent or in combination with Tamoxifen against breast cancer xenografts.	(Sachdev, Li et al. 2003)

A new approach regarding the use of antibodies is represented by the generation of bispecific antibodies (Bs-Ab). They are referred to as Di-diabodies.
One such antibody, BsAb-IGF-IR-EGFR targets IGF-IR and EGFR and proved to have in vitro and in vivo antitumor effects (Lu, Zhang et al. 2004; Lu, Zhang et al. 2005).
Another novel antibody is mAb KM 1468 (rat IgG2b) directed against human IGF-I and IGF-II (KM1468) and reported to suppress the growth of human prostate cancer cells in human adult bone (Goya, Miyamoto et al. 2004).

Small molecules tyrosine kinase inhibitors

The catalytic function of IGF-IR is vital for its activation and function, thus modulating the tyrosine kinase activity by targeting the intracellular kinase domain with small molecules tyrosine kinase inhibitors appears to be one of the best approaches. In addition, the proof-of-concept of the potential therapeutic benefit of using small molecules was demonstrated by the clinical success of Imatinib (Cohen, Johnson et al. 2005).
The identification of specific low molecular mass kinase inhibitors of IGF-IR kinase activity has always been a major challenge for medical chemistry, because of the high sequence homology at the kinase domains of IGF-IR and insulin receptor. At the ATP-binding pocket the two receptors share a 100% homology (Ullrich, Gray et al. 1986). Advances in the drug discovery field and in the characterization of the three-dimensional structure of the IGF-IR and insulin receptor facilitated the design of more or less specific IGF-IR inhibitors. To date several TKRI are described and classified into two major groups: ATP antagonists and non-ATP antagonist.

ATP antagonists:

- Tyrphostins are a family of synthetic protein tyrosine kinase inhibitors, derived from a benzylidene malononitrile nucleus, which resembles the phenolic group of tyrosine, with additional substitutions. Tyrphostins inactivate the IGF-IR tyrosine kinase by blocking the substrate binding site. Several such compounds were identified: AG1024, AG 1034, AG 538, I-OMeAG. Crossreactivity with insulin receptor was reported, but

AG1024 and AG1034 had significant lower IC_{50} for inhibition of IGF-IR phosphorylation than for insulin receptor. They were the first described and represented an excellent tool to examine the IGF-IR functions (Parrizas, Gazit et al. 1997; Blum, Gazit et al. 2000; Blum, Gazit et al. 2003).

- 6-5 Ring-fused compounds are a group of 19 chemically modified pyrrolo[2,3-d]pyrimidines modified substances. Being inspired by Imatinib 's mechanism of action, which effectively inhibits the Bcr-Abl tyrosine kinase by binding to the unphopshorylated form (Schindler, Bornmann et al. 2000), one group tested these substances for their ability to target the various phospho-forms of the IGF-IR. These compounds were first reported to selectively inhibit the unphosphorylated form of IGF-IR (Li, Favelyukis et al. 2004).
- NVP-ADW742 and NVP-AEW541 belong to a new series of pyrrolo[2,3-d]pyrimidines derivates. They are optimized IGF-IR kinase inhibitors that selectively distinguished between the native IGF-IR and the closely related insulin receptor. The selectivity toward the IGF-IR was observed at the cellular level (27-fold), but not in the *in vitro* kinase assays (Garcia-Echeverria, Pearson et al. 2004). NVP-ADW742 efficacy was demonstrated *in vitro* and *in vivo* using a multiple myeloma model, a hematological malignancy critically dependent on IGF-1R function. The authors demonstrated significant antitumor activity both as a single agent, as well as in combination with cytotoxic chemotherapy, without significant treatment-related toxicity (Mitsiades, Mitsiades et al. 2004).
- BMS-536924 and BMS-554417, belong to a group of benzimidazol derivates. They almost equipotently inhibit IGF-IR, the insulin receptor and the focal adhesion kinase. Several studies that investigated their antitumor activity (Wittman, Carboni et al. 2005; Haluska, Carboni et al. 2006), followed the concept that inhibition of both IGF-IR and insulin receptor might be necessary for inhibiting IGF-mediated proliferation (Kalli, Falowo et al. 2002; Pandini, Frasca et al. 2002).

Non-ATP antagonist

- The cyclolignan derivative picropodophyllin (PPP) inhibits phosphorylation of IGF-IR at an IC_{50} of 0.04μM without interfering with insulin receptor activity (Girnita, Girnita et al. 2004)and it is proved to interfere with the conformational state of IGF-IR and not with the ATP binding site. It blocks the phosphorylation of Tyr1136 in the activation loop of the kinase while sparing the other two kinases Tyr 1131 and 1135 (Vasilcanu, Girnita et al. 2004). Its antitumor activity is demonstrated in several tumor models (Girnita, All-Ericsson et al. 2006; Menu, Jernberg-Wiklund et al. 2006).

INSM-18 is a small molecule tyrosine kinase inhibitor that demonstrated selective inhibition of both IGF-IR and HER2 as reported by the pharmaceutical company Insmed (http://www.drugresearcher.com/news/ng.asp?id=55546-insmed-trials-encourage). The structure and the biological activity of INSM-18 were not disclosed. According to the company reports, this inhibitor demonstrated anti-tumor activity in preclinical studies of breast, lung, pancreatic and prostate tumors. Two single dose Phase I clinical studies in patients with relapsed prostate cancer were completed with INSM-18. In both studies, INSM-18 was safe and well tolerated (http://www.drugresearcher.com/news/ng.asp?id=55546-insmed-trials-encourage).

Dominant-negative mutants

Designed to interfere with the function of wild-type protein, IGF-IR dominant-negative receptors successfully suppressed in many studies the receptor function, resulting in reduced growth and/or tumorigenicity (Bahr and Groner 2004). Different mutants were constructed: proteins that were truncated within the β-subunit, forming inactive heterodimers of mutant and wild-type receptors, unable to transduce downstream signals (Brodt, Fallavollita et al. 2001); other IGF-IR dominant-negatives lacked the transmembrane region and were secreted from the cell to compete with wild-type receptors for ligand binding (D'Ambrosio, Ferber et al. 1996). This approach was not tested in clinical settings because it faced the common problem to all plasmids, the efficient *in vivo* delivery.

