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IDENTIFICATION AND EVALUATION OF GROWTH HORMONE ANTAGONISTS

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Stockholm 2005

ABSTRACT

States in which Growth hormone (GH) levels are elevated have been implicated in several physiological disorders such as acromegaly, diabetic complications and progression of malignancies. Classical treatments for acromegaly are surgery and radiotherapy but also pharmacological therapies exist. There is, however, a need for improved remedies and in the development of new drugs reliable preclinical *in vitro* and *in vivo* models are crucial.

GH, secreted from the pituitary, acts both directly and indirectly through induction of insulin-like growth factor–I (IGF-I) to promote tissue growth and regulate metabolism. GH induces IGF-I expression in the liver and peripheral target tissues but hepatic-derived IGF-I accounts for most circulating IGF-I. Therefore, the liver constitutes a valuable target organ for studies on GH-induced IGF-I production.

In the first part of this study, we used rodent primary hepatocytes for studies on pharmacological intervention of GH-induced IGF-I mRNA expression. We developed a 96-well non-radioactive IGF-I mRNA quantification assay based on hybridization of sense and antisense RNA probes to replica membranes with crude hepatocyte lysates. The sense hybridization was used as an internal standard. The antagonistic properties of a set of GH-receptor binding compounds were evaluated and two compounds were found to reduce GH-induced IGF-I mRNA expression. Effects due to metabolic inhibition or toxicity were excluded using a cell proliferation assay. To investigate possible unspecific transcriptional effects, the mRNA levels of the housekeeping genes β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were determined. The antagonistic property of one compound, BVT-A, was confirmed by showing mitigated GH-induction of two additional GH regulated genes using RNase protection assays. Accordingly, this direct filter hybridization assay of hepatocyte lysates using non-radioactive sense and antisense probes can be used for quantitative mRNA measurements and could constitute a valuable tool in screening for pharmacologically active compounds. Using other probe sets, the assay can have a wider application in cellular screening for drugs affecting gene transcription.

In the second part of the study, we used GH-treated, hypophysectomized (HX) rats to circumvent the feedback mechanisms of the hypothalamo-pitutary axis on the GH/IGF-I system, to study *in vivo* effects of BVT-A. In addition to hepatic IGF-I expression, GH also regulates production of major binding proteins for IGF-I in serum; IGF binding protein-3 (IGFBP-3) and the acid labile subunit (ALS). As expected, one week of GH treatment of HX rats induced serum IGF-I, body weight and hepatic mRNA levels of IGF-I, IGFBP-3, ALS, and of IGF-I receptor and GH receptor. Co-treatment with the GH antagonist BVT-A suppressed the GH-induced rise in serum IGF-I and body weight and also reduced hepatic mRNA levels of IGF-I, IGFBP-3, ALS, IGF-I receptor and GH receptor. Thus, the GH substituted HX rat is a useful model for studies of GH receptor antagonists. Together, the results show that BVT-A is a GH antagonist acting both *in vitro* and *in vivo*.

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., and Fhölenhag K. A rat in vivo model for evaluation of growth hormone receptor antagonists. *Manuscript*

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LIST OF ABBREVIATIONS

A1BG	α1B-glycoprotein
ALS	acid-labile subunit
cDNA	complementary deoxyribonucleic acid
CYP	cytochrome P450
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
HX	hypophysectomized
IGF-I	insulin-like growth factor I
IGF-IR	insulin-like growth factor I receptor
IGFBP	insulin-like growth factor binding protein
IR	insulin receptor
IRMA	immuno radiometric assay
IRS	insulin receptor substrate
JAK	janus kinase
MAPK	mitogen activated protein kinase
mRNA	messenger ribonucleic acid
PI3K	phosphoinositol-3-kinase
PRL	prolactin
PRLR	prolactin receptor
RIA	radio immuno assay
RPA	RNase protection assay
RT-PCR	real-time polymerase chain reaction
SH2	src homology 2
STAT	signal transducers and activators of transcription

GENERAL INTRODUCTION

Growth Hormone synthesis and secretion

Growth hormone (GH) is a 191 amino acid peptide hormone (MW 22,000), produced by somatotroph cells of the anterior pituitary (1). 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 (2, 3). These hypothalamic influences are tightly regulated by an integrated system of neural, metabolic and hormonal factors (4, 5). Ghrelin, a peptide hormone that is mainly produced in the stomach was recently discovered as a natural ligand of the GH secretagog receptor and acts synergistically with GHRH on GH release (6, 7). In addition, ghrelin and somatostatin seem to be involved in a feedback loop in humans (8, 9). Ghrelin is a functional antagonist of somatostatin and somatostatin antagonizes the actions of ghrelin at the level of the pituitary gland in rat (10).

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 (11). Insulin-like growth factors, produced in response to GH, inhibit GH release via negative feedback on the pituitary, and via stimulation of somatostin release from the hypothalamus. There is also a direct negative feedback by which GH stimulates hypothalamic somatostatin release (12). GH secretion is low in infancy, peaks at puberty and thereafter declines with ageing (13, 14). 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 (15, 16).

