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TARGETING THE GH/IGF-1 AXIS WITH NOVEL, SMALL MOLECULE INHIBITORS

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ABSTRACT

The growth hormone (GH) / insulin-like growth factor (IGF) family of ligands, binding proteins and receptors play multiple roles in cell growth, metabolism and development. In addition, numerous studies have demonstrated the pathophysiological importance of the GH/IGF-1 axis. In particular, the impact of IGF-1 receptor (IGF-1R) in cancer has attracted increasing attention during the last decade. Several classes of pharmacological agents that inhibit GH/IGF-1 signaling at different levels have shown anticancer activity both *in vitro* and *in vivo*. GH receptor (GHR) antagonists have proven the most effective and safe way to pharmacologically treat overproduction of GH (acromegaly) and antibodies against the IGF-1R cause massive apoptosis in vitro and tumor regression in animal models. However, there is a need to develop low molecular weight compounds targeting the GH/IGF-1 axis which can be administered perorally and have increased bioavailability compared to protein drugs. In paper I, we present a new mRNA quantification method which was used to test a number of low molecular weight compounds for their ability to reduce GH-induced IGF-1 mRNA in primary hepatocytes. One such potential GHR antagonist, BVT-A, was selected, and in paper II its attenuating effect on several markers for GH/IGF-1 overactivity was verified in an animal model of acromegaly. Picropodophyllin (PPP) was discovered some years ago as an effective inhibitor of IGF-1R signaling in cell lines and tumor repressor *in vivo*. The mechanism by which PPP caused this effect has not been fully delineated. In paper III, we show that IGF-1R knockout cells at late passages can acquire IGF-1R expression and dependency and therefore become sensitive to PPP treatment. Paper IV describes the inhibitory effect of PPP on IGF-1 induced vascular endothelial growth factor (VEGF) production as well as on neovascularization of the choroid in a model of macular degeneration, the most common cause of blindness. In paper V we show that PPP induces downregulation of the IGF-1R. This effect is important since it is known that downregulation, not only deactivation of the receptor is necessary for induction of massive apoptosis. Finally, in paper VI we show that PPP recruits the E3 ligase Mdm2 and β-Arrestin1 to the IGF-1R. This action was found to be involved in receptor downregulation and inhibition of AKT signaling and suggests that PPP acts as a β-Arrestin1-biased IGF-1R agonist. Hopefully, the substances identified and evaluated in this thesis will function as lead compounds in the development of improved pharmaceutical agents against GH/IGF-1 dependent diseases.

LIST OF PUBLICATIONS

I. **Rosengren L**., Simko H., Aryan L., Axelsson-Lendin P., Chmielewska J., Mode A., and Parrow V. (2005) Antisense- and sense RNA probe hybridization to immobilized crude cellular lysates – a tool to screen GH antagonists. *J. Biomol. Screen.* 10, 260-269

II. **Rosengren L,** Parrow V, Chmielewska J, Mode A, Fholenhag K. (2007) In vivo evaluation of a novel, orally bioavailable, small molecule growth hormone receptor antagonist. *Growth Horm IGF Res.* Feb;17(1):47-53.

III. **Rosengren L,** Vasilcanu D, Vasilcanu R, Fickenscher S, Sehat B, Natalishvili N, Naughton S, Yin S, Girnita A, Girnita L, Axelson M, Larsson O. (2006) IGF-1R tyrosine kinase expression and dependency in clones of IGF-1R knockout cells (R-). *Biochem Biophys Res Commun.* Sep 8;347(4):1059-66.

IV. Economou MA, Wu J, Vasilcanu D, **Rosengren L**, All-Ericsson C, Van der Ploeg I, Menu E, Girnita L, Axelson M, Larsson O, Seregard S, Kvanta A. (2007) Inhibition of VEGF secretion and choroidal neovascularization by picropodophyllin (PPP), an inhibitor of the insulin-like growth factor-1 receptor. *Invest Ophtamol Vis Sci*. In press

V. Vasilcanu R, Vasilcanu D, **Rosengren L,** Natalishvili N, Sehat B, Yin S, Girnita A, Girnita L, Axelson M, Larsson O. (2007). Picropodophyllin induces downregulation of the insulin-like growth factor 1 receptor: potential mechanistic involvement of Mdm2 and β-Arrestin1. *Oncogene*. Sept 10

VI. **Rosengren L**, Girnita L, Axelson M, Larsson O. (2007). Picropodophyllin (PPP) acts as a β-Arrestin1-biased IGF-1R agonist. *Submitted*

POPULAR SCIENTIFIC SUMMARY

The Growth Hormone / Insulin-like Growth Factor axis is a complex system of ligands, binding proteins and receptors which the cells use for communication of growth stimulating signals. GH is released from the pituitary and by binding to the GH receptor it stimulates longitudinal growth and has anabolic effects on metabolism. Most of the growth-promoting effects of GH are mediated by IGF-1 which is produced mainly in the liver after GH stimulation. IGF-1 has crucial functions especially for embryonic growth and development. On the cellular level, IGF-1 stimulates cell growth and multiplication and inhibits programmed cell death. There is also increasing evidence that overactivity of the GH/IGF-1 axis is associated with the development of several human cancers. In particular, a high level of IGF-1 in serum is associated with increased risk of developing cancer in the prostate, colon, and breast. Overproduction of GH by a pituitary tumor causes acromegaly, a serious disease with high mortality as well as an increased risk of developing other cancers. Increased activity of the GH/IGF-1 system is also involved in pathological blood vessel growth in the kidneys and retina as well as in psoriasis.

There is increasing interest in developing GH/IGF-1 inhibitors for use as cancer treatment. Preferably the drugs developed should be small molecules, possible to administer as tablets. There are several ways to interfere with the GH/IGF-1 system. The secretion of GH can be inhibited by affecting the normal control mechanism for hormone release. The most effective and safe way to medically treat acromegaly is to inhibit binding of GH to its receptor. However, the only existing therapy with this mechanism today is a protein drug with the well-known disadvantages off this class of drugs; expensive production and costly and painful injections. In the studies presented in this thesis we first describe a method for identifying inhibitors of the GH receptor that reduce the production of IGF-1. After identifying one such low molecular weight inhibitor, we continued to test the activity and specificity of this molecule in cells and in an animal model of acromegaly. We could conclude that the compound was effective in decreasing several parameters of the overactive GH/IGF-1 system in the acromegaly model and this drug therefore seems to be a good candidate for further studies and potential clinical trials.

IGF-1R activity can be reduced by several means such as inhibition of synthesis of receptor protein, degradation of the receptor, competitive inhibition of ligand binding or

inhibition of receptor activation. Several of these strategies show promising results in cell-lines and animals. However, the IGF-1R and the insulin receptor (IR) are very similar in structure and it has been difficult to find a selective inhibitor that does not affect IR and thereby cause diabetic symptoms. Some years ago it was discovered that the low molecular weight compound Picropodophyllin (PPP) decrease IGF-1R activity but does not affect the IR. PPP decreased tumor cell growth and reduced tumor size in animal models of cancer. The mechanism by which PPP caused this effect has not been fully understood. In these studies we show that PPP induce degradation of the IGF-1R and that this is an important feature of its effect on tumor cells. We also show that the IGF-1R degradation is responsible for the PPP effect on IGF-1R signaling. In another part of these studies we also show why cells that lack the IGF-1R can become sensitive to PPP-treatment, even though this drug seems so specific. Finally we show that PPP treatment can decrease pathological growth of new blood vessels in the eye, a common cause of blindness. Hopefully, the drugs identified and evaluated in this thesis will in the future be tested and evaluated in patients and developed into improved drugs against GH/IGF-1 dependent diseases.

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LIST OF ABBREVIATIONS

A1BG	α1B-glycoprotein
AKT	protein kinase B
ALS	acid-labile subunit
AMD	age related macular degeneration
cDNA	complementary deoxyribonucleic acid
CNV	choroidal neovascularization
CYP	cytochrome P450
EGF	epidermal growth factor
ELISA	enzyme-linked immuno-sorbent assay
ERK	extracellular signal-regulated kinase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GH	growth hormone
bGH	bovine growth hormone
hGH	human growth hormone
rGH	rat growth hormone
GHBP	growth hormone binding protein
GHR	growth hormone receptor
GHRH	growth hormone releasing hormone
GPCR	G-protein coupled receptor
HX	hypophysectomized
IGF-1	insulin-like growth factor 1
IGF-1R	insulin-like growth factor 1 receptor
IGFBP	insulin-like growth factor binding protein
IR	insulin receptor
IRMA	immuno radiometric assay
IRS	insulin receptor substrate
JAK	janus kinase
kDa	kilo Dalton
MAPK	mitogen activated protein kinase
Mdm2	mouse double minute 2
mRNA	messenger ribonucleic acid
PBS	phosphate buffered saline
PDGF	platelet derived growth factor

PI3K	phosphatidylinositol-3-kinase
PPP	picropodophyllin
PRL	prolactin
PRLR	prolactin receptor
RIA	radio immuno assay
RPA	RNase protection assay
RTK	receptor tyrosine kinase
RT-PCR	real-time polymerase chain reaction
SCID	severe combined immuno deficiency
SH2	src homology 2
siRNA	small interfering RNA
SPA	scintillation proximity assay
STAT	signal transducers and activators of transcription
TKI	tyrosine kinase inhibitor
Ub	ubiquitin
VEGF	vascular endothelial growth factor
wt	wild type

GENERAL INTRODUCTION

THE GROWTH HORMONE SYSTEM

GH synthesis and secretion

Growth hormone (GH) is a single polypeptide chain of 191 amino acids, 22 kDa, containing 2 disulphide bridges (Li and Dixon 1971). GH is synthesized in the anterior pituitary, where it is stored in secretory granules. It is the most abundant hormone in the pituitary accounting for 25% of the gland's hormones (Matsuzaki, Irie et al. 1971). The synthesis and release of GH is predominantly controlled by the opposing actions of two hypothalamic peptides, the stimulatory growth hormone releasing hormone (GHRH) and the release inhibiting hormone somatostatin (Brazeau, Vale et al. 1973; Mayo, Godfrey et al. 1995). These hypothalamic influences are tightly regulated by an integrated system of neural, metabolic and hormonal factors (Jansson, Eden et al. 1985; Devesa, Lima et al. 1992). Ghrelin, a peptide hormone that is mainly produced in the stomach is a natural ligand of the GH secretagogue receptor and acts synergistically with GHRH on GH release (Kojima, Hosoda et al. 1999; Takaya, Ariyasu et al. 2000). In addition, ghrelin and somatostatin seem to be involved in a feedback loop in humans (Arosio, Ronchi et al. 2003; Barkan, Dimaraki et al. 2003). Ghrelin is a functional antagonist of somatostatin and somatostatin antagonizes the actions of ghrelin at the level of the pituitary gland in rat (Tannenbaum, Epelbaum et al. 2003). Ghrelin levels are independent of gender and pubertal status but are negatively associated with obesity and insulin levels (Higgins, Gueorguiev et al. 2007). Insulin-like growth factors, produced in response to GH, inhibit GH release via negative feedback on the pituitary, and via stimulation of somatostatin release from the hypothalamus. There is also a direct negative feedback by which GH stimulates hypothalamic somatostatin release (Clark, Carlsson et al. 1988). GH release in humans is promoted by stress, particularly hypoglycemia, surgery and extensive exercise, deep sleep and some dietary amino acids, while in the rat most of these factors have inhibitory effects on GH secretion (Giustina and Veldhuis 1998). GH secretion is low in infancy, peaks at puberty and thereafter declines with ageing (Ho, Evans et al. 1987; Westgren 1989). GH is released in a pulsatile, gender specific manner in all species although the sex difference in secretion pattern is more pronounced in rodents than in humans (Eden 1979; Jaffe, Turgeon et al. 2002).

Biological effects of GH

GH plays a crucial role for postnatal somatic growth, but is not essential for intra-uterine growth and development (Laron 1993; Takahashi, Kaji et al. 1996). GH stimulates proliferation of chondrocytes and osteoblasts thereby promoting linear bone growth (Isaksson, Lindahl et al. 1987). Even after epiphyseal closure, GH is active in bone remodeling by increasing bone mass, bone density and strength (Ohlsson, Bengtsson et al. 1998). GH also exerts many metabolic effects that persist throughout life. The overall metabolic effects of GH are anabolic, leading to increased lean body mass. GH enhances amino acid uptake in skeletal muscle and stimulates protein synthesis (Kostyo 1968; Mauras, O'Brien et al. 2000). GH also reduces fat mass by stimulating lipolysis in adipose tissue and muscle (Ottosson, Vikman-Adolfsson et al. 1995; Oscarsson, Ottosson et al. 1999). Acute administration of GH causes a temporary insulin-like effect on glucose uptake. In contrast, chronic exposure to GH leads to insulin resistance, hyperglycemia and increased hepatic gluconeogenesis and glycogenolysis (Jorgensen, Krag et al. 2004). The latter effects may be indirectly caused by the GH-induced lipolysis and elevated plasma free fatty acids that inhibit insulin activity. Central effects of GH in neuroprotection, cognitive function and well-being are increasingly recognized. GH receptors are expressed in many brain areas and GH is able to pass the blood-brain barrier although the mechanism is not yet understood (Schneider, Pagotto et al. 2003).