Triple helix

Oligonucleotide-directed triple helix formation is an approach to block transcription of specific genes by inhibiting the passage of RNA polymerase along target DNA. The third effector strand, oligoribonucleotide contains oligopurine sequences potentially capable of forming a triple helix with oligopurine and /or oligopyrimodine sequenced in target DNA. A homopurine RNA sequence designed to form a triple helix with a homopurine-homopyrimidine sequence 3`to the termination codn of the IGF-IR encoding gene was reported to induce dramatic reduction of IGF-IR transcripts and IGF-IR expression and inhibited tumor formation in nude mice (Rininsland, Johnson et al. 1997; Shevelev, Burfeind et al. 1997).

Antisense strategies

Antisense oligodeoxynucleotides (AS ODN) are short pieces of synthetic DNA or RNA, designed to interact with mRNA in order to block transcription and thus the expression of the specific target protein. There are many studies describing AS mediated IGF-IR down-regulation using AS RNA or AS ODN, most of them using ODNs containing sequences complementary to the IGF-IR translation initiation site. Tumor cells survival was inhibited both *in vitro* and *in vivo* in a wide range of tumor types such as melanoma (Resnicoff, Coppola et al. 1994), glioblastoma (Ambrose, Resnicoff et al. 1994; Resnicoff, Sell et al. 1994), prostate (Hellawell, Ferguson et al. 2003), and breast (Salatino, Schillaci et al. 2004). Antisense strategy also enhanced tumor cell chemosensitivity (Hellawell, Ferguson et al. 2003) and radiosensitivity (Macaulay, Salisbury et al. 2001). It was also suggested that IGF-IR down-regulation by AS ODN might induce a systemic immune response capable of protecting the host from tumor cell rechallenge. The mechanism was correlated to the upregulation of Class I and co-stimulatory B-7 molecules (Trojan, Duc et al. 1996) for brain tumors and to the inducement of CD86 and heat shock protein 70 molecules expression for breast cancer (Schillaci, Salatino et al. 2006). However, antisense agents have their drawbacks: they cause only modest IGF1R down-regulation (Nakamura, Hongo et al. 2000) (Hellawell, Ferguson et al. 2003) and can affect the insulin receptor (Bohula, Salisbury et al. 2003).

si-RNA

RNA interference has rapidly displaced antisense and ribozymes as the preferred means for sequence-specific gene inhibition in cell culture studies. RNAi involves the

targeted post-transcriptional degradation of messenger RNA thereby inhibiting the synthesis of the desired protein. This effectively leads to silencing of gene expression. The effectors of this process are short interfering RNA (siRNA) duplexes (approximately 21-23nt) that are key intermediaries in the specific degradation of target mRNA following incorporation into the RNA-induced silencing complex (RISC) in the cytosol (Elbashir, Harborth et al. 2001).

RNA interference (RNAi) represents a promising new gene silencing technology for functional genomics and could have tremendous therapeutic potential that will be realized when clinically feasible methods of delivery will be developed. Several articles evaluating the prospects of siRNA for therapeutics have been recently published (Lu, Xie et al. 2005; Gartel and Kandel 2006; Tong 2006).

IGF-IR siRNA treatment induced profound gene silencing, blocked the IGF-mediated signaling (Bohula, Salisbury et al. 2003; Yeh, Bohula et al. 2006) and sensitized tumors to DNA damaging agents (Rochester, Riedemann et al. 2005).

Other molecular approaches

- Peptide aptamers are a class of genetically selected molecules that consist of a conformational constrained random peptide sequence integrated into E. coli thioredoxin or other scaffold proteins. Peptide aptamers specifically interacting with the COOH-terminus or the kinase domain of IGF-IR were reported (Bahr and Groner 2004).

- Inhibitors of N-linked glycosylation, a process required for IGF-IR translocation to the cell surface (Carlberg, Dricu et al. 1996). Tunicamycin (Carlberg, Dricu et al. 1996) or lovastatin, a HMG-CoA reductase inhibitor (Girnita, Wang et al. 2000) were used to block IGF-IR N-linked glycosylation and induced receptor downregulation and tumor cell survival.

1.3.3 IGF-IR and the resistance to radiotherapy

1.3.3.1 Molecular mechanism of radiotherapy

Radiation therapy (RT) has proven to be of paramount importance in the treatment of cancer since its first use in late 1890`s.

Radiotherapy is today a complex scientific discipline based on the interplay of oncology, biology, physics, and mathematics.

This thesis approaches radiotherapy from a radiobiological point of view. The knowledge of the basic concepts of radiobiology is essential for daily radiotherapy practices and for all oncologists. From the early observation of Becquerel or from Pierre Curie 's experiments studying ionizing radiation action on living things, radiobiology has considerably developed. Many of the biological pathways underlying radiation response have been elucidated and there is a constant effort in the research community to find better strategies for improving the outcome of curative radiotherapy. A comprehensive discussion of radiobiological principles is a very complex one and beyond the scope of this thesis. For a broad lecture please see the following reference (Hall Eric J. 2006).

The most common form of RT employs ionizing radiation (IR) and aims to deliver the radiation in doses that kill cancer cells while preserving normal tissue. The biological effects of IR vary with dose, the physical nature of IR and the type of cell being irradiated. The latter is a very important aspect that influences the outcome of RT. It is a well known clinical observation that certain types of tumors, like seminomas, lymphomas and small cell lung cancer respond well to RT while others like glioblastomas and NSCLC are radioresistant. Our knowledge of cellular and molecular biology today shows that molecular pathways in a cell are determinants of the intrinsic and induced cellular response to IR: cell death, cell cycle arrest or DNA repair and adaptive responses.

Ionizing radiation induces a complex response and importantly all molecules in a cell may be affected. DNA has long been considered the major target of IR. However, there is increasing evidence that other sources have critical contributions in initializing signaling responses upon cell exposure to IR. A simplified illustration is shown in Figure 2.