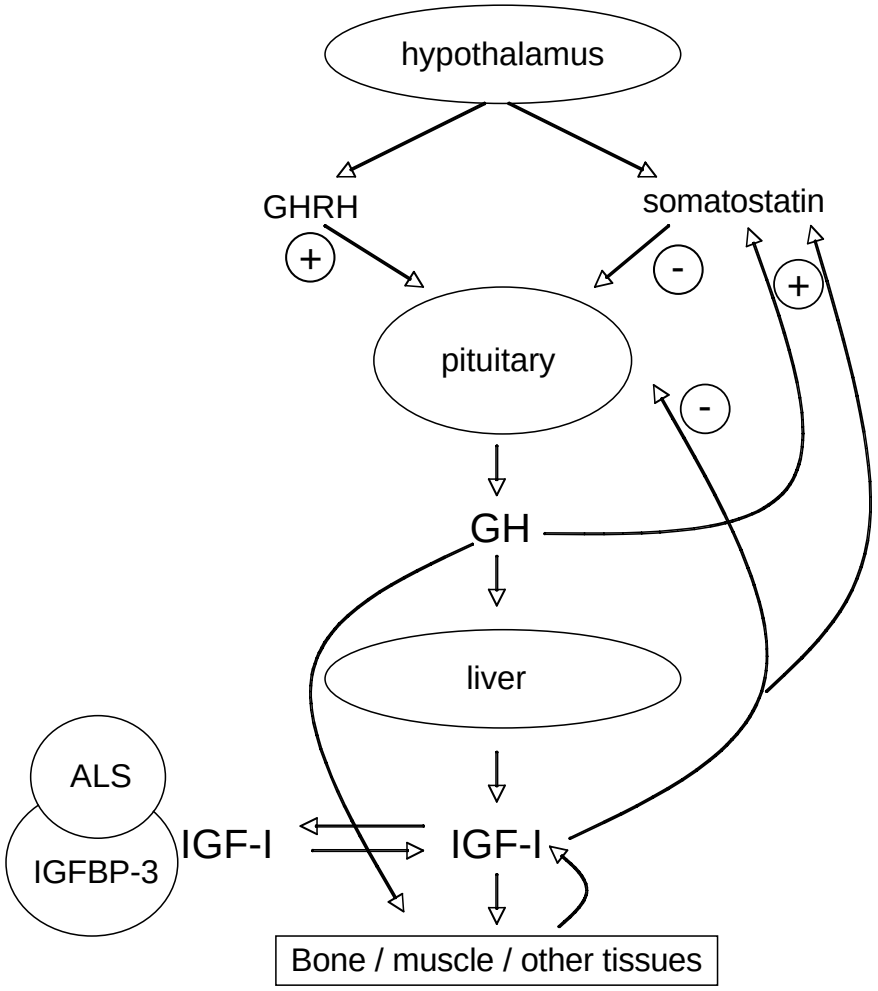

Figure 1. Interplay in the GH/IGF-I axis

Biological effects of GH

GH plays a crucial role for postnatal somatic growth, but is not essential for intra-uterine growth and development (17, 18). GH stimulates proliferation of chondrocytes and osteoblasts thereby promoting linear bone growth (19). Even after epiphyseal closure, GH is active in bone remodeling by increasing bone mass, bone density and strength (20). 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 (21, 22). GH also reduces fat mass by stimulating lipolysis in adipose tissue and muscle (23, 24). 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 (25). 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 (26).

GH receptor

GH exerts its biological effects by binding to specific cell surface growth hormone receptors (GHR) (27). GHR mRNA is found in most tissues and cell types, with the highest expression detected in liver (28). 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 (29). 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 (30, 31). 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 down regulation (32). The human GH (hGH) and hPRL receptors show high sequence identity, particularly in the ligand binding domain (33). hGH is able to bind both the hGHR and the hPRL receptor (hPRLR), while hPRL only binds the hPRLR (34). 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 (35).

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 mainly occurs by alternative splicing of the primary receptor transcript (36-38). This high affinity GHBP binds roughly 50 percent of the GH in plasma

in man (39). 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) (40).

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 (41). However, some evidence suggest the existence of preformed, unliganded receptor dimers that are activated by a ligand-induced change in receptor conformation (42, 43). In accordance with the receptor dimerization mechanism, dose-response experiments give rise to biphasic, bell-shaped kinetics (44). 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 (45) and phosphorylates cytoplasmic domains of GHRs on tyrosine residues, thereby creating docking sites for SH2 (src homology 2) containing proteins (46). 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 (47). Activation of the GH receptor can also initiate signal transduction via the Ras-MAPK (mitogen activated protein kinase) pathway leading to effects on gene transcription and metabolism (48). 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 (49).

Insulin-like growth factors

Many of the growth-promoting actions of GH are mediated indirectly by the GH-induced production of insulin-like growth factor I (IGF-I) (50). As the name implies, IGF-I has a strong structural resemblance with proinsulin (51). Circulating IGF-I is produced primarily

in the liver, while local production in bone and muscle exerts important paracrine and autocrine effects in these tissues (52, 53). Expression of IGF-I is primarily regulated by GH, but also influenced by other hormones, growth factors, and nutritional status (54). During fasting, insulin production decreases, causing reduction in GHR expression and thereby