The GH receptor

GH exerts its biological effects by binding to specific cell surface growth hormone receptors (GHR) (Kelly, Djiane et al. 1991). GHR mRNA is found in most tissues and cell types, with the highest expression detected in liver (Mathews, Enberg et al. 1989). Expression of GHR is regulated by age, nutritional intake, GH itself, steroid hormones and insulin. Regarding regulation by GH, previous reports have shown increased GHR expression in rat liver by continuous, female specific GH secretion (Ahlgren, Norstedt et al. 1995). The GHR belongs to the cytokine/hematopoietin receptor family that includes the receptors for prolactin (PRL), erythropoietin, leptin, interferons, granulocyte colony stimulating factor and interleukins (Cosman, Lyman et al. 1990; Horseman and Yu-Lee 1994). These receptors are single-pass, transmembrane proteins that contain an extracellular ligand-binding domain, a transmembrane region and a cytoplasmic domain with elements responsible for signal transduction, receptor internalization and downregulation (Kopchick and Andry 2000). The human GH (hGH)

and hPRL receptors show high sequence similarity, particularly in the ligand binding domain (Nicoll, Mayer et al. 1986). hGH is able to bind both the hGHR and the hPRL receptor (hPRLR), while hPRL only binds the hPRLR (Cunningham and Wells 1991). hGH can also bind both the rat GHR (rGHR) and the rPRLR, thereby eliciting both somatogenic and lactogenic effects in the rat. In rodents, rat or bovine GH, often used in animal experiments, are considered to bind only to the GHR and thereby having only somatogenic effects (Ranke, Stanley et al. 1976).

GH binding protein

A soluble form of the GHR corresponding to the extracellular domain of the transmenbrane receptor is found in the circulation. In human, this so called GH binding protein (GHBP) is predominantly produced by proteolytic cleavage of the membrane-anchored GHR, while in rodents formation of GHBP occurs mainly by alternative splicing of the primary receptor transcript (Leung, Spencer et al. 1987; Baumbach, Horner et al. 1989; Schantl, Roza et al. 2004). This high affinity GHBP binds roughly 50 percent of the GH in plasma in man (Baumann, Amburn et al. 1988). The physiological role of GHBP is not completely clear. Experimental evidence suggest both agonistic effects (by delaying renal GH clearance) and antagonistic effects (by sequestering GH away from cellular GHR) (Baumann 2001).

GHR signaling

Initiation of GH signaling requires that a single GH molecule binds to two GHR monomers causing receptor dimerization. Proper dimerization occurs by sequential binding of the receptor to a high affinity site in GH followed by recruitment of the second receptor to a low affinity site (Cunningham, Ultsch et al. 1991). However, some evidence suggest the existence of preformed, unliganded receptor dimers that are activated by a ligand-induced change in receptor conformation (Behncken and Waters 1999; Frank 2002). In accordance with the receptor dimerization mechanism, dose-response experiments give rise to biphasic, bell-shaped kinetics (Ilondo, Damholt et al. 1994). GHR dimerization and ligand-induced conformational change bring the intracellular receptor domains into close proximity whereby downstream signaling events are triggered. Receptor-associated tyrosine kinase JAK2 (Janus kinase 2) is activated (Argetsinger, Campbell et al. 1993) and phosphorylates cytoplasmic domains of GHRs on tyrosine residues, thereby creating docking sites for SH2 (src homology 2) containing proteins (Herrington and Carter-Su 2001). Signal transducers and activators

of transcription (STAT) proteins are cytoplasmic transcription factors containing SH2 domains that bind to the activated GHR and become phosphorylated by JAK2. Phosphorylated members of the STAT family homo- or heterodimerize and translocate to the nucleus where they bind to specific DNA target elements and activate gene transcription (Herrington, Smit et al. 2000). Activation of the GH receptor can also initiate signal transduction via the Ras/Raf/mitogen-activated protein (MAP) kinase pathway leading to effects on gene transcription and metabolism (Vanderkuur, Butch et al. 1997). GH also stimulates phosphorylation of the insulin receptor substrates 1, 2, 3 (IRS-1, 2, 3) with subsequent activation of the phosphoinositol-3-kinase (PI3K) resulting in stimulation of glucose transport (Souza, Frick et al. 1994).

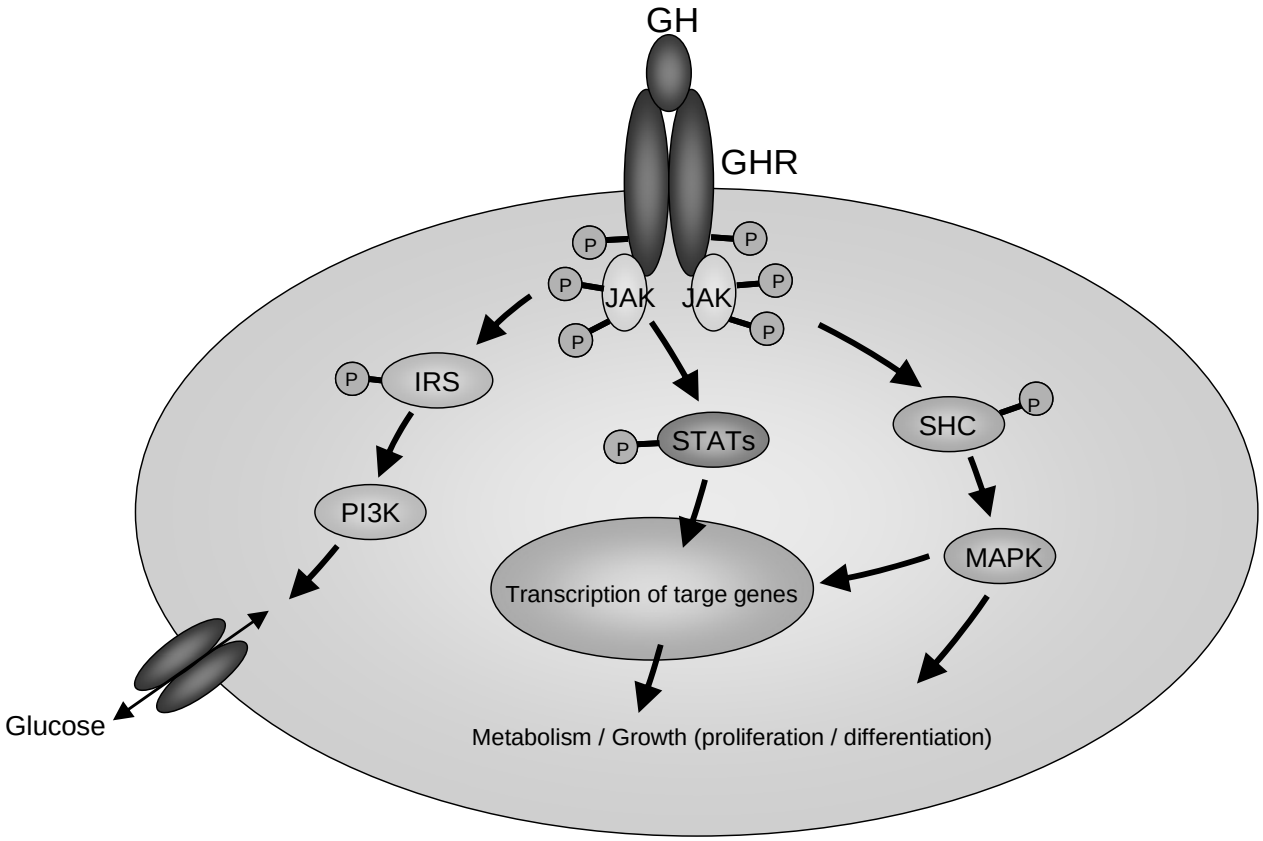

Figure 1: GH signaling

THE INSULIN-LIKE GROWTH FACTOR SYSTEM

IGF:s

Many of the growth-promoting actions of GH are mediated indirectly by the GH-induced production of insulin-like growth factor 1 (IGF-1) (Le Roith, Bondy et al. 2001). The IGFs were originally entitled sulfation factors due to their ability to restore the capacity of hypophysectomized rat serum to fully stimulate the incorporation of sulfate into rat chondrocytes *in vitro* (Salmon and Daughaday 1957). This function was not observed by addition of GH alone and demonstrated that IGFs in serum were GH-dependent. Later it was demonstrated that only a small component of the insulin-like activity of normal serum could be blocked by addition of anti-insulin antibodies. The remaining activity was therefore termed non-suppressible insulin-like activity (NSILA) and subsequently two low molecular weight proteins (~7 kDa) were identified to cause this effect (Burgi, Muller et al. 1966). A few years later it was discovered that the mitogenic effect of bovine serum could be substituted by a factor secreted from cultured hepatocytes, and this factor was now termed multiplication-stimulating activity (MSA) (Dulak and Temin 1973). The name Somatomedin was formulated about the same time, originally including all insulin-like growth factors (Daughaday, Hall et al. 1972). Even nowadays IGF-1 is still sometimes referred to as Somatomedin-C. Eventually, when their chemical structures were characterized, it was apparent that IGF-1 and IGF-2 share close structural homology to pro-insulin, and thus, they acquired their present names, (Rinderknecht and Humbel 1978; Daughaday and Rotwein 1989). The IGF family of peptides includes more than 10 different but structurally similar proteins including IGF-1, IGF-2, pro-insulin, relaxin and a number of relaxin-like peptides. IGF-1, the most intensely studied member of this family, is a basic peptide of 70 amino acids with a molecular mass of 7,5 kDa. The IGF-1 protein is built up by an A and B domain connected by disulfide bonds, a linking C-peptide and a carboxy terminal D-peptide. The IGF-1 molecule is synthesized as a prohormone containing a carboxy-terminal E-domain which is cleaved in the golgi apparatus before secretion (Daughaday and Rotwein 1989).

IGF-2, which shares 62% sequence identity with IGF-1 (Daughaday and Rotwein 1989), is important for fetal growth and development while postnatal growth, at least in rodents, is IGF-2 independent. The cellular actions of IGF-2 are propagated by the IGF-1 receptor while the IGF-2/mannose-6-phosphate receptor does not appear to function as a traditional signaling receptor. The IGF-2R is thought to act mainly as a clearance

receptor for IGF-2 and disruption of IGF-2R gene expression in mice results in elevated IGF-2 levels and overgrowth (Jones and Clemmons 1995). The IGF-2 gene displays parental imprinting. Thus, in most tissues of normal subjects, IGF-2 is produced only from the paternal allele, the maternal allele being transcriptionally silent (DeChiara, Robertson et al. 1991). Unlike IGF-1, the expression of IGF-2 is not regulated by GH but by other hormones and tissue specific growth factors (Nielsen 1992). IGF-2 seem to be involved in the progression of tumors by an autocrine mechanism, but expression of IGF-2 alone is not sufficient to induce full malignant transformation (Yakar, Leroith et al. 2005).

IGF-1 synthesis and secretion

Circulating IGF-1 is produced primarily in the liver, while local production in bone and muscle exerts important paracrine and autocrine effects in these tissues (D'Ercole, Stiles et al. 1984; Daughaday and Rotwein 1989). Multiple mRNA species exist for IGF-1 and this permits remarkable complexity in the regulation of gene expression, allowing for both tissue-specific expression of transcripts as well as both ontogenic and hormonal regulation. GH is the most prominent postnatal regulator of IGF-1 expression, but also other hormones, growth factors as well as nutritional status affects IGF-1 levels (Sara and Hall 1990). The levels of IGF-1 are low in human fetal serum, rise during childhood, peak at puberty and thereafter decline with ageing (Cohen 2006). During fasting, insulin production decreases, causing reduction in GHR expression and thereby GH resistance - that is, low IGF-1 secretion despite high concentrations of GH (Merimee, Zapf et al. 1982).

Biological effects of IGF-1

IGF-1 is classified as an anabolic and mitogenic hormone, it stimulates protein and glycogen synthesis, increases DNA synthesis, stimulates cell cycle progression and inhibits apoptosis (Jones and Clemmons 1995). Mice carrying null mutations of the IGF-1 gene are born small and grow poorly postnatally (Baker, Liu et al. 1993). Conditional liver knockout of the IGF-1 gene in mice results in significantly suppressed serum levels of IGF-1, but without any corresponding decrease in growth (Sjogren, Liu et al. 1999). This suggests that paracrine/autocrine IGF-1 effects might be more important than circulating IGF-1 for longitudinal bone growth. However, when these conditional liver-knockout mice were crossed with acid-labile subunit (ALS) gene-deleted mice, the total levels of circulating IGF-1 levels were further reduced. In the

circulation, ALS forms a complex with IGF-1 and IGFBP-3 (see below). This double knockout animal exhibits growth retardation, suggesting that circulating IGF-1 contributes to growth, but that local production of IGF also is of importance (Yakar, Rosen et al. 2002). In addition, these studies revealed that IGF-1 has dual effects on bone development; circulating IGF-1 plays a major role for bone density, whereas both circulating and local IGF-1 is involved in linear bone growth.