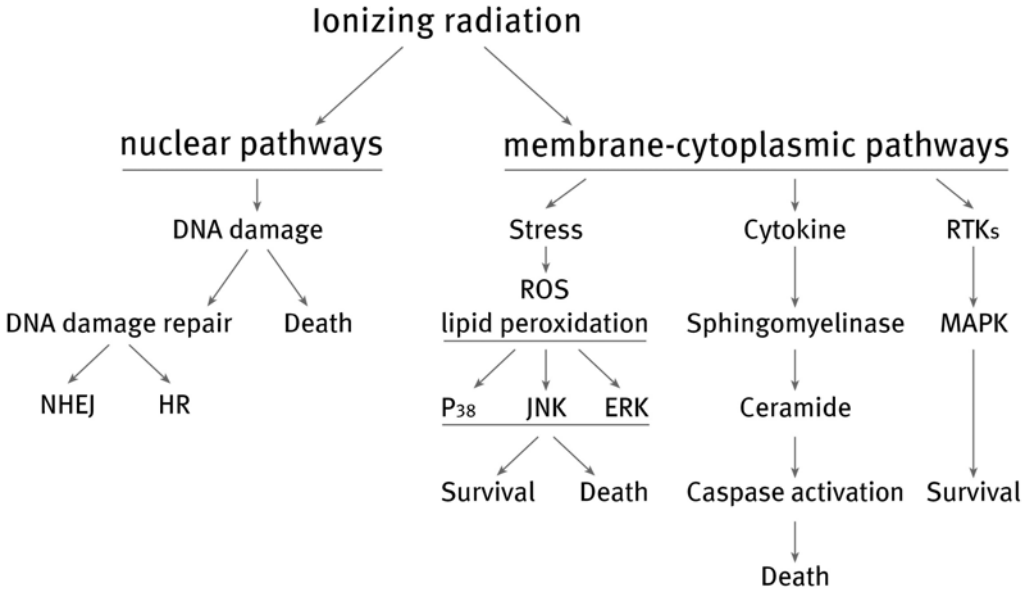

Figure 2. Ionizing radiation activates both nuclear pathways and membrane cytoplasmic signal transduction pathways. There is a considerable cross-talk among the pathways and all of them converge on multiple downstream factors that initiate transcriptional and non-transcriptional mechanisms in a complex combinatorial fashion. The outcome, cell death or survival, depends on the cell type

DNA damage

The concept of DNA being the most important target of IR comes from early experiments showing that irradiation of the nucleus, but not the cytoplasm result in cell death. The incidence of radiation-induced chromosome aberrations also correlates with cell death. Each grey of ionizing radiation causes in each cell thousands of single-strand DNA breaks (SSB), cross-links in DNA and with DNA-associated proteins, and 30-40 double-strand DNA breaks (DSB). Most of the DNA damage is repaired. Cells respond to DSBs, the most significant lesions, through a cascade of proteins ranging from sensors, which recognize the damage, through signal and mediator proteins to a series of downstream effectors that induce cell-cycle arrests, complete repair by homologous or nonhomologous mechanisms, or alternatively trigger cell death by apoptosis (Abraham 2001; Willers, Dahm-Daphi et al. 2004).

Other initiators and sensors of radiation damage

Damage to cytoplasmic, mitochondrial and membrane structures, changes in the homeostatic redox balance, alterations in protease activity and the production of lipid peroxides in the membranes following ionizing radiation were all suggested to influence the cellular response to irradiation. Even if the molecular pathways mediating these effects were described for certain cell types it is becoming obvious that the response to radiation vary with the cell type and its microenvironmental context.

Well characterized is the sphingomyelin pathway that is initiated by the radiation-induced cleavage of the membrane sphingomyelin by acidic and neutral sphingomyelinases, resulting in the formation of ceramide, a lipid second messenger (Haimovitz-Friedman, Kan et al. 1994; Haimovitz-Friedman, Kolesnick et al. 1997). The consequences of ceramide production depend on the cell type, but it commonly results in apoptosis initiation. This pathway is not IR specific, but it is shared with other stimuli such as cytokine death signals (TNFα, Fas ligand) or exposure to glucocorticoids (Kolesnick, Haimovitz-Friedman et al. 1994).

Many cytokines together with their receptors are believed to play an important role in cellular radiation responses. TNFα, IL6, urokinase-type plasminogen activator and TGFβ were all proposed to control cell survival responses after irradiation (Legue, Guitton et al. 2001; Ma, Webb et al. 2001; Eichholtz-Wirth and Sagan 2002; Dent, Yacoub et al. 2003).

Another signal transduction cascade generated at the plasma membrane by radiation involves RTKs. Several groups showed that all ErbB receptors stimulated both unique and redundant cellular programs that protected carcinoma cells from radiation-induced cell death and generated cytoprotective responses, at least in certain cell types (Schmidt-Ullrich, Dent et al. 2000; Dent, Yacoub et al. 2003; Schmidt-Ullrich, Contessa et al. 2003). ErbB-dependent signaling, typically stimulated through autocrine/juxtacrine ligand-receptor activation, receptor homo- and heterodimerizations, and tyrosine kinase activation (Schlessinger 2002) was stimulated by IR too. The receptor autophosphorylated shortly after irradiation of tumor cells both *in vitro* and *in vivo* (Schmidt-Ullrich, Mikkelsen et al. 1997; Akimoto, Hunter et al. 1999; Todd, Mikkelsen et al. 1999; Bowers, Reardon et al. 2001). The mechanism of receptor activation is not clear, but several hypotheses were suggested: ROS action on cysteine residues might lead to cross-linking and receptor

aggregation or inactivation of phosphatases could be also involved. In addition to causing ligand-independent activation of ErbB receptors, IR induced the synthesis and the release from tumor cells of autocrine growth factors (Dent, Reardon et al. 1999; Albanell, Codony-Servat et al. 2001). In this way the ErbB receptors were maintained active even hours after the initial exposure to IR. Upon irradiation, ErbB receptors activation resulted in activation of multiple downstream pathways providing radioprotective signals, as described in many studies. K-/H-Ras, PI3K, MAPK and NFKB were considered key pathways in many cell types, protecting cells against the radiation stress (Dent, Yacoub et al. 2003). Nevertheless, the signal transduction pathways in radiation response cannot be generalized due to the strong dependence on the molecular profile of the tumor cell. All these pathways were proved to have a dual nature in the control of cell survival after irradiation by either protecting against IT-induced cell death in certain cell types or enhancing the radiation sensitivity in others (Dent, Yacoub et al. 2003).

Other RTKs are also probably activated after radiation exposure, but there are not many studies covering this subject. For fibroblasts, IR was proved to induce the autocrine and paracrine PDGF expression and significantly increase the PDGFR phosphorylation (Li, Ping et al. 2006).

Another RTK correlated with the responses following cellular exposure to IR is IGF-IR, one of the subjects of this research project.

1.3.3.2 Rationale for targeting the IGF-IR in radiotherapy

Evidence accumulated in recent years supports the hypothesis that IGF-IR targeting may be of benefit when combined with radiation. Up to date only preclinical data are published.

First suggestions came from studies on mouse embryo fibroblast cell lines lacking IGF-IR (R- cells) that were more sensitive to ionizing radiation than cells overexpressing the human IGF-IR (R+ cells) (Nakamura, Watanabe et al. 1997). A study that associated IGF-IR expression with the outcome to RT of patients with breast cancer was published around the same time. High levels of IGF-IR correlated with early relapse within 4 years of lumpectomy and irradiation (Turner, Haffty et al. 1997). Moreover, the addition of IGF-IR AS ODN reversed the radioresistant phenotype (Turner, Haffty et al. 1997). Following same line of evidence, a specific

inhibitor of the IGF-IR tyrosine kinase, tyrphostin AG1024, significantly radiosensitized human breast cancer cells (Wen, Deutsch et al. 2001). The radiosensitizing effect of IGF-1R inhibition was demonstrated in several tumor cell types. In colon cancer cells using a mouse monoclonal antibody αIR-3 (Perer, Madan et al. 2000) or in NSCLC cells using a fully human antibody A12 (Allen G.A. 2005) the investigators obtained enhanced cytotoxicity and a potentiation of radiation induced apoptosis. After transfection of AS ODN against the IGF-IR the mouse melanoma cells displayed an enhanced radiosensitivity (Macaulay, Salisbury et al. 2001). Two other recent studies used adenoviruses expressing a truncated dnIGF-IR, which up-regulated radiation-induced apoptosis in pancreatic adenocarcinoma cells (Min, Adachi et al. 2003) and in gastric carcinoma cells (Min, Adachi et al. 2005). Prostate cancer cells became more radiosensitive by silencing IGF-IR with siRNA (Rochester, Riedemann et al. 2005).

Signaling mechanisms whereby IGF-IR leads to radioresistance have been always of interest. How are they triggered? Ionizing radiation by itself activates other TKRs, which in turn activate specific downstream pathways. However, no prior studies observed activation of IGF-IR, although undetectable levels of activation could not be ruled out (Yu, Watanabe et al. 2003). In a study on primary malignant brain tumor cells, the investigators suggested that increased IGF-IR expression after irradiation might be one of the responses that aided cell survival (Kim, Xiao et al. 2003). Despite having a different response to IR, R+ and R- cells showed similar cell cycle distribution after irradiation and failed to induce p53. The cause of this difference in response to IR was unclear. It was suggested at that time that the signals from IGF-IR might be involved in IR-induced necrosis and apoptosis without affecting the cell cycle arrest and being p53-independent (Nakamura, Watanabe et al. 1997). Later on, it was possible a more differentiated description of the downstream pathways involved in the IGF-IR mediated radioresistance (Yu, Watanabe et al. 2003). Similar to the studies that described the complex and redundant signaling network of activated IGF-IR (Peruzzi, Prisco et al. 1999) the authors used a series of mutant IGF-IRs, expressed in R- cells, relevant to PI3-K and MEK/ERK activation. Their results delineated a complex mechanism with IGF-IR being involved in clonogenic radioresistance through a number of redundant signals of differently weighted relevance, including PI3-K, MEK/ERK and signals stemming from C terminus domain, through 14-3-3 proteins. When describing the signal transduction mechanism, the cell type was of

great importance because IGF-IR signaling and function vary from one cell type to another, possibly depending on the availability of the substrates and transducing molecules in each cell type (Petley, Graff et al. 1999). In accordance, radiation-induced IGF-IR signaling via PI3-K was not occurring in all contexts. For example, IGF-IR-mediated radioresistance in 3T3 cells was described as PI3-K-independent (Dong, Watanabe et al. 2002).

Based on our current understanding, upon irradiation, IGF-IR initiates a cytoprotective response composed of pro-proliferative and antiapoptotic responses. IGF-IR may prevent radiation-induced cell death by interfering with proteins involved in the repair of DNA lesions (Heron-Milhavet, Karas et al. 2001; Trojanek, Ho et al. 2003). Indirect evidence, consistent with the role of IGF-IR in DNA damage response, were brought by the experiments in which IGF-IR silencing was associated with enhanced sensitivity to DNA-damaging agents (Rochester, Riedemann et al. 2005). Other reports indicated that IGF-IR activation could rescue NWTb3 cells overexpressing IGF-IR from overall DNA-damage induced by UV-mimetic agents, by a mechanism that involved p38 kinase (Heron-Milhavet, Karas et al. 2001). In keratinocytes IGF-IR provided protection against UV-DNA damaging induced apoptosis through activation of Akt signaling pathway (Decraene, Agostinis et al. 2002). Nevertheless, it was not fully understood if either the receptor affected DNA repair directly or its strong antiapoptotic properties (Sell, Baserga et al. 1995; D'Ambrosio, Valentinis et al. 1997) simply increased the resistance to genotoxic agents.

Hence, little is known about the mechanism of action. The only established connection was made between the ATM kinase and the IGF-IR, which shared a reciprocal link. This idea was demonstrated by studies in which phenotypes with similar radiosensitivity levels were induced by either ATM mutations or IGF-IR downregulation (Macaulay, Salisbury et al. 2001; Peretz, Jensen et al. 2001). A more recent study suggested that ATM ability to coordinate the DNA damage response might depend on its capacity to control IGF-IR gene expression (Shahrabani-Gargir, Pandita et al. 2004). Regarding the mechanism of DNA damage repair, the signals from activated IGF-IR enhanced homologous recombination repair (HRR) by a mechanism that controlled the translocation of Rad51 to the sites of damaged DNA, a crucial step in the process of DNA repair by homologous recombination (Trojanek, Ho et al. 2003). More specific, a direct involvement of IGF-1R signaling in HRR but not

in non-homologous end joining repair (NHEJ) was reported as well (Reiss, Khalili et al. 2006).

Some of the findings presented in this thesis focused on IGF-IR activation and on the requirement for IGF-IR-mediated signaling in survival-response to radiation. These results add to and point out once more the important role IGF-IR can play in the response of tumor cells to irradiation.

2 THE PRESENT STUDY

2.1 AIMS

The general aim of this thesis was to investigate the role of IGF-IR as a potential target for cancer treatment and to analyze the benefit that such an approach would have when it is used in combination with conventional cancer therapy i.e. radiotherapy.

There were several reasons for choosing NSCLC and HGG cell lines. NSCLCs account for approximately 75% to 85% of all lung neoplasms, the leading cause of cancer-related mortality for both men and women. Thoracic radiotherapy for both curative and palliative purposes is a traditional treatment for patients with locally advanced NSCLC. Malignant gliomas represent among the most treatment-refractory tumors encountered by clinicians. For several decades, the standard of care represented maximal total resection, followed by radiation ± nitrosurea-based chemotherapy.