IGF binding proteins

IGF-1 is transported in plasma bound to a family of specific high-affinity IGF binding proteins (IGFBP-1 to IGFBP-6) that are unrelated to the cell surface receptor (Jones and Clemmons 1995; Firth and Baxter 2002; Mohan and Baylink 2002). These IGFBPs are closely related to each other structurally, although they are distinct gene products and they all have very distinct functional properties. The circulating IGFBPs bind IGF-1 with higher affinity than the IGF-1R and exert their main function in protecting IGF-1 from degradation and prolonging its half-life in plasma. The IGFBPs also sequester IGF-1 away from the IGF-1R, thereby inhibiting mitogenesis, differentiation, survival and other IGF-1 stimulated effects. Proteolysis of IGFBPs can reverse this inhibition or generate IGFBP fragments with novel bioactivity. There exist specific IGFBP proteases which in turn are controlled by specific inhibitors. In addition, IGFBP-interaction with cell surface components may concentrate IGF-1 near its receptor, enhancing IGF-1 activity. IGF-1-independent actions of the IGFBPs are also increasingly recognized (Baxter 2000). An explanation for the somewhat odd combination of IGFBP-effets could be that they evolved as part of a large family of cell regulatory proteins and then acquired the ability to bind IGFs and modulate IGF activity which complemented their original functions (Holly and Perks 2006). This is analogous to other binding proteins, for example corticosteroid-binding globulin, which evolved as part of a large family of proteinase inhibitors and subsequently acquired an ability to bind corticosteroids.

The majority of IGF-1 (about 75 percent) in serum is transported in a 150 kDa complex with IGFBP-3 and the acid labile subunit (ALS) that is present in excess (Clemmons 1998). This trimeric complex forms a reservoir of IGF-1 in circulation by extending the half-life from about 10 minutes when in free form, to 30 - 90 minutes in binary complexes, up to more than 12 hours when bound in the ternary complex and also by preventing passage of the complex across the vascular endothelial barrier (Rajaram, Baylink et al. 1997). ALS is expressed predominantly in the parenchymal liver cells,

and its expression is tightly regulated by GH (Dai and Baxter 1994). IGFBP-3 is expressed in the hepatic nonparenchymal cells as well as in endothelial cells. Hepatic expression of IGFBP-3 is also regulated by GH either directly (Gucev, Oh et al. 1997) or indirectly via IGF-1 production in adjacent hepatocytes (Chin, Zhou et al. 1994; Scharf, Ramadori et al. 1996). The binding of IGF-1 in a ternary complex also results in the build-up of very high concentrations such that in adult humans, the total IGF-1 concentration in the circulation is around 100 nM. This is approximately 1,000 times higher than insulin and most other peptide growth factors and hormones. Since at the cellular level, optimal regulation of the IGF-1R is achieved with just 1 to 2 nM IGF-1, there is obviously a vast excess in the circulation. In the tissues, IGF-1 concentrations are around a third of that in the circulation, but this is still much higher than the concentration needed for cell regulation (Holly 2004). Therefore, since IGF-1 is not stored within cells, there appears to be a large extra-cellular amount maintained in complexes with IGFBPs.

Most studies on the IGF independent actions of IGFBPs have focused on IGFBP-3 and IGFBP-5 (Mohan and Baylink 2002). Growth inhibitory and proapoptotic IGF-1 independent effects of IGFBP-3 have been reported both *in vitro* and *in vivo* (Hong, Zhang et al. 2002; Lee, Chun et al. 2002). A circulating protease that acts specifically on IGFBP-3 has been described in many different conditions (Maile and Holly 1999). Limited cleavage of IGFBP-3 can shift the complex equilibrium resulting in IGF-1 re-equilibrating to other IGFBPs that only form binary complexes and therefore have greater ability to transport IGF-1 out into target tissues. Numerous studies have reported increased IGFBP-3 proteolysis during pregnancy and other catabolic states where increased availability of an anabolic metabolic regulator could be an advantage (Hossenlopp, Segovia et al. 1990; Davies, Wass et al. 1991).

IGFBP-5 shares considerable structural homology with IGFBP-3 and is also involved in trimeric complexes with IGF-1 and ALS (Twigg, Kiefer et al. 1998). IGFBP-5 has been reported to have both IGF-1 stimulatory and inhibitory effects. It binds to extracellular matrix proteins, stimulates cell migration, and enhances bone formation and osteoblast proliferation (Firth and Baxter 2002). There have been reports of putative IGFBP-3 and IGFBP-5 cell surface receptors (Oh, Muller et al. 1993; Andress 1998) that could mediate their intrinsic actions. In addition to cell surface interactions, much interest has been generated by observations of nuclear targeting of IGFBPs and in identification of potential nuclear proteins as binding partners. IGFBP-3 and IGFBP-5 were observed possess nuclear localization sequences and to translocate to the nucleus

via an importin β-dependent pathway (Schedlich, Le Page et al. 2000), raising the question if these IGFBPs could directly modulate transcriptional activity. Subsequently it has been shown that IGFBP-3 can bind to the nuclear retinoid RXR receptor (Liu, Lee et al. 2000). However, the physiological consequence of the nuclear translocation remains to be established.

Animal experiments have shown that insulin reduces hepatic secretion of IGFBP-1 (Ooi, Tseng et al. 1992). Also in humans, elevated IGFBP-1 occurs universally in conditions associated with decreased insulin levels, including fasting, exercise, and insulin-dependent diabetes (Suikkari, Sane et al. 1989; Batch, Baxter et al. 1991; Cotterill, Holly et al. 1993). In the circulation, IGFBP-1 levels undergo a circadian variation due to this dynamic insulin regulation (Holly, Biddlecombe et al. 1988). This appears to provide an additional acute control to ensure that IGF-1 activity is appropriate to nutritional conditions. When nutrition is limited, insulin levels decrease, resulting in increased IGFBP-1 levels and reduced IGF-1 activity (Taylor, Dunger et al. 1990). A clearly defined mechanism has been established for the intrinsic actions of IGFBP-1 and -2. These are the only IGFBPs to possess an arginine-glycine-aspartic (RGD) motif which is a classical integrin receptor recognition sequence. IGFBP-1 has been shown to stimulate cell migration by engaging $\alpha_5\beta_1$ integrin, the fibronectin receptor (Jones, Gockerman et al. 1993). After integrin-binding, IGFBP-1 is reported to exert IGF-1 independent, proapoptotic effects in human breast cancer cells (Perks, Newcomb et al. 1999).

Several studies describe IGFBP-4 as a potent inhibitor of the anabolic effects of IGF-1 by regulating IGF-1 bioavailability. However, specific proteases belonging to the pregnancy-associated plasma protein A system, present in serum from pregnant women, can cleave IGFBP-4 into fragments with low affinity for IGF-1 thereby increasing the anabolic IGF-1 effects (Parker, Gockerman et al. 1995).

The IGFBPs thus maintain a readily available extracellular store of IGF-1 and provide a mechanism for integrating IGF-1 activity with many other regulators via a sophisticated interplay of multiple components which appears to provide a means of conferring specificity such that cell functions can be very finely controlled in a tissue-specific manner.

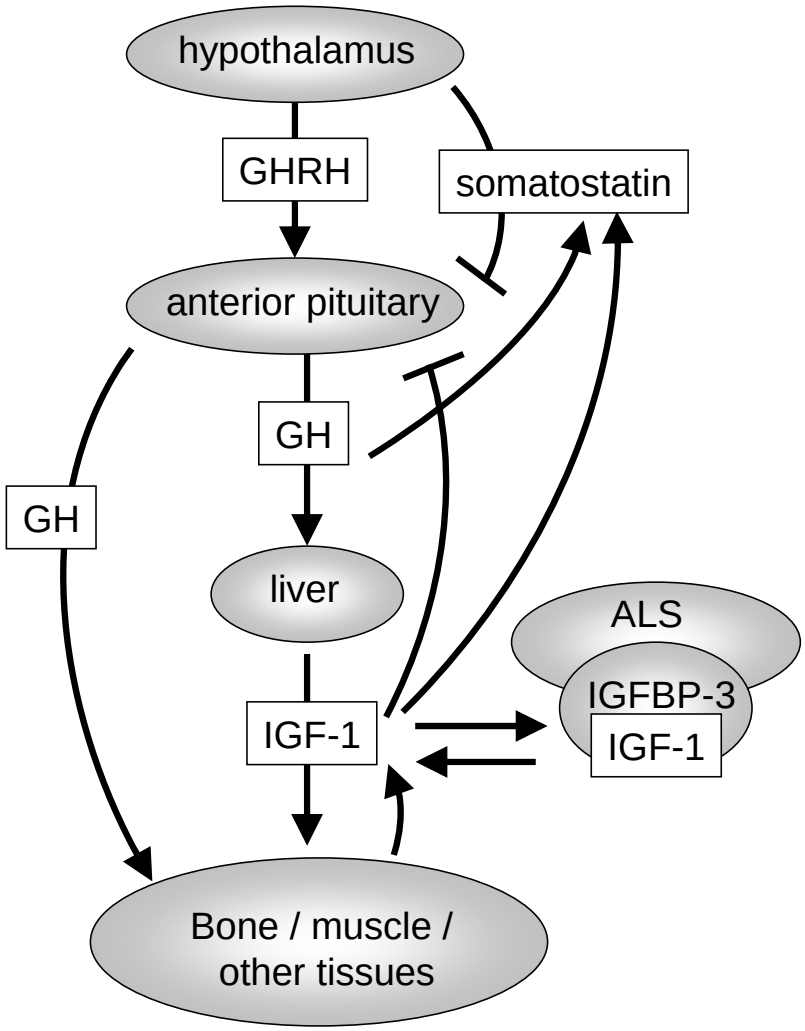

Figure 2: Interplay in the GH / IGF-1 axis

IGF-1 receptor

The biological actions of IGF-1 are mediated by the IGF-1 receptor (IGF-1R), a member of the receptor tyrosine kinase family. The functional IGF-1R is a transmembrane glycoprotein formed as a tetramer consisting of two identical α-subunits and two identical β-subunits connected by disulfide bonds (LeRoith, Werner et al. 1995; Sepp-Lorenzino 1998) The α-chains are exclusively extracellular and contain the ligand binding domain while the β-chains comprise a short extracellular segment, a transmembrane region and an intracellular tyrosine kinase domain (Adams, Epa et al. 2000). The IGF-1R binds IGF-1 with high affinity while IGF-2 is bound with 20% the affinity of IGF-1 and insulin is bound at less than 1% (Denley, Bonython et al. 2004). Virtually every tissue and cell type expresses IGF-1R mRNA, although it is a low-abundant transcript. In the adult rat, the highest levels of IGF-1 mRNA are

observed in the central nervous system, with intermediate levels found in kidney, stomach, testes, lung, heart and liver (LeRoith, Werner et al. 1995; Novosyadlyy, Tron et al. 2004). Expression of the IGF-1R is stimulated by hormones including estrogens, corticosteroids and growth factors such as GH, basic fibroblast growth factor, platelet-derived growth factor and epidermal growth factor. Oncogenes such as the Hepatitis B virus oncoprotein and Ewing Sarcoma fusion protein also induce IGF-1R transcription. In contrast, IGF-1R expression is inhibited by tumor suppressors, such as p53, Wilms' tumor-1 and BRCA-1. The expression of IGF-1R declines with ageing and is subject to negative feedback regulation by high IGF-1 levels (LeRoith, Werner et al. 1995; Werner, Shalita-Chesner et al. 2000). IGF-1R knockout results in more severe growth failure than deletion of IGF-1 and the IGF-1R null mice die of respiratory failure at birth (Liu, Baker et al. 1993), showing the importance of IGF-1R for embryonic development. However, strong evidence has been provided that IGF-1R is not an absolute requirement for normal growth of adult cells, only under anchorage-independent conditions (LeRoith, Werner et al. 1995; Ludwig, Eggenschwiler et al. 1996; Baserga 1997; Baserga 1999).