Central nervous system and lung cancers constitute sites that are particularly difficult to irradiate combining a large number of conceptual difficulties, allowing them to be considered as two particularly interesting study models.

Specific aims

- to investigate the expression levels and the importance for the cell survival of IGF-IR in a panel of NSCLC and HGG glioma cell lines
- to evaluate the IGF-IR involvement in the molecular mechanism of resistance to ionizing radiation, in relation to the downstream partners involved and the DNA repair mechanisms
- to find appropriate strategies for improving the response to radiation, which include inhibition of IGF-1R function alone or in combination with other targeted therapies.

2.2 MATERIAL AND METHODS

Cells and cell culture

For the studies we analyzed six NSCLC cell lines (U1810, U1752, H157, H125, H23 and A549) and two HGG cell lines (18, 38).

The U1810, U1752, 18 and 38 cell lines were established at the University of Uppsala and have been previously characterized (Carney, Mitchell et al. 1983; Brodin, Lennartsson et al. 1991) (Hagerstrand, Hesselager et al. 2006). The H157, H125, H23 and A549 cell lines were purchased from American Type Culture Collection, USA.

NSCLC cells were cultured in RPMI 1640 medium and HGG cells in 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% CO2 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-IRα (1H7, N20), IGF-IRβ (C20), IGF-I (H70), IGF-II (H103), PDGFR-β (958), JNK1 (FL), actin (C-2), anti-phosphotyrosine (PY99), p38 (H147), phosphop38 (D8) and Protein A/G Plus Agarose were purchased from Santa Cruz Biotechnology, Scandinavian Diagnostic Services. Phycoerythrin labelled, anti-IGF-IR antibody (clone 1H7) and relevant isotype controls were commercially purchased from Becton Dickinson AB. The antibodies against KU (p70) and KU (p86) were purchased from NeoMarkers. Anti-rabbit, anti-mouse, anti-goat secondary antibodies conjugated to horseradish peroxidase and the ECL Western blotting analysis system were purchased from Amersham Pharmacia Biotech UK Limite. Normal goat serum was purchased from Dako, goat-anti-rabbit FITC from Jackson and DAPI from Sigma-Aldrich.

The radiolabeled I^{125}-IGF-I was obtained from Amersham Pharmacia Biotech UK Limited. Recombinant human IGF-I and IGF-II was purchased from R&D Systems Inc. Recombinant human PDGF-BB was obtained from Biosource. Tyrphostin AG1024, AG538, AG1433, Ly294002, and SB 202190 were purchased from Calbiochem. They 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.
The MTT Cell proliferation Kit was purchased from Roche Diagnostics GmbH.
DNA Repair Kit Ku70/86, p38, and JNK Elisa Kit were purchased from Active Motif.
Non-extraction IGF-I and IGF-II ELISA were from Diagnostic System Laboratory.

TKRs and other proteins detection

1. Binding study

Cells were grown in 35-mm dishes in regular growth media. Confluent cells (80 %) were rinsed twice with ice-cold PBS and once with binding buffer (1 mM Hepes pH 7.4, 1% BSA, 135 mM NaCl, 4.8 mM KCl, 1.7 mM MgSO4, 2.5 mM CaCl2 × 2H2O). Finally, each dish was incubated for 30 min at 20°C with 1 ml binding buffer containing 60,000 DPM of I^{125}-labeled IGF-1. Thereafter, the cells were washed twice with PBS to remove unbound ligand and then lysed in a buffer solution (20 mM Hepes, 1% Triton-X, 10% glycerol, and 0.1% BSA), transferred to scintillation vials and counted in a scintillation counter. The nonspecific binding was determined by coincubation with unlabeled IGF-I (1000 ng/ml). For Scatchard analysis the cells were incubated with I^{125}-IGF-I together with increasing concentrations of unlabeled IGF-I (between 10 and 1000 ng/ml). The density of receptors was calculated as described (Clark 1977).

2. Flow Cytometry for protein detection

Cells were trypsinized, washed with FACS buffer (PBS containing 3% FBS and 0.02% NaN3), blocked in 10% FBS and stained with the antibodies or isotype control for 30 minutes on ice. Cells were analyzed using a FACScalibur flow cytometer and the CellQuest™ software.

3. Immunofluorescence

Double staining for nuclei (DAPI, 4',6-diamidine-2-phenylindole dihydrochloride) and JNK1 or IGF-IRβ or PDGFR-β (FITC) was performed on 18 and 38 paraffin embedded cells. Deparaffinised and rehydrated 4-µm sections were microwave heated 6 minutes in 0,1 M citrate buffer (pH 6,0) for antigen retrieval. Antibodies were diluted in Tris Buffer Saline (TBS) containing 1% bovine serum albumin and 0,05%

sodium azide. Sections were blocked for nonspecific antibody binding with normal goat serum (30 minutes). After the incubation with the primary antibody, at 4°C, overnight, cells were incubated with 1: 100 goat anti-rabbit streptavidin-FITC for 40 min. Nuclei were co-stained with 1 μg/ml DAPI for 10 min. Between 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 were used to document specific FITC and DAPI fluorescence images which were edited and overlaid by Adobe Photoshop 6.0.

4. Western Blotting

Cell lysis

To prepare whole cell extract, cells were collected in PBS/PIB 5% (Phosphatase Inhibitor Buffer: 125 mM NaF; 250 mM β-glycerophosphate; 250 nM para-nitrophenyl phosphate; 25 mM NaVO3) and lysed in Lysis buffer (20 mm Tris-HCl, pH 7.5; 150 mm NaCl; 1 mM CaCl2; 1 mM MgCl2; 10% glycerol; 1% Nonidet P-40). Solubilized proteins were separated from the cell debris by centrifugation.

To prepare the nuclear and the cytoplasmic extracts, the cells were collected in ice-cold PBS in the presence of Phosphatase Inhibitors and washed 2 times. Then, the cells were resuspended in Hypotonic Buffer (20 mM Hepes, 5 mM NaF, 10 μM Na2MoO4, 0.1 mM EDTA, pH 7,5) and incubated for 15 minutes on ice. The homogenate was centrifuged for 30 seconds at 4°C. The cytoplasmic fraction was collected and stored at -80°C. The nuclear pellet was resuspended in Lysis buffer, in the presence of Protease Inhibitor Cocktail (2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride) and incubated for 30 minutes on ice, on a shaking platform. The nuclear extract was obtained by centrifugation for 10 minutes at 14000g.

To prepare mitochondrial extract cells were washed twice with cold PBS, and lysed in buffer A (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol, 1 mM PMSF, 10 μg of leupeptin/ml, 10 μg of aprotinin/ml, 10 mM benzamidine, 0.2 mM sodium orthovanadate) at 4°C for 30 min. After homogenization samples were centrifuged two times (2,500 × g for 5 min) to remove the nuclei and centrifuged again at 13,000 × g for 30 min to obtain the heavy membrane pellet. This fraction was resuspended in buffer A and centrifuged again at

13,000 × g for 30 min. Finally, the pellet was resuspended in lysis buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100, 10 µg of aprotinin/ml, 1 mM PMSF, 1 mM sodium orthovanadate). Samples were then centrifuged at 13,000 × g for 20 min.

Immunoprecipitation

A total of 500 µg of total cellular proteins was first precleared with 25 µl of protein A/G Plus-agarose for 30 minutes and then incubated overnight with 1-2 µg of primary antibodies. 25 µl of protein A/G Plus-agarose were added for additional 4 h. All steps were done on a rocker at 4°C. Immune complexes were collected by centrifugation at 12,000 x g for 1 minute. Immunoprecipitates were washed five times with 200 µl Lysis Buffer, by resuspension and centrifugation.

Immunoblotting

25-100 µg of cellular proteins were subjected to reducing SDS-PAGE on 7.5-12.5% polyacrylamide and then the proteins were transferred to nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20 (TBST) (0.15 M NaCl; 0.01 M Tris-HCl pH 7.4; 0.05% Tween 20) for 1 h at room temperature with gentle shaking. Membranes were then washed 3 times with TBST. For detecting proteins expression or activation, membranes were incubated with different primary antibodies, in TBST for 1 h at room temperature or overnight at 4°C, followed by 3 times washing with TBST and incubation with HRP conjugated secondary antibody, for 1 h at room temperature. Proteins were detected by chemoluminiscense.

Protein concentrations were determined by a dye-binding assay (Bradford) using bovine serum albumin as a standard.

Cellular proliferation assessment

1. DNA synthesis

Treated cells were incubated for different time periods and [3H] thymidine (1 Ci/mmol) was added for the final 3h of the incubation period. The culture media was removed, and the cells were washed three times with PBS, treated for 15 min with ice-cold 10% trichloroacetic acid, and then solubilized by treatment with 0.5M sodium

hydroxide at 25°C for 4h. Cell-associated radioactivity was then quantitated by liquid scintillation counting.

2. MTT assay

The anti-proliferative effect of different inhibitors was examined using MTT assay. The 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. Tests were conducted with 10^3-10^4 cells $well^{-1}$, plated in 200 µl media in 96-well plates, with six replicates. Cell proliferation was quantified after treatment. 10µl MTT reagent was added to each well, incubated for 4h at 37°C and lysed by addition of 100µl solubilization buffer. Optical density (OD) was measured using a spectrophotometer at 595 nm and relative cell viability was expressed as a percentage of that in untreated control cultures.

3. Cell counting by trypan blue excision

To investigate the effect of inhibitors, cells were seeded in 6-well plates at a density of 10^5 cells /well. The cell growth was evaluated at the indicated times, on harvested cultures by trypan blue vital cell count. The relative cell viability was expressed as a percentage of that in control cultures.

4. Colony-forming assay

Cells were trypsinized, resuspended in complete medium and counted. Known numbers of cells were cultured on 60-mm agar coated tissue culture dishes and treated. The number of cells per dish initially inoculated varied with the treatment dose so that the number of colonies surviving was in a range that could be counted conveniently. Cells were allowed to grow for two or three weeks until the surviving cells produced macroscopic colonies (of more than 50 cells). After staining with methylene blue the colonies were counted under magnification.

Apoptosis assessments

The number of apoptotic cells was quantified by calculating the percentage of cells with fragmented or condensed nuclei. 72h after treatment, cells were fixed in 4% paraformaldehyde (PFA) and smears were prepared on slides. The slides were stained with 4,6′-diamidino-2-phenylindole dihydrochloride (DAPI) and the percentage of

cells with fragmented nuclei was scored. Two hundred nuclei were assessed in each sample.

ELISA

1. Non-extraction IGF-I/IGF-II ELISA

The standards, the controls and the pretreated undiluted samples were incubated in microtitration plates wells, precoated with anti-IGF-I or anti-IGF-II antibodies. It was followed by an incubation step with HRP labeled anti-IGF-I or anti-IGF-II antibodies, then by washing and incubation with the substrate tetrametylbenzidine. An acidic stopping solution was added and the degree of enzymatic turnover of the substrate was determined by dual wavelength absorbance measurement at 450 and 620nm. Sets of IGF-I or IGF-II standards were used to plot a standard curve of absorbance from which the IGF-I or IGF-II concentrations from the samples were calculated. The DSL kit sensitivity was 0,01ng/ml for the IGF-I ELISA and 2,2ng/ml for the IGF-II.

2. Face ELISA for protein-specific phosphorylations: p38 and JNK.

Cells were cultured in a 96 well plate, treated as described above and then assayed with the antiphospho p38 kinase or JNK kinase antibody, using the following procedure: at the desired time, cells were fixated in formaldehyde 4% for 20 minutes at room temperature. A buffer containing 1% H2O2 and 0,1% sodium azide was added for another 20 minutes, 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 dilution and incubated overnight, at 4ºC. The 1:1000 diluted HRP conjugated secondary antibody was incubated for 1 h at room temperature. Each of the above step described was followed by a 15 minutes of washing. The colorimetric reaction was started by adding 100 μl of Developing Solution, protected from direct light. After 10-15 minutes, when the darkest stained wells were medium to dark blue, the reaction was stopped with 100 μl Stop Solution. The absorbance was read within 5 minutes at 450nm wavelength. In the same experiment, the cell number was quantified by using Crystal Violet staining. After 30 minutes incubation at room temperature with 100 μl of Crystal Violet stain, 100 μl of 1% SDS were added per well and incubated on an orbital shaker. The absorbance was read 1 hour later at 595 nm

wavelengths. The p38 kinase or JNK kinase phosphorylation was evaluated relative to cell number.