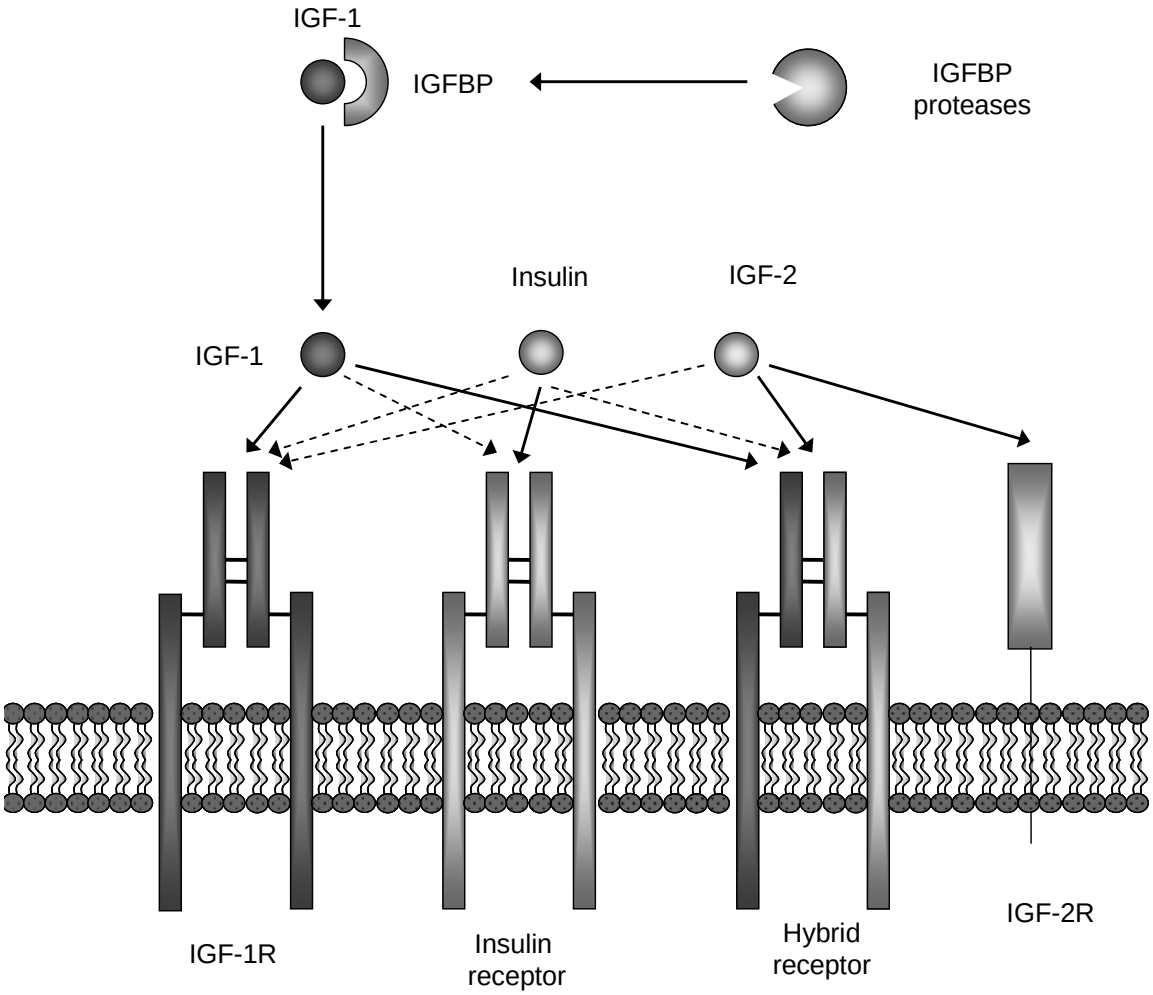

Figure 3: The IGF / insulin receptor family

IGF-1R signaling

Binding of IGF-1 to the extracellular domain of IGF-1R induces a conformational change in the receptor that triggers trans-autophosphorylation of three tyrosine residues within the kinase domain resulting in increased catalytic activity (Kato, Faria et al. 1993; Favelyukis, Till et al. 2001). Next, tyrosine residues within the juxtamembrane region and C-terminal serine residues are phosphorylated, creating docking sites for downstream signal transduction molecules. This induces recruitment and phosphorylation of the adaptor proteins insulin receptor substrates (IRS)-1 and -2, Shc and Grb10 and subsequently activation of the phosphatidylinositol 3-kinase (PI3K)-AKT pathway and the Ras/Raf/mitogen-activated protein (MAP) kinase pathway (Navarro and Baserga 2001). The signaling cascades emanating from the IGF-1R

ultimately lead to cell proliferation (predominantly mediated via the MAP kinase pathway) and inhibition of apoptosis (mainly via the PI-3 kinase pathway) but can also induce differentiation and malignant transformation. Moreover, the activated IGF-1R regulates cellular adhesion, cytoskeletal organization and migration through interaction with adhesion molecules and integrins (Gray, Stenfeldt Mathiasen et al. 2003). In normal physiology the IGF-1R stimulates linear body growth, promotes neuronal survival and myelination, postnatal mammary gland development and lactation and is implicated in bone formation and renal function (Jones and Clemmons 1995).

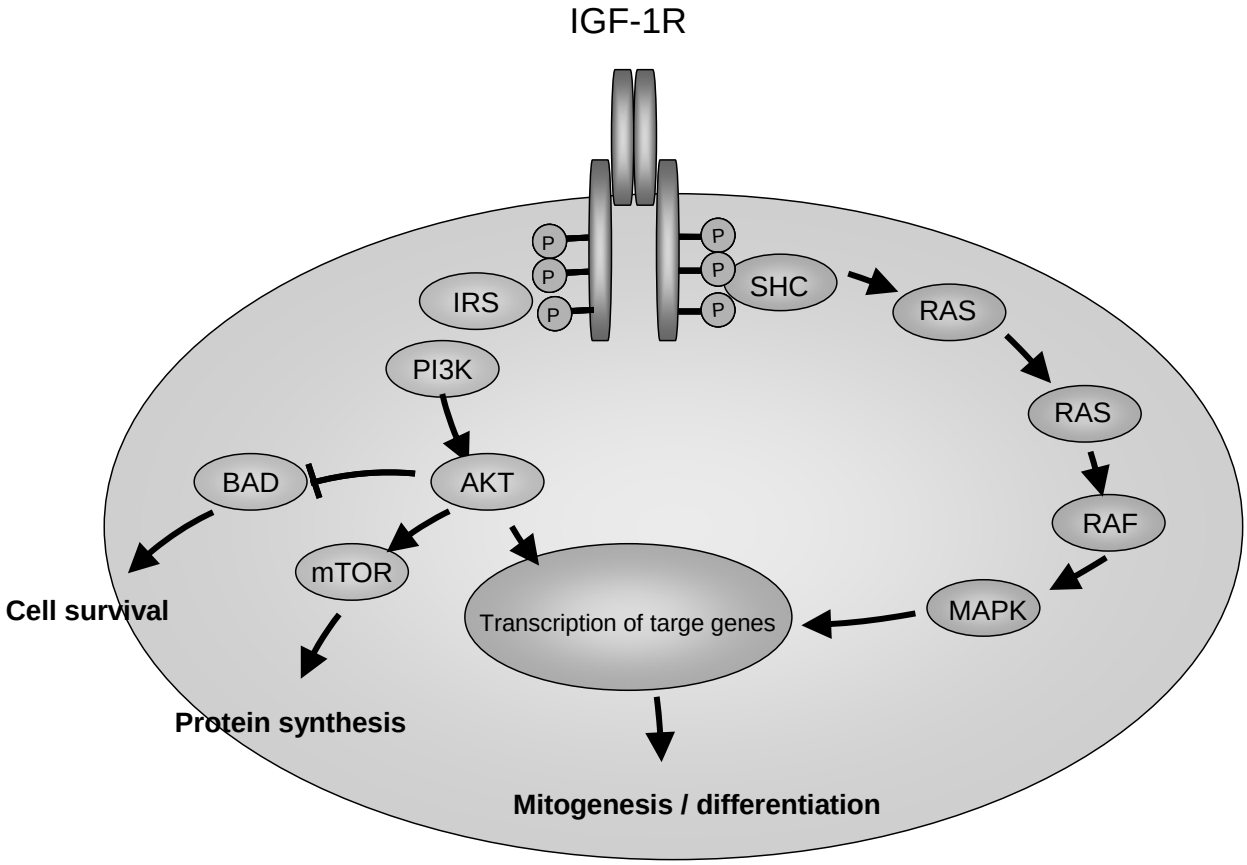

Figure 4: IGF-1 signaling

IGF-1R internalization / degradation

Many cell surface receptors undergo endocytosis via clathrin coated vesicles. Either the receptors recycle continuously or, as for the IGF-1R, they are internalized after ligand-stimulation (Chow, Condorelli et al. 1998). Receptor internalization leads to attenuation of some forms of extracellular signaling by reducing the number of cell surface receptors. In addition, growing evidences are now suggesting a very important cross-talk between receptor endocytosis, ubiquitination and ligand-mediated receptor

signaling (Di Fiore and De Camilli 2001; Weissman 2001; Seto, Bellen et al. 2002). For example, MAP kinase signaling from the IGF-1R and subsequent mitogenic response, has been shown to require receptor endocytosis (Chow, Condorelli et al. 1998).

The β-Arrestins (1 and 2) were originally discovered as desensitizers of G protein coupled receptor signaling. Later, β-Arrestins were also shown to be involved in endocytosis and signal transduction of GPCRs (Reiter and Lefkowitz 2006). Ubiquitination of β-Arrestin has been found to play a role in several of these functions (Lefkowitz, Rajagopal et al. 2006). β-Arrestins have also been demonstrated to bind to ligand-stimulated IGF-1R and promote IGF-1R internalization (Lin, Daaka et al. 1998).

Ubiquitination; the post-translational covalent attachment of one or more ubiquitin (Ub) molecules to lysine residues in the substrate protein, was originally identified as a protein tag for proteosomal degradation (Hershko, Ciechanover et al. 1980). Lately, ubiquitination has been discovered to play a role in protein trafficking and signal transduction (Hicke and Dunn 2003; Welchman, Gordon et al. 2005). Mono-ubiquitination of proteins seems to be required as signal for internalization of various membrane proteins whereas poly-ubiquitination normally targets them to the endocytotic pathway for rapid degradation by either the protasome or the lysosomal pathway, to mediate receptor desensitization (Hicke 2001). The ubiquitination process is highly regulated and requires the sequential action of three different enzymes; an E1-Ub activating enzyme, an E2-Ub carrying enzyme and an E3-Ub ligating enzyme (Hochstrasser 1996; Hershko and Ciechanover 1998). The specificity of the ubiquitin system is due primarily to the many families of E3 ligases, which recognize specific protein substrates through specific recognition sequences (Hershko and Ciechanover 1998).

It has been shown that the IGF-1R is a substrate for ubiquitination, and two E3 ligases; Mdm2 (Girnita, Girnita et al. 2003) and Nedd 4 (Vecchione, Marchese et al. 2003), have been demonstrated to be involved in this process. In Mdm2-mediated ubiquitination, β-Arrestin1 acts as a molecular scaffold by bridging the ligase to the receptor (Girnita, Shenoy et al. 2005). Similarly, Nedd4-mediated IGF-1R ubiquitination requires Grb10 to function as an adapter protein (Vecchione, Marchese et al. 2003). The interaction between IGF-1R, Mdm2 and β-Arrestin1 appears to be essential for receptor desensitization as well as ERK activation and cell cycle progression (Girnita, Shenoy et al. 2005; Girnita, Shenoy et al. 2007). Mdm2 has also been demonstrated to serve as the E3 ligase responsible for ubiquitination of β-Arrestin

and agonist stimulated β-Arrestin ubiquitination is necessary for rapid β-Arrestin-dependent receptor internalization (Shenoy, McDonald et al. 2001). The time course of β-Arrestin ubiquitination correlates with the stability of receptor-β-Arrestin interaction, i.e. transient ubiquitination is associated with transient association (Shenoy and Lefkowitz 2003).

Insulin and the insulin receptor

IGF-1, its receptor and signaling pathways are very similar to those of insulin, but still they have evolved to function as very different communication systems in mammals. Generally, the expression of insulin is restricted to the pancreas where it is stored in secretory granules within the islet β-cells from which it is secreted via a regulated pathway in response to stimuli such as glucose. IGF-1 on the other hand, is expressed widely throughout most tissues in the body. In addition, like most other peptide growth factors and cytokines, IGF-1 is not stored in secretory granules within cells, but instead secreted as it is produced, via the secretory pathway and stored outside of cells with soluble binding proteins.

Insulin Receptor (IR) is structurally and functionally related to IGF-1R. The kinase domains share the highest homology with overall 84 % sequence identity and 100 % sequence identity at the ATP-binding pocket (Ullrich, Gray et al. 1986; LeRoith, Werner et al. 1995). Upon insulin binding to IR, IRS proteins are phosphorylated and activated, which lead to activation of downstream pathways, including MAPK and PI3K pathways (Taniguchi, Emanuelli et al. 2006). Although similar pathways are activated, the physiologic effects mediated by IR and IGF-1R differ such that IGF-1R is mainly involved in regulation of cell proliferation, antiapoptosis, differentiation and cell motility while IR has most impact on glucose uptake and metabolism. High levels of IR are found in liver and adipose tissue, whereas IGF-1Rs are almost absent in liver and found at lower levels in adipose tissue. This partially explains some of the preferential effects of insulin on metabolic homeostasis (Dupont and LeRoith 2001).

Human IR exists in two isoforms determined by alternative splicing of exon 11 located at the carboxy-terminus of the receptor α-subunit: isoform A (IR-A) lacks exon 11, whereas isoform B (IR-B) contains a 12 amino acid extension encoded by this small exon (Moller, Yokota et al. 1989). The relative expression of these two isoforms varies in a tissue-specific manner. The IR-A isoform is expressed in fetal tissues and malignant cells and promotes cell growth in response to IGF-1 and IGF-2 (Frasca, Pandini et al. 1999; Pandini, Medico et al. 2003).