3. Ku-DNA repair protein ELISA.

After cell treatment, nuclear extract from each sample was prepared. The assay used a 96 well plate, provided by the manufacturer, each well having mobilized a linear oligonucleotide with a blunt end. Positive controls, provided by the manufacturer were included. The Ku competitor oligonucleotides, provided by the manufacturer were also included, in order to monitor the specificity of the assay. All reactions took place at room temperature, with mild agitation. Between all steps, the wells were washed 3 or 4 times. 10 μg of nuclear extract on each well were added in triplicates, in combination with 40 μl Binding Buffer. After 1 h, the primary antibody was added in a dilution of 1:200, followed by the secondary HRP conjugated antibody diluted 1:1000. Each antibody was incubated for 1 h. The colorimetric reaction was started by adding 100 μl of Developing Solution protected from direct light. After 2-4 minutes, when the color in the positive control wells turned medium to dark blue, the reaction was stopped with 100 μl Stop Solution. The absorbance was read within 5 minutes at 450 nm wavelength.

Transfection

IGF-1Rβ siRNA was transfected using a commercial siRNA kit, according to the manufacturer's instructions. U1810 and A549 cell lines were seeded one day before, at an optimal density for high transfection efficiency, transfected with 100nm IGF-IR si-RNA and incubated overnight. After the transfection mixture was replaced with fresh normal growth medium, the cells were subjected to different experiments.

For the dnJNK1 plasmid, 18 and 38 HGG cells, plated at subconfluent density were 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.

Cell cycle distribution

Cells were treated and fixed in 4% buffered formaldehyde for 18 hours at room temperature. Formaldehyde was removed by 95% ethanol for 1 hour followed by rehydration in distilled water for 1 hour. After treatment with Carlsberg solution

[0.1% Sigma protease XXIV, 0.1 M Tris and 0.07 M NaCl (pH 7,5)] and staining with DAPI-Sulforhodamine solution [8 M DAPI, 50 M Sulforhodamine 101, 0.1 M Tris and 0.07 M NaCl (pH 7.5)], samples were analysed using a PAS II flowcytometer (Partec, Münster, Germany) equipped with a 100 W mercury arc lamp HBO 100. DAPI fluorescence was measured above 435 nm. The multicycle program for cell cycle analysis (Phoenix flow systems, San Diego, CA) was used for histogram analysis; the number of nuclei/histogram was 40,000.

Irradiation

Cells were irradiated in a culture medium at room temperature using a ^{137}Cs source at a dose rate of 1,55 Gy/min.

2.3 SUMMARY OF THE PAPERS

Paper1

This study compared the effects of IGF-IR inhibition on viability and apoptosis of two NSLCL cell lines, using three different methods for the impairment of IGF-IR function: αIR3, an anti-IGF-IR antibody; tyrphostin AG1024, a tyrosine kinase inhibitor and IGF-IR-siRNA. IGF-IR inhibition led to a decrease of cell survival and induced apoptosis in a manner depending on the approach used for the receptor inhibition. To find an explanation, we analyzed the effects of these treatments on three major antiapoptotic pathways evoked by IGF-IR signaling: IRS-1, Shc and 14.3.3-dependent mitochondrial translocation of Raf-1 kinase (mitRaf). αIR3 downregulated IRS-1 phosphorylation while AG1024 and IGF-IR-siRNA treatment decreased both IRS-1 and Shc phosphorylation. A significant decrease in the mitRaf was observed after IGF-IR-siRNA treatment. Neither αIR3 nor AG1024 had any effect on Raf-1 kinase translocation. In addition, the IGF-IR-siRNA proved to be the most potent inducer of apoptosis suggesting that more than one antiapoptotic pathway in IGF-IR signaling should be inhibited to effectively induce apoptosis in lung cancer cells.

Paper2

In this study, we evaluated the cell surface expression of IGF-IR and the antitumoral effect of IGF-IR blockade in combination with irradiation in 6 non-small cell lung cancer (NSCLC) cell lines. Our results indicated that the IGF-IR was present on NSCLC cells as evaluated by competitive binding assay and the amount of binding sites ranged from 118 fmol/mg to 377 fmol/mg protein. In one cell line (U1810), the combined treatment led to synergistic cell death and it was associated with an accumulation of cells in the G_2 phase. IGF-IR activation was able to obstruct serum starvation/radiation-induced cell death in U1810 cell line. Additive interactions were found for four cell lines (A549, H157, H23 and H125) whereas only subadditive effects were observed in U1752 cell line. Our results indicated that the presence of IGF-IR on NSCLC cell surface and the receptor involvement in the modulation of radiosensitivity in lung cancer cells.

Paper3

Based on the results from Paper 2, we chose a NSCLC cell line to evaluate the ionizing radiation exposure effects with regard to IGF-IR involvement. This study demonstrates for the first time that ionizing radiation activates IGF-1R in U1810 NSCLC cell line and inhibition of IGF-1R signaling via p38 kinase induces radiosensitivity by a novel mechanism involving nuclear Ku86.

Ionizing radiation activates IGF-1R within 10 minutes, with a maximal activation effect 2 hours post-irradiation. Impairment of IGF-1R tyrosine kinase activity enhances human lung cancer cells radiosensitivity by a mechanism that involves phosphatidylinositol 3-kinase (PI3-K) and p38 kinase. In an active form, IGF-1R binds and activates p38 kinase, promoting receptor signaling. Conversely, inhibition of IGF-1R phosphorylation results in IGF-1R/p38 complex disruption and p38 kinase inactivation. We have also demonstrated that in IGF-1 stimulated cells, Ku-DNA-binding activation is induced by ionizing radiation within 4 hours, reaches a maximum level at 12 hours and remains active up to 72 hours. Blockade of IGF-1R activity or its downstream signaling through p38 kinase induces a decrease in radiation-mediated Ku-DNA-binding activation and downregulates the level of Ku86, without affecting Ku70 expression in the nucleus of U1810 cells. The IGF-1R signaling *via* PI3-K does not interfere with the p38 signaling, the Ku-DNA-binding activity or the level of Ku86.

Paper 4

In this study, we found co-expression of IGF-1R and PDGFR in two high-grade gliomas (HGG) cell lines 18 and 38. Dual targeting of IGF-1R and PDGFR increased cell death in both 18 and 38 cell lines 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 HGG 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, HGG cells were treated with dominant negative JNK1 (dnJNK1) and the response to radiation was assayed in presence or absence of receptors co-inhibition. Indeed dnJNK protected 18 cells against γ-irradiation induced cell death. dnJNK treatment did not influence radiation response of the 38 cell line, which expressed low levels of JNK1.

2.4 DISSUSSION AND CONCLUSIONS

Our studies are placed in the general setting of IGF-IR demonstrated to be a major survival factor for tumor cells and a promising therapeutic target in cancer.