The structural similarities allow formation of hybrid receptors in which an IGF-1R αβ-chain is connected to an IR-A or –B αβ-chain. When IGF-1R and IR are co-expressed on the same cell, receptor hybrids can randomly assemble and the least abundant receptor is drawn predominantly into hybrid receptors (Kasuya, Paz et al. 1993; Siddle, Urso et al. 2001). In addition, hybrid receptors can be overexpressed in a variety of tumor cells as a result of both IGF-1R and IR overexpression (Papa, Gliozzo et al. 1993; Pandini, Vigneri et al. 1999). The hybrid receptors, particularly those incorporating IR-A moieties, bind IGF-1 and IGF-2 with high affinity resulting in both proliferative and anti-apoptotic responses (Pandini, Frasca et al. 2002).

In plasma IGF-1 circulates in up to thousand-fold higher concentrations compared to insulin. Despite the fact that the affinity of IGF-1 for the IR is only 5 percent of insulin's affinity for the IR, IGF-1 could in theory have a huge impact on glucose levels because of its abundance (Juul 2003). Some studies do point to a direct role of circulating IGF-1 in maintaining glucose homeostasis (Yakar, Liu et al. 2001). IGF-1R can partially compensate for loss of metabolic functions of the IR (Di Cola, Cool et al. 1997) and IGF-1 can interact with the IR although with a lower affinity than that of insulin (Steele-Perkins, Turner et al. 1988). Circulating IGF-1 indirectly confers important metabolic effects by controlling GH secretion and thereby modifying the biological role of insulin in maintaining normal metabolic homeostasis (Le Roith, Scavo et al. 2001). However, the effect of IGF-1 on IR is limited since the activity of IGF-1 is inhibited by binding to specific binding proteins (Juul 2003).

CANCER AND THE GH/IGF-1 SYSTEM

The involvement of IGF-1R in malignant transformation was first recognized in fibroblasts derived from mouse embryos carrying homozygous deletions of the IGF-1R gene (Sell, Dumenil et al. 1994). Normally, mouse embryonic cells are prone to transformation, but in the absence of IGF-1R they become resistant to malignant transformation by a number of oncogenes (e.g. Simian Virus 40T antigen, Ewings Sarcoma fusion protein, H-ras and c-*src*) (Valentinis, Morrione et al. 1997). Reexpression of the IGF-1R restored the susceptibility to transformation in these cells (Morrison, Tognon et al. 2002). Since homozygous IGF-1R knockouts are not viable (Liu, Baker et al. 1993), the in-vivo effects of decreased IGF-1R signaling have been studied in IGF-1 deficient animals. Low IGF-1 levels were associated with reduced growth and metastasis of tumors and xenografts and increased resistance to carcinogen-

induced tumorigenesis (Yakar, Leroith et al. 2005). On the other hand, several in-vivo studies in mice have shown increased tumor development after overexpression of IGF-1R (DiGiovanni, Bol et al. 2000; DiGiovanni, Kiguchi et al. 2000). In addition, IGF-1R overexpression in a mouse model of cancer led to more invasive tumors and increased amount of distant metastasis compared to parental mice (Lopez and Hanahan 2002). Together, these studies provided compelling evidence that active IGF-1R signaling facilitates malignant transformation, drives growth and progression of established tumors and enhance capability to invade and metastasize. Transgenic mice for both GH and agonists for the IGF-1R show an increased incidence of breast tumor development (Tornell, Carlsson et al. 1992; Bates, Fisher et al. 1995). In contrast, almost no growth of transplanted human breast cancer cells is seen in transgenic mice with a nonfunctioning GHRH receptor and therefore only have 10% of normal GH and IGF-1 levels (Yang, Beamer et al. 1996). Elevated GHR expression has been observed in colorectal, prostate, and breast cancer tissues (Lincoln, Kaiser et al. 2000; Laban, Bustin et al. 2003; Weiss-Messer, Merom et al. 2004; Yang, Liu et al. 2004). Illustrated in a review by Khandwala et al. are the cumulative findings of 20 years of research that the growth of virtually every type of human neoplasm, be it solid or hematological, can be modulated by altering the GH/IGF axis, at least in the controlled environment of the laboratory (Khandwala, McCutcheon et al. 2000)

Over the past decade, several prospective epidemiological studies have shown correlation of high serum IGF-1 levels together with low levels of IGFBP-3 and increased risk of developing several of the most common cancers such as colon, breast, and prostate cancer (Hankinson, Willett et al. 1998; Giovannucci 1999; Ma, Pollak et al. 1999; Manousos, Souglakos et al. 1999; Giovannucci, Pollak et al. 2000). It has even been suggested that serum IGF-1 levels have a stronger association than most other risk factors for these cancers (Burroughs, Dunn et al. 1999). Additional support for IGF-1 being a positive regulator of tumors comes from the numerous reports of increased cancer risk, especially colorectal, in acromegaly patients in which hypersecretion of GH is accompanied by elevated IGF-1 levels (Jenkins, Mukherjee et al. 2006). Furthermore, the risk of colon neoplasia in acromegalics was significantly related to both serum GH and IGF-1 (Cats, Dullaart et al. 1996; Jenkins, Frajese et al. 2000). Although this risk represents circumstantial evidence for an association between IGF-1 and neoplasia, it is surprisingly modest in magnitude. The very high IGF-1 levels in acromegalics are not associated with an extreme cancer risk. One explanation for this could be that the levels of IGFBP-3 are raised in acromegalics, such that the free and

active IGF-1 concentration might not be dramatically changed. In addition, there are few long-term follow-up studies with untreated acromegaly to determine cancer incidence.

Overexpression of IGF-1R in tumor compared to normal tissue is reported in several solid as well as hematological malignancies (Hakam, Yeatman et al. 1999; Xie, Skytting et al. 1999; Kanter-Lewensohn, Dricu et al. 2000; All-Ericsson, Girnita et al. 2002; Hellawell, Turner et al. 2002; Bataille, Robillard et al. 2005; Chng, Gualberto et al. 2006; Chong, Colston et al. 2006; Cunningham, Essapen et al. 2006). Recently, upregulation of IGF-1R signaling has been implicated in the development of resistance to anti-cancer treatment such as radiotherapy, hormonal therapy and human epidermal growth factor receptor 2 (HER2) targeting (Milano, Dal Lago et al. 2006; Nahta, Yu et al. 2006).

On the molecular level there are multiple pathways emanating from he IGF-1R that lead to tumor cell growth and protection against apoptosis. The main antiapoptotic pathway is through IRS-1, PI3K and the phosphorylation, by AKT, of BAD, one of the members of the Bcl-2 family of proteins. In its phosphorylated form, BAD cannot bind to antiapoptotic proteins of the Bcl-2 family and therefore cannot induce cell death (Yang, Zha et al. 1995). This antiapoptotic mechanism is also used by the insulin receptor, at least in mouse embryo fibroblasts (Prisco, Romano et al. 1999). The stabilization of phosphorylated BAD seems also to be induced by IGF-1 via the MAPK pathway as well as through association of 14.3.3 proteins to the IGF-1R and subsequent translocation of Raf-1 to the mitochondria (Peruzzi, Prisco et al. 1999). Moreover, expression of the anti-apoptotic Bcl-xL protein is upregulated by IGF-1 (Parrizas and LeRoith 1997). IGF-1 can also protect from apoptosis through down-regulation of the pro-apoptotic tumor suppressor protein p53 via AKT-mediated phosphorylation of Mdm2 (Kurmasheva and Houghton 2006).

In addition to its proliferative and antiapoptotic actions, IGF-1 is involved in several stages of the metastatic process such as cell adhesion, migration, invasion, angiogenesis and metastatic growth in distant organ sites (Samani and Brodt 2001; Bustin, Dorudi et al. 2002; Wu, Yakar et al. 2002).

ANGIOGENESIS AND THE GH/IGF-1 SYSTEM

Recruitment of new vasculature is a crucial process both for tumor growth and metastatic propagation in virtually every kind of cancer known to man (Folkman 1990;

Folkman 1992). Studies have conclusively proved that a primary tumor cannot grow beyond a few cubic millimeters in the absence of neovascularization (Hanahan and Folkman 1996). Angiogenesis is also involved in numerous other pathological conditions such as proliferative retinopathy, diabetic nephropathy, rheumatoid arthritis, psoriasis, inflammation, cardiovascular, and cerebral ischemia (Griffioen and Molema 2000; White, Atley et al. 2004; Kiselyov, Balakin et al. 2007). All of these conditions are characterized by excessive angiogenesis, resulting in development of vessels in an uncontrolled or disorganized manner. Proliferative retinopathies, involving the pathological growth of new blood vessels as a result of retinal ischemia, account for majority of the cases of vision loss throughout the world (Grant and Caballero 2002). Proliferative retinopathies include diabetic retinopathy, retinopathy of prematurity and age-related macular degeneration.

Consequently, inhibition of angiogenesis is considered to be one of the most promising strategies for the development of antineoplastic therapies and such treatment would also be of use for other neovascularization diseases (Talks and Harris 2000; Hlatky, Hahnfeldt et al. 2002).

Vascular Endothelial Growth Factor (VEGF) is the major pro-angiogenetic factor responsible for neovascularization in tumors. High levels of VEGF expression alone are capable of initiating angiogenesis in a quiescent vasculature (Ferrara, Houck et al. 1992; Thomas 1996). VEGF and VEGF receptors are overexpressed in almost all tumors and tumor vasculature, implicating its role in as a paracrine mediator which localizes in target cells (Bergers and Benjamin 2003). VEGF is also secreted by retinal pigment epithelium (RPE) cells in diabetic retinopathy and synovial fibroblasts (in autoimmune diseases like rheumatoid arthritis). Hence, the inhibition of VEGF production and action is a promising strategy to abrogate inappropriate angiogenesis.

Signaling through the IGF-1R has been shown to increase VEGF transcription and also to stabilize VEGF mRNA and protein (Akagi, Liu et al. 1998). The IGF-1 effect on VEGF transcription has recently been attributed to the MAPK pathway activating the transcription factor Hypoxia-inducible factor-1 (Fukuda, Hirota et al. 2002). *In vivo* experiments have shown increased metastatic potential of tumors together with increased VEGF expression after IGF-1 treatment, confirming the biological relevance of IGF-1 effects on angiogenesis (Wu, Yakar et al. 2002). Decreased VEGF production and disturbed angiogenesis has been demonstrated in various models following IGF-1R inhibition (Manara, Landuzzi et al. 2007; Wu, Zhou et al. 2007). Taken together, anti GH/IGF-1 therapy may in addition to tumor growth and survival also target tumor

neovascularization and thereby exert dual anti-tumor effect. In addition, these agents could also have therapeutic value in other pathological neovascularization diseases.

ADDITIONAL GH/IGF-1 PATOPHYSIOLOGY

Abnormal secretion of GH and IGF-1 is observed in multiple disease processes. In children, hyposecretion of GH results in short stature, whereas hypersecretion before epiphyseal closure yields gigantism. GH deficiency in adulthood is associated with reduced wellbeing, central obesity, increased fat mass, dyslipidemia and increased mortality. Low levels of IGF-1 in serum has been associated with risk of cardiac disease (Juul, Scheike et al. 2002; Vasan, Sullivan et al. 2003). Hypersecretion of GH in adults is usually caused by a pituitary somatotroph tumor and results in acromegaly. This leads to excessive GHR activation and overproduction of IGF-1, causing a series of serious complications including acral and soft tissue overgrowth, severe debilitating arthritic features, skin thickening, thyroid enlargement, impaired cardiac function, sleep apnea, glucose intolerance and diabetes (Brabant 2003). Acromegaly has been associated with a two- to three-fold increased mortality, most commonly due to cardiovascular, cerebrovascular, and respiratory disease (Orme, McNally et al. 1998). The development of cardiovascular disease in acromegaly is in some respect paradoxical since GH is beneficial in models of heart failure (Cittadini, Grossman et al. 1997). Also, patients with heart failure have improved cardiac function when treated with GH (Spallarossa, Rossettin et al. 1999) and the cardiac abnormalities associated with GH deficiency are corrected after GH therapy (Colao, di Somma et al. 2002). Administration of supraphysiological doses of GH to healthy subjects is associated with ventricular hypertrophy and increased cardiac output (Cittadini, Berggren et al. 2002). Acromegalics frequently develop cardiomyopathy, but why and how this turns in to a maladaptive process is yet incompletely revealed (Sacca, Napoli et al. 2003). Hypertension caused by GH/IGF-1-induced hypertrophy and fibrosis of smooth muscle cells as well as diabetes are both frequent in acromegaly and known risk factors for coronary heart disease (Matta and Caron 2003). Similar effects as those seen in acromegalics on the cardiovascular system and on glucose and lipid metabolism are known long-term side effects of GH doping, although controlled studies on GH overdosing are limited due to ethical reasons (Birchard 1998). Elevated GH and IGF-1 levels have further been correlated with diabetic complications and increased renal and glomerular growth (Doi, Striker et al. 1988; Flyvbjerg, Bennett et al. 1999).