IGF-IR is ubiquitously expressed in most normal tissue (Werner H 1991) and many types of cancer. Nevertheless no pattern of expression and no correlation with any marker could be found for cancers. Instead, IGF-IR presence on the cell surface was suggested as a minimum requirement for the receptor targeting (Yee 2006). In this regard, we chose for our study a panel of cell lines, representative for both NSCLC and HGG in which IGF-IR was expressed. IGF-IR proved to be a molecule of importance in the survival of NSCLC cell lines. The receptor inhibition decreased cell proliferation and induced apoptosis. Interestingly, we found these effects depending on the type of IGF-IR inhibition. The mechanism by which IGF-IR protects tumor cells from apoptosis was extensively investigated and well described (Peruzzi, Prisco et al. 1999; Navarro and Baserga 2001), thus we analyzed how the inhibitors targeting the receptor influenced the proapoptotic downstream pathways emerging from IGF-IR. Out of many approaches available for receptor inhibition, we used three of them: the mAb and the TKIs were chosen for their clinical applicability and siRNAs, although used in preclinical experiments only, were chose for their powerful inhibition of the protein expression. Our results suggested that IGF-IR siRNA induced a dramatic decrease in cell survival probably by efficiently inducing the apoptosis machinery through blocking all described IGF-IR signaling pathways. Unfortunately, in further studies we encountered technical problems related to the siRNAs delivery into different cell types. We tried to use as delivery techniques, electroporation or vector delivery and tried to optimize the transfection parameters from one cell type to another. However transfection was not successful for all cell lines. In fact this is one of the main disadvantages of siRNAs in general, raising the question if siRNA therapy could be translated into clinical praxis. Against this background, for the other studies that explored the IGF-IR function in the tumor cells response to IR we used either a mAb or a TKI.

For the NSCLC cell lines studied, combination of IGF-IR inhibition with irradiation induced a greater degree of cell death, in comparison to either of them alone. This cooperative antiproliferative effect was valid for five out six cell lines. One cell line, (U1752) failed to demonstrate any additive effect after combined treatment and is

currently under investigation for other potential mechanisms involved in resistance to ionizing radiation. In contrast, another cell line (U1810) showed high synergistic cytotoxic effect after the combination therapy. Therefore, the consecutive, more detailed studies were focused on this cell line. In addition, previous results obtained by colleagues in our research group found U1810 to be an interesting radioresistent cell line. It harbored an irradiation-specific block in an apoptotic pathway potentially associated with SAPK signaling, which failed to activate p38MAPK or JNK upon IR (Viktorsson, Ekedahl et al. 2003). Our results showed that IGF-IR activation counteracted serum starvation/radiation induced cell death and IGF-IR inhibition combined with IR led to an accumulation of cells in G2 phase with a concurrent reduction of S phase. These results indicated IGF-IR involvement in the modulation of radiosensitivity in U1810 cells in particular and in other NSCLC cell lines in general.

The HGG cell lines responded differently to IGF-IR inhibition and a cross talk between IGF-IR and PDGFR was found. The IGF-IR and PDGFR concomitant inhibition caused increased cell death in both cell lines studied in comparison to either inhibitor alone and augmented radiation induced cell death only in one cell line.

The next step was to elucidate the IGF-IR-mediated signal transduction pathways in response to radiation and as expected we saw that the mechanism was very complex.

For the NSCLC cells, we used as a model U1810 cells that were most affected by the IGF-IR inhibition in combination with IR. In this cell line, we were able to demonstrate for the first time that irradiation activates IGF-IR. IGF-IR responded to IR with receptor activation, as reflected by the two to six fold increase in tyrosine phosphorylation. We wanted to be sure that the activation was induced by ionizing radiation only. Therefore we first excluded the existence of a potential IGFs autocrine loops. Secondly, all experiments were conducted under serum free conditions in order to exclude the interference with other ligands. Our result was further confirmed by the finding that radiation-induced IGF-IR activation had a two-fold increase upon IGF-I stimulation. The role of radiation-induced IGF-IR activation and the downstream consequences were studied in more detail through the selective inhibition of IGF-IR, PI3-K and p38 kinase. PI3-K and p38 kinase were previously identified as IGF-IR-mediators of cytoprotective responses after radiation (Heron-Milhavet, Karas et al. 2001; Wan, Wang et al. 2001; Wen, Deutsch et al. 2001). We found that both PI3-K and p38 kinase are part of IGF-IR mediated radioprotection in U1810 cells. Interestingly IR -induced activation of IGF-IR resulted in binding of IGF-IR to p38

kinase, inducing p38 kinase phosphorylation. Moreover, IGF-IR inhibition decreased Ku-DNA-binding capacity and caused the reduction of Ku86 nuclear level in U1810 irradiated cells, a mechanism that was mediated by p38 kinase, but PI3-K independent. This was in accordance with the hypothesis that IGF-IR might also prevent radiation-induced cell death by interfering with proteins involved in the repair of DNA lesions (Heron-Milhavet, Karas et al. 2001; Trojanek, Ho et al. 2003). Thus impaired IGF-1R function increased radiosensitivity by a novel mechanism involving decrease in Ku-DNA-binding activity and nuclear Ku86 downregulation. This novel mechanism was provided to be p38 kinase-dependent.

In HGG cell lines the radiosensitisation mechanism was different and the key protein involved was JNK1. Only the 18 cell line, highly expressing JNK1 was radiosensitized upon IGF-IR and PDGFR inhibition. Corroborating all the results of this study with our previous knowledge regarding RTKs response to radiation, we speculated for these cells that the mechanism of radioresistance might involve two different signals: one triggered by the RTKs activation after IR and the other involving JNK1 directly.

In conclusion the major findings of this research project are:

- IGF-IR is present and important for cell survival in the panel of cell lines studied
- IGF-IR inhibition modulates the response to radiation.
- Ionizing radiation activates IGF-IR in a NSCLC cell line
- For NSCLC, impaired IGF-IR function increased radiosensitivity by a novel mechanism, involving decrease in Ku-DNA-binding activity and nuclear Ku86 downregulation *via* p38.
- For HGG, impaired IGF-IR function increased radiosensitivity when combined with PDGFR inhibition and only in JNK1 expressing cells

Collectively, our results suggest that IGF-IR targeting may be of benefit when combined with radiation, but the results are highly dependent on the cell type. In this regard of great importance is the improvement of the molecular characterization of different tumors, which will lead to a better understanding of the complex biological processes underlying the therapeutic response. Future research will clarify many of these questions and hopefully allow a biologically optimized and patient individualized therapy.

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