GHR TARGETING STRATEGIES

Current treatment modalities for acromegaly consist of surgery, radiation therapy, and medical treatment with dopamine agonists, somatostatin analogs as well as the FDA approved GH antagonist Pegvisomant (Somavert®, Pfizer, Sandwich Kent, UK). A significant proportion of acromegalics require long-term medical therapy for control of their GH/IGF-1 levels (Melmed, Vance et al. 2002). Dopamine agonists (Bromocriptine, Quinagolide and Cabergoline) suppress growth hormone secretion in acromegalics but have limited efficacy and tolerability and patient often develop resistance. Dopamine agonists and are in general less effective than the somatostatin analogues (Jaffe and Barkan 1992). Somatostatin is a neuropeptide widely distributed in the central and peripheral nervous system, most recognized for its effect on inhibition of GH secretion from the pituitary (Brazeau, Vale et al. 1974; Ferjoux, Bousquet et al. 2000). Long-acting somatostatin analogues (Octreotide and Lanreotide) are given every 2-4 weeks and normalize serum IGF-1 levels in about 65% of patients (Chanson, Boerlin et al. 2000). However the somatostatin analogues also inhibit thyroid-stimulating hormone, insulin, glucagon and neuropeptide secretion and the relatively modest effect on serum IGF-1 still leaves at least one third of patients eligible for a more effective medical therapy.

Pegvisomant is a genetically engineered analog of hGH conjugated with polyethylene glycol (PEG) moieties to increase its biological half-life (Pradhananga, Wilkinson et al. 2002; Kopchick 2003). Pegvisomant competes with endogenous GH for binding to the GHR but does not activate the receptor, thereby blocking the GH-initiated signaling and IGF-1 production (Kopchick 2003). In contrast to other treatments for acromegaly, Pegvisomant inhibits GH action instead of GH secretion. Clinical studies report reduced serum IGF-1 into the age-related reference range in up to 90 % of the patients treated with Pegvisomant. In addition, Pegvisomant seems also to be very well tolerated in patients (Kopchick, Parkinson et al. 2002; van der Lely 2002; Stewart 2003; Muller, Kopchick et al. 2004; Paisley, Trainer et al. 2004). GH antagonist treatment in acromegaly could potentially lead to increased tumor volume since the reduced feedback inhibition from IGF-1 leads to elevated serum GH. However, the rise in GH was not accompanied by an increase in tumor volume in a 12-wk placebo-controlled study with Pegvisomant (Trainer, Drake et al. 2000). Nevertheless, attention to this possible side effect is warranted in future studies of GHR antagonists.

Pegvisomant, being a mutated hGH analogue, binds to rat and mouse GHR with much lower affinity than to hGHR (Mode, Tollet et al. 1996), making results from animal studies difficult to extrapolate to humans. At high doses however, Pegvisomant was able to reduce circulating IGF-1 concentrations in mice by approximately 75-80%. This is considerably more IGF-1 suppression than would be obtained by other agents designed to down-regulate the GH axis. Moreover, studies in animal models of metastatic colon cancer showed that Pegvisomant in combination with conventional chemotherapy, practically eliminated metastatic disease (Maple, Fernandes et al. 2001).

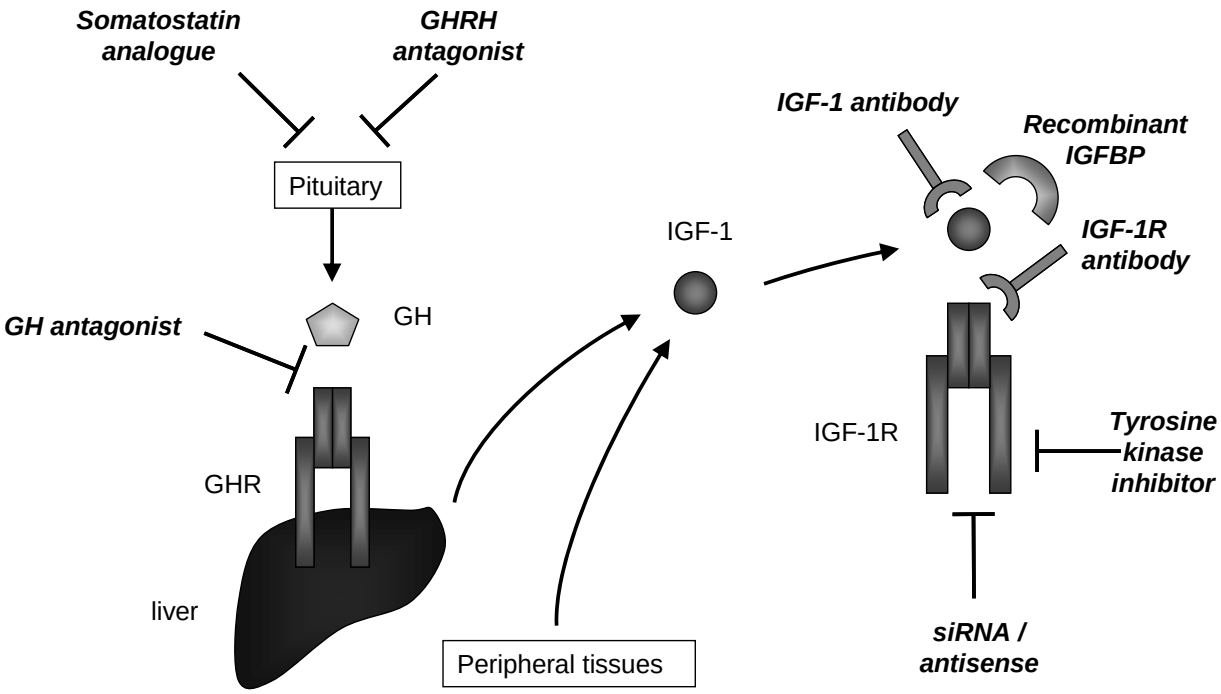

Figure 5: GH / IGF-1 targeting strategies

IGF-1R TARGETING STRATEGIES

Given the evidence to support an important role for the IGF-1R in development and progression of cancer, together with the fact that IGF-1R is not an absolute requirement for normal growth but plays crucial roles in cell growth under anchorage-independent

conditions or *in vivo* in transplanted tumors, the IGF-1R has emerged as an attractive target for cancer therapy (LeRoith, Werner et al. 1995; Baserga, Hongo et al. 1997; Baserga 1999). Response to IGF-1R inhibitors are anticipated in a wide range of tumors based on in vitro and in vivo results. Numerous approaches to therapeutically interfere with IGF-1R signaling have been explored (Hofmann and Garcia-Echeverria 2005); recombinant IGFBPs and antibodies against IGF-1 can be used to reduce ligand availability, antisense and RNA interference can reduce IGF-1R expression, small molecule tyrosine kinase inhibitors or IGF-1R antibodies can inhibit IGF-1R activation. Inhibition of IGF-1R signaling *in vitro* and *in vivo* have resulted in massive apoptosis in malignant cells growing under anchorage-independent conditions and dramatic inhibition of tumor development after injection in nude mice (Hartog, Wesseling et al. 2007). Since metastatic spread of tumors is ultimately responsible for 90% of all deaths from cancer in humans (Sporn 1996), this is an area of interest for chemical intervention. It was recently demonstrated that silencing of endogenous IGF-1R expression with a truncated IGF-1R in a metastatic breast cancer cell line resulted in complete inhibition of metastatic spread after injection of the transfected cells into nude mice (Sachdev, Hartell et al. 2004). IGF-1R antibodies exert dual effect on IGF-1R signaling in that they both prevent ligand-induced activation and induce internalization and degradation of the receptor (Baserga, Peruzzi et al. 2003). IGF-1R downregulation seems to be of great importance for induction of apoptosis and antitumor activity (Baserga, Peruzzi et al. 2003; Jackson-Booth, Terry et al. 2003). Antibodies as well as tyrosine kinase inhibitors are currently tested in clinical trials. Treatment with IGF-1R antibodies has proven to be effective against a large number of solid tumors as well as multiple myeloma *in vivo*. Recent clinical studies with IGF-1R antibodies also report activity in cancer patients (Hartog, Wesseling et al. 2007). Moreover, combining IGF-1R antibodies with chemotherapy, targeted therapy and radiation induced additional tumor regression (Beltran, Lu et al. 2006; Wu, Haugk et al. 2006; Allen, Saba et al. 2007; Wu, Zhou et al. 2007).

Tyrosine kinase inhibitors (TKIs) act by directly binding to and blocking the catalytic kinase domain. Since the IGF-1R and IR kinase domains are nearly identical, specific targeting of IGF-1R activity has been a challenge for medicinal chemists (Hartog, Wesseling et al. 2007; Sarma, Tandon et al. 2007). Coinhibition of IR could cause diabetogenesis which is an unacceptable side effect of a drug. IGF-1R TKIs have proved effective against a panel of solid tumors *in vitro* and in several cancer models *in vivo* (Garcia-Echeverria, Pearson et al. 2004; Mitsiades, Mitsiades et al. 2004;

Scotlandi, Manara et al. 2005; Girnita, All-Ericsson et al. 2006; Haluska, Carboni et al. 2006; Menu, Jernberg-Wiklund et al. 2006; Tanno, Mancini et al. 2006; Manara, Landuzzi et al. 2007). Synergistic effects have also been seen when combining TKIs with conventional chemotherapy (Mitsiades, Mitsiades et al. 2004; Scotlandi, Manara et al. 2005).

The low molecular weight cyclolignan picropodophyllin (PPP) was identified some years ago as an inhibitor of IGF-1R activation and AKT signaling in tumor cell lines (Girnita, Girnita et al. 2004; Vasilcanu, Girnita et al. 2004; Conti, Regis et al. 2007). In contrast to many other TKIs, PPP did not affect signaling of the highly homologous IR (Girnita, Girnita et al. 2004; Fulzele, Digirolamo et al. 2007; Vasilcanu, Vasilcanu et al. 2007). PPP was also found to efficiently repress tumors in animal models (Girnita, Girnita et al. 2004; Girnita, All-Ericsson et al. 2006; Menu, Jernberg-Wiklund et al. 2006; Menu, Jernberg-Wiklund et al. 2007). The exact molecular mechanism of PPP is under investigation but recent data has demonstrated that PPP-induced IGF-1R downregulation (Vasilcanu, Vasilcanu et al. 2007) and transient ERK activation (Vasilcanu et. al. 2007b, submitted manuscript) are important components of the mechanism.

AIMS OF THIS THESIS

Given the evidence to support an important role for the GH/IGF-1 axis in cancer as well as in neovascularization diseases, low molecular weight compounds targeting this axis have good potential to become effective treatments.
A GHR antagonist offers the possibility of higher IGF-1 normalization rates and greater specificity of effect compared to other current therapies for acromegaly. Although Pegvisomant effectively normalizes IGF-1 in most acromegalics, a small molecule, nonpeptidyl drug would have superior pharmacokinetic properties including solubility, stability and oral bioavailability.
Blockade of the IGF-1R in various ways has proven to be effective as antitumor treatment. Strategies that lead to IGF-1R down regulation and not only tyrosine kinase inhibition, have resulted in the strongest anti-tumor effects.

The specific aims of this study were:

To establish a non-radioactive IGF-1 mRNA quantification method and to use this assay in a screen for potential small molecule GH antagonists in cultured primary hepatocytes.

To study the *in vivo* effects of one selected GH antagonist (BVT-A) on different markers of the GH/IGF-1 axis in hypophysectomized (HX) GH-substituted rats, a model system of Acromegaly.

To explore potential expression and dependency of the IGF-1R tyrosine kinase in clones of IGF-1R knockout cells.

To investigate the effects of Picropodophyllin (PPP) on expression level of IGF-1R and delineate the compounds mechanism of action

To study effects on VEGF production and choroidal neovascularization *in vivo* after IGF-1R inhibition by PPP.

To identify the primary binding site for PPP and to characterize the sequential steps of the PPP mechanism.

RESULTS AND DISCUSSION

This thesis is based on six papers with the overall aim to identify and evaluate antagonists of the GH /IGF-1 axis.

PAPER I

Antisense- and sense RNA probe hybridization to immobilized crude cellular lysates – a tool to screen GH antagonists

Changes in gene expression levels are important markers of biological activity of drugs and chemical compounds. It is of interest to develop and optimize minimally laborious and non-radioactive methods for specific mRNA quantification in cell cultures in the standard microplate format. Using a suitable internal standard is a prerequisite for correct comparison between samples and care has to be taken since expression of housekeeping genes, commonly used as standards, has been shown to vary between different experimental setups. In this study, the aims were to set up a non-radioactive mRNA quantification assay for analyzing IGF-1 expression in cultured primary rodent hepatocytes, to correlate the determined mRNA levels to an adequate internal standard and to use this assay for investigation of the IGF-1 mRNA response to hGH and potential hGH antagonists.

As previously shown by others (Kaabache, Barraud et al. 1995), the RNA purification step was possible to circumvent by solubilizing the hepatocytes in guanidine thiocyanate directly in the culture wells. Furthermore, the 96-well format for culturing of the hepatocytes was shown by conventional RNase Protection Assay (RPA) to give adequate amount of IGF-1 mRNA for detection and that the cells responded to GH in this format. By using two RNA probes, transcribed from opposite directions of a cDNA template of the mouse IGF-1 gene, we were able to use the antisense probe for detection of transcript formation and the sense probe hybridization signal as an internal standard. This method for normalization ascertained that only differences in gene expression of the particular gene of interest were detected. Various techniques for labeling of RNA probes, including transcriptional labeling with fluorescein or biotin, were compared and we concluded that RNA probes labeled by UV-crosslinking with intercalating psoralen-biotin followed by detection with alkaline phosphatase generated a good signal to noise. The assay was used to quantify IGF-1 mRNA expression after GH treatment of primary hepatocytes cultured in the 96-well format, and the level of

induction was concordant with previous observations using large scale cultures and RPA in solution for detection (Tollet, Enberg et al. 1990). Similar results were obtained using hepatocytes from both rat and mice.

Next, we examined the effect of a set of low molecular weight compounds, previously selected by their ability to bind to the GH receptor, on GH-stimulated IGF-1 expression. Two potential antagonists termed BVT-A and BVT-B were found. After analyzing possible general effects of the compounds on transcription and viability of hepatocytes in culture, BVT-A remained as a GH antagonist. The mRNA levels of two additional GH regulated genes; CYP 2C12 and A1BG, were determined with RPA and the GH induction of both genes was reduced by BVT-A, confirming the GH antagonistic effect of the compound.

In conclusion, the filter hybridization assay we have developed can be a useful tool in screening for GH antagonists or agonists. Compared to existing techniques such as RT-PCR and Branched DNA assays, the assay does not require any special or costly reagents and equipment. Using other sets of probes, the assay could be applied to other drug targets affecting gene transcription in the liver. In the future, the use of microtiterplates with nucleotide-binding surfaces together with probes labeled with two differently colored fluorophores could further simplify and improve the sensitivity and throughput of this hybridization assay.

PAPER II

In vivo evaluation of a novel, orally bioavailable, small molecule growth hormone receptor antagonist

Studies *in vivo* on the effects of GH receptor antagonists are preferentially performed in animal models in which the feedback mechanisms of the GH/IGF-1 system and the hypotalamo-pituitary axis can be avoided. The hypophysectomized (HX) rat is an established model with reduced growth and IGF-1 levels that can be restored by GH treatment (Simpson ME 1949). Furthermore, by administration of GH to the animal, the GH levels can be regulated in order to resemble different disease states. In this study, we have used HX rats infused with GH to study the *in vivo* effects of the GH antagonist BVT-A (N-[5-(aminosulfonyl)-2-methylphenyl]-5-bromo-2-furamide) presented in paper I. The animals were treated for 7 days with GH and in addition given the low molecular weight compound BVT-A the last 6 days. We measured body weight, serum IGF-1 and hepatic mRNA levels of components of the GH/IGF-1 axis.

Serum IGF-1 levels as well as body weight increased markedly after GH treatment and both markers were significantly and dose dependently reduced by treatment with BVT-A. Notably, BVT-A was able to reduce serum IGF-1 levels almost down to control (HX) levels. Food intake was monitored and was not considerably affected by BVT-A.

Hepatic mRNA expression of the GH target genes encoding IGF-1, IGFBP-3, ALS, IGF-1R and GHR was determined by RT-PCR. The expression of all these genes was induced by GH, and as expected the most pronounced induction was seen for IGF-1, in accordance with its tight regulation by GH. Treatment with BVT-A markedly reduced the GH-induced levels of all genes, with most significant effect on IGFBP-3, perhaps due to its regulation by both GH and IGF-1 (Chin, Zhou et al. 1994; Scharf, Ramadori et al. 1996; Gucev, Oh et al. 1997). The kinetics of IGF-1 stimulated IGFBP-3 expression appears to follow the same time course as GH induction of IGF-1 mRNA. IGFBP-3 mRNA is reported to increase by 3 h treatment with IGF-1, with maximum increases observed between 8–12 h of treatment (Cohick, Wang et al. 2000). If as some studies suggest, the induction of IGFBP-3 is secondary to GH stimulation, via IGF-1 (Chin, Zhou et al. 1994; Scharf, Ramadori et al. 1996), there will be a delay in both onset of induction, repression by antagonist and re-establishment of induced levels after removal of antagonist. Since the tissue samples in this study were taken 6 hours after the last dose of compound but with continued GH treatment, the IGF-1 (as well as ALS) mRNA levels probably had time to rise, while the rise in IGFBP-3 had just started. The reduction of IGF-1 was not as marked at the hepatic mRNA level as at the level of protein in serum. This could be explained by the reduced expression of the binding proteins IGFBP-3 and ALS, which stabilize IGF-1 in serum. The IGF-1 mRNA levels could also have had time to rise after the last dose of antagonist, with the effect on IGF-1 protein level not being evident yet. Moreover, the effect on IGF-1 mRNA *in vivo* was not as pronounced as *in vitro*. This could relate to the time factor discussed above, the cultured hepatocytes were continuously exposed to both BVT-A and GH. It could also be due to the direct exposure of the hepatocyte *in vitro* and/or to differences in metabolism. In addition, mRNA stability can be affected by factors that the cells in culture are not exposed to.

In conclusion, our results demonstrate that the novel low molecular weight compound, BVT-A (N-[5-(aminosulfonyl)-2-methylphenyl]-5-bromo-2-furamide), is a GH receptor antagonist *in vivo* and that the GH substituted HX rat is a useful model for studying the action of compounds that inhibit activation of the GHR. Currently available pharmacological therapies for patients suffering from hypersecretion of GH,

leading to gigantism and/or acromegaly, is limited to dopamine agonists, somatostatin analogues and to a GHR antagonist, Pegvisomant, which is a genetically engineered analogue of human GH (Fuh, Cunningham et al. 1992; Ezzat 2004) that has to be administered by injections. Although Pegvisomant effectively lowers IGF-1 levels in acromegalics (Trainer, Drake et al. 2000; Kopchick, Parkinson et al. 2002), orally available small molecule GHR antagonists, such as BVT-A, would certainly improve patient acceptability and compliance. Other therapeutic areas for GHR antagonists might also be considered, e.g. for diabetic kidney disease (Schrijvers, De Vriese et al. 2004) and for certain cancers; it has been shown that mammary epithelial expression of GH, i.e. non-pituitary derived, is associated with pathological proliferation (Raccurt, Lobie et al. 2002) and that autocrine human GH stimulates oncogenic transformation of immortalized human mammary epithelial cells (Xu, Emerald et al. 2005). Thus, this work could have future clinical applications. It would be of interest to compare the effect of BVT-A with the GH antagonist on the market, Pegvisomant, but since this mutated hGH analogue binds to rat and mouse GHR with much lower affinity than to hGHR (Mode, Tollet et al. 1996), rodents are not an ideal model for GHR blockade by Pegvisomant. Even though rodent models are not ideal, several studies have proved the concept of GHR inhibition in rodents with other peptide GH antagonists (nonpegylated molecules with a single amino acid change) that in addition to reducing serum IGF-1, also inhibit diabetes-induced glomerusclerosis (Flyvbjerg, Bennett et al. 1999), reduce hypoxia-induced retinal neovascularization (Smith, Kopchick et al. 1997), and decrease size and progression of dimethyl butyric acid-induced breast tumors (Pollak, Blouin et al. 2001).

PAPER III

IGF-1R tyrosine kinase expression and dependency in clones of IGF-1R knockout cells (R-)

An IGF-1R knockout mouse model (homozygous igf-1R-/-) was generated some years ago by targeted deletion of a part of exon 3 which encodes the major portion of the cysteine-rich ligand-binding domain (Liu, Baker et al. 1993). Using this knockout model Baserga and coworkers developed IGF-1R null fibroblasts, denoted R- (Sell, Rubini et al. 1993; Sell, Dumenil et al. 1994). The R- cells represent a useful tool for molecular mapping of biological properties of the IGF-1R. In contrast to wild-type mouse embryonic fibroblasts (MEFs) the R- cells were found be very difficult to

transform by a number of viral and cellular oncogenes, highlighting the requirement of IGF-1R for transformation (Valentinis and Baserga 2001). Reintroduction of the IGF-1R in R- cells also restored their ability to transform (Morrison, Tognon et al. 2002). However, over time it has become clear that R- cells can transform spontaneously, but at a rate 3 logarithms lower than MEFs expressing the receptor (Baserga, Peruzzi et al. 2003). This observation raises the question as whether R- cells over the years have acquired some properties mimicking those of IGF-1R. In addition, we have over the last years observed R- cell cultures that are sensitive to treatment with the IGF-1R inhibitor Picropodophyllin (PPP). In most cases PPP responsive R- cells (here denoted R-s) exhibited a higher proliferative rate compared to the resistant ones (denoted R-r). This study aimed at investigating the cause of responsiveness of R- cells to PPP. One theory was that PPP cause cell death by other mechanisms than IGF-1R inhibition although previous studies have ruled out PPP interactions with the highly homologous insulin receptor and other major tyrosine kinase receptors involved in tumor cell growth. However, PPP is chemically closely related to podophyllotoxin (PPT), a potent microtubule inhibitor (Kelleher 1977). PPP has previously been described to cause weak inhibition of microtubules (Loike, Brewer et al. 1978), and this could explain why rapidly proliferating R- cells are more sensitive to PPP than the normal, slow-growing R- cells. We performed binding competition experiments with tubulin protein immobilized on Scintillation Proximity Assay (SPA) beads and [^{3}H]Colchicine (a known tubulin binder). As expected, PPT efficiently displaced [^{3}H]Colchicine with an IC_{50} of approximately 0,2 μM, demonstrating that it binds to the colchicine binding site on tubulin. Meanwhile, we could not detect a 50% displacement of [^{3}H]Colchicine using PPP doses up to 50 μM, implying that IC_{50} of PPP on microtubule binding exceeds that of PPT > 250-fold. Neither could we detect any displacement of [^{3}H]PPP binding by addition of cold PPP, indicating that no binding sites for PPP exist on tubulin. Previous reports claim PPP activity on microtubules (Loike, Brewer et al. 1978), but since the purity of PPP was not clearly described in these studies, the PPP solutions used could potentially be contaminated with PPT. PPP and PPT normally exist in equilibrium in a 97.5 to 2.5 ratio (Gensler and Gatsonis 1966). To produce PPP with more than 99.5% purity including less than 0.2% PPT, a laborious process of 1–4 times recrystallization or chromatography is needed. Because of the very strong tubulin binding capacity of PPT, very little contamination in the PPP batch used can cause substantial effects on microtubule. Taken together, highly purified PPP (99.7%) used in this study did not or only slightly interfere with microtubule binding implying that PPP

does not cause cell death through microtubule inhibition. Although PPP could have other mechanisms of action, our and others' results suggest that inhibition of IGF-1R is the predominating mechanism behind its apoptotic effects (Girnita, Girnita et al. 2004; Colon, Svechnikov et al. 2005; Ulfarsson, Karstrom et al. 2005; Colon, Strand et al. 2006; Menu, Jernberg-Wiklund et al. 2006; Fulzele, Digirolamo et al. 2007; Menu, Jernberg-Wiklund et al. 2007; Shields, Nicola et al. 2007).

The next possibility was that the R-s cells had acquired expression of other growth regulating protein(s) that is/are targeted by PPP. We discovered that R-s express a 90 kDa protein reactive to IGF-1R β-subunit antibodies but defective in ligand binding. This protein was weakly but constitutively tyrosine phosphorylated and downregulated by siRNA targeting IGF-1R, paralleled by decreased R-s survival. All together, these results suggest that clones of R- express IGF-1R activity and dependency, which in turn could explain why R- can undergo spontaneous transformation.

R- cells were created by targeting the ligand-binding domain of the IGF-1R, not the whole receptor, why R- cells may express a mutant IGF-1R lacking the ligand binding domain. Our present results suggest that the 90 kDa receptor protein might be important for survival of R- cells. Interestingly, others have demonstrated that truncated IGF-1R, lacking ligand binding domain, can signal anchorage-independent growth (and increased PI3K activity (Himmelmann, Terry et al. 2001). We speculate that IGF-1R lacking the ligand binding domain could become biologically active because of increased concentration of IGF-1R. Autophosphorylation of the cytoplasmic domain of IGF-1R is known to be concentration dependent (Lopaczynski, Terry et al. 2000; Favelyukis, Till et al. 2001), consistent with a trans-phosphorylation mechanism. Here, we also have to consider the fact that lack of ligand-receptor interaction would also decrease IGF-1R degradation resulting in increased amount of IGF-1R. Another hypothesis refers simply to a Darwinian selection based on growth/survival advantages of R- cells expressing IGF-1R over cells not expressing it. Consistent with our observations it was recently reported that late passages of R- cells could be transformed by SV40 T antigen, while early R- passages were completely refractory to transformation (Spence, Shaffer et al. 2006). Collectively our present study suggests that restoration of IGF-1R functions in R- cells may explain why they over time started to transform spontaneously and have become responsive to IGF-1R inhibitors.

PAPER IV

Inhibition of VEGF secretion and choroidal neovascularization by picropodophyllin (PPP), an inhibitor of the insulin-like growth factor-1 receptor

VEGF is a major angiogenic stimulant in the development of new vasculature and is also found important for neovascularization of the choroid (Campochiaro 2000; Witmer, Vrensen et al. 2003), the hallmark of age-related macular degeneration (AMD), the major cause of blindness among the elderly in the western world (Ambati, Ambati et al. 2003; Fine 2005). IGF-1 is known to stimulate VEGF production and a potential role of IGF-1 and its receptor in choroidal neovascularization (CNV) has been suggested. Retinal pigment epithelium (RPE) cells secrete VEGF and this is known to be stimulated by IGF-1.

In his study we explored the therapeutic role of IGF-1R inhibition by PPP as a treatment for AMD. Mice were treated with krypton laser in one eye to induce CNV according to already published procedures (Mori, Ando et al. 2001). PPP or vehicle was administered intraperitoneally (i.p.) or orally for 2 weeks after the CNV induction. Flat-mounts of the choroids were prepared and stained with the endothelial cell-specific marker FITC-isolectin-B4 to identify vessels. Both i.p and peroral PPP-treatment was found to reduce the size of laser-induced CNV lesions by 22 and 31,5 % respectively. In a parallel experiment, formalin-fixed eyes from PPP- or vehicle-treated animals were cut in thin sections and stained with haematoxylin-eosin for histological examination. This experiment confirmed the results from the flat-mounts that PPP treatment reduced the CNV area and that both i.p. and peroral administration was effective.

The effects of PPP on VEGF secretion in RPE cells was studied *in vitro* by ELISA. Treatment with IGF-1 strongly induced VEGF secretion while co-treatment with PPP reduced the VEGF-levels almost down to control level. To study if PPP affects VEGF on a transcriptional level, a VEGF luciferase reporter gene construct was transiently transfected into RPE cells. IGF-1 treatment vastly increased the VEGF promoter activity and co-incubation with PPP decreased the IGF-1 induced luciferase activity by 25 %. VEGF expression was also studied *in vivo* in RPE-choroid–sclera eyecups from rats treated i.p or perorally with PPP according to above. VEGF protein levels were detected by western blotting and normalized to GAPDH. PPP significantly decreased the laser-induced VEGF expression, the VEGF levels were actually reduced below the levels of the no-laser treated group. In the same experiment we also observed a significant decrease in IGF-1R expression in the PPP- and laser-treated animals

compared to laser-treated alone. No significant effects on IGF-1 levels were seen by the different treatments.

This study is to our knowledge, the first to describe *in vivo*-effects on VEGF-expression and CNV after IGF-1R inhibition. The seemingly modest PPP-effect (30% reduction) on CNV area *in vivo* could be explained by low bioavailability because of the blood retina barrier. Intravitreal administration, which is the current administration route for treatments against neovascular AMD, would most certainly enhance bioavailability. Peroral dosage, however could still be a clinical option in a combination therapy regime.

PAPER V

Picropodophyllin induces downregulation of the insulin-like growth factor 1 receptor. Potential mechanistic involvement of Mdm2 and β-Arrestin1

It has been shown that strategies leading to downregulation of the IGF-1R, and not only inhibition of its tyrosine kinase activity, are associated with the strongest anti-tumor effect (Baserga 2005), suggesting that down-regulation of IGF-1R is necessary to produce a complete inhibition of its functions. The cyclolignan PPP display high anti-tumor efficacy both *in vitro* and *in vivo* and together with the notion that IGF-1R downregulation is required for induction of massive apoptosis, we aimed to investigate whether PPP treatment might affect expression levels of IGF-1R. We discovered that PPP mediated a moderate but significant ligand-independent downregulation of IGF-1R. The decrease in receptor protein was 30-50% after 12h treatment of a number of human tumor cell lines and 50% *in vivo* in tumors allografted into SCID mice. By using [^{35}S]Methionine chase and semiquantitative RT-PCR, we confirmed that PPP caused degradation and not inhibition of synthesis. The specificity of downregulation of IGF-1R was also studied and neither insulin receptor, VEGFR, EGFR, Kit (stem cell growth factor receptor), PDGFR-α and -β, were downregulated in cell systems expressing detectable amounts of these receptors. Further, PPP did not affect the uptake of transferrin, suggesting that it does not generally interfere with receptor internalization.

The ubiquitin ligase Mdm2 was recently demonstrated to associate with, ubiquitinate and induce ligand-dependent degradation of the IGF-1R. Herein, we show that the PPP-induced IGF-1R downregulation also is dependent on expression of Mdm2 since over-expression of a dominant-negative Mdm2 construct eliminated this response. On the other hand, receptor downregulation following IGF-1 stimulation was not fully

decreased by dominant negative Mdm2. This may suggest that IGF-1 induced downregulation may also require another ubiquitin ligase, while the PPP induced degradation is mainly due to Mdm2-controlled ubiquitination. The scaffold protein β-Arrestin1, known to bring Mdm2 to the IGF-1R was also shown to be important for PPP-induced downregulation of IGF-1R. We could also demonstrate *in vitro* that reduction of PPP induced IGF-1R downregulation (by blocking β-Arrestin1) decreased PPP-induced cell death more than 4 fold.

Inhibition of IGF-1R phosphorylation, without accompanying downregulation, leads only to decreased proliferation but not to apoptosis. It has been shown that 30,000 IGF-1 receptors/cell was a minimum for anchorage-independent growth (Rubini, Hongo et al. 1997). Even though the PPP mediated IGF-1R downregulation seen in our experiments was moderate, a 50% reduction of IGF-1R number in tumors is likely to be below this critical level. In previous studies using antisense strategies the apoptotic effect was associated with a 50-70% IGF-1R downregulation (Resnicoff, Coppola et al. 1994; Shapiro, Jones et al. 1994; Lee, Wu et al. 1996). The downregulating effect of PPP on IGF-1R is slightly lower, but since PPP has dual effects (induction of partial degradation and inhibition of IGF-1R signaling), PPP could act synergistically with itself.

The causal relationship between the two actions of PPP on IGF-1R is, however, a kind of "chicken versus egg" dilemma. One possibility is that the IGF-1R downregulation induced by PPP is causing the impaired IGF-1R phosphorylation. Alternatively, PPP may act as a phosphorylation inhibitor of IGF-1R that primarily targets some specific residues (e.g. Tyr 1136) eliciting internalization and degradation. Actually it was recently demonstrated that IGF-1R with mutated Y1136 has an accelerated degradation (Sehat, Andersson et al. 2007). It is of course also possible that PPP-mediated inhibition of autophosphorylation and increase in downregulation of IGF-1R represent two independent events.

This study adds a new aspect on the mechanism of action of PPP, demonstrating that it besides inhibiting IGF-1R phosphorylation induces degradation of the receptor. This degradation, although partial, may be very important since downregulation of IGF-1R is necessary for induction of massive apoptosis in tumor cells.

PAPER VI

Picropodophyllin (PPP) acts as a β-Arrestin1-biased IGF-1R agonist

Activation of the IGF-1R results in receptor-recruitment of the multifunctional adapter protein β-Arrestin1 (Lin, Daaka et al. 1998). Originally described as desensitizers of G protein coupled receptor (GPCR) signaling, the β-Arrestins (1 and 2) are now also known to be involved in endocytosis and signal transduction of GPCRs (Reiter and Lefkowitz 2006). Ubiquitination of β-Arrestin has been found to play a role in several of these functions (Lefkowitz, Rajagopal et al. 2006) and agonist stimulated β-Arrestin ubiquitination is necessary for rapid β-Arrestin-dependent receptor internalization. The kinetics of β-Arrestin ubiquitination correlates with the stability of receptor-β-Arrestin interaction such that transient ubiquitination is associated with transient association (Shenoy and Lefkowitz 2003).

Mdm2 has been demonstrated to serve as the E3 ligase responsible for ubiquitination of both β-Arrestin and IGF-1R (Shenoy, McDonald et al. 2001; Girnita, Girnita et al. 2003), The IGF-1R ubiquitination was shown to require the interaction with β-Arrestin1 (Girnita, Shenoy et al. 2005; Girnita, Shenoy et al. 2007). The interaction between IGF-1R, MDM2 and β-Arrestin1 appears to be required for receptor desensitization as well as ERK activation and cell cycle progression (Girnita, Shenoy et al. 2005; Girnita, Shenoy et al. 2007).

Picropodophyllin (PPP) was recently showed to induce ubiquitination and downregulation of the IGF-1R and cause transient IGF-1R-dependent activation of ERK (Vasilcanu, Vasilcanu et al. 2007) (Vasilcanu et. al. 2007b, submitted manuscript). However, PPP does not affect signaling or expression of the highly homologous insulin receptor. The exact molecular mechanism of action of PPP is still not known and it has not been clarified if the primary site of action is the IGF-1R itself or any of its associated proteins. In this study we aimed to identify the primary binding site for PPP, to characterize the sequential steps of the PPP mechanism and to delineate what are the causes or consequences of the effects seen. We could demonstrate that PPP binds directly to the IGF-1R and induces receptor-association of β-Arrestin1 and Mdm2. PPP also induced poly-ubiquitination of both IGF-1R and β-Arrestin1 and internalization of the IGF-1R-β-Arrestin1 complex into endosomal vesicles. Importantly, we could also show that the inhibitory effect of PPP on IGF-1 dependent AKT signaling is at least partly a consequence of internalization/downregulation of the IGF-1R since knockdown of β-Arrestin1 recovered phosphorylation of AKT.

Normally β-Arrestins recognize and are recruited to the activated and phosphorylated cytoplasmic C-terminal region of the receptor (Shenoy and Lefkowitz 2003). However, at least for some receptors, β-Arrestins recruitment can take place also in the absence of receptor phosphorylation (Min, Galet et al. 2002; Terrillon and Bouvier 2004). It has also been described that mutant forms of β-Arrestins, presumably in a state that mimics the activated conformation, can interact with unphosphorylated receptors (Kovoor, Celver et al. 1999). The existence of β-Arrestin "biased" ligands for G-protein coupled receptors has been demonstrated in numerous publications. (Blanpain, Vanderwinden et al. 2002; Holloway, Qian et al. 2002; Azzi, Charest et al. 2003; Wei, Ahn et al. 2003; Kohout, Nicholas et al. 2004; Gesty-Palmer, Chen et al. 2006). These biased ligands act as antagonists on the G-protein signaling pathway while stimulating the β-Arrestin–dependent MAPK pathway. The theory is that different ligands stabilize distinct active receptor-conformations and that some of these conformations may favor activation of one signaling pathway over another and/or induce differential recruitment of β-Arrestin (Berg, Maayani et al. 1998; Ghanouni, Gryczynski et al. 2001; Kenakin 2005; Yee, Suzuki et al. 2006; Violin and Lefkowitz 2007). We propose that PPP is a β-Arrestin1 "biased" IGF-1R ligand that after binding to the IGF-1R presumably induces changes in receptor conformation that leads to association of β-Arrestin1 and Mdm2, ultimately leading to inhibition of signaling and degradation of IGF-1R.

The PPP mechanism described in this paper may explain its specificity to IGF-1R as opposed to the IR. Even though PPP might bind to the IR and induce β-Arrestin recruitment, β-Arrestin1 seems not to affect IR-mediated metabolic or mitogenic signaling (Dalle, Ricketts et al. 2001; Dalle, Imamura et al. 2002). Neither are there any published reports on β-Arrestins being involved in IR endocytosis. Furthermore, PPP does not induce ubiquitination (shown in present study) or degradation (Vasilcanu, Vasilcanu et al. 2007) of the IR. Accordingly, we speculate that the PPP specificity to IGF-1R compared to the IR is due to the involvement of β-Arrestin1 and Mdm2.

SUMMARY AND FUTURE PERSPECTIVES

This thesis aimed to identify and evaluate inhibitors of the GH/IGF-1 axis. The ability of a GH antagonist to effectively normalize IGF-1 concentrations and reduce signs and symptoms of acromegaly, even in patients resistant to previous treatments, has been demonstrated with Pegvisomant. The effects of BVT-A, not only on hepatic mRNA expression of IGF-1, but also on IGFBP-3 and ALS expression, can as observed in this study cause a substantial decrease in serum levels of IGF-1. The prospects are good for a new GH antagonist being an effective treatment alternative for acromegaly. A non-peptide drug would be a less costly alternative to Pegvisomant, improve patient acceptability and compliance as well as reduce the risk of developing antibodies. Targeting IGF-1R has in numerous recent studies led to impressive antineoplastic activity in many *in vitro* and *in vivo* models of common human cancers. IGF-1R inhibition has also led to sensitization of cancer cells to conventional chemotherapy or irradiation. Several strategies have been employed but methods inducing downregulation, not only signal attenuation, have been associated with the strongest anti tumor activity. PPP, being a low molecular weight IGF-1R inhibitor with receptor downregulatory effect, seemingly well tolerated *in vivo*, holds high promise for future drug development.

The future of cancer treatment will largely depend on the successful development of targeted therapies. Considerable antitumor effect in combination with manageable side effects is seen with several of the targeted therapies used in clinic today (e.g. Herceptin®, Gleevec®, Avastin®). An important issue for targeted therapy research in the future will be identification of reliable biomarkers to assess which patients are likely to gain the most benefit from these drugs and to evaluate tumor response.

Although the concept of targeting the GH / IGF-1 axis seems a promising strategy to combat malignancies, clinical trials must be performed to completely evaluate the usefulness and risks of inhibiting this axis. In addition to treatment for malignancies, inhibitors of the GH / IGF-1 axis could potentially also be used as therapy for neovascularization diseases.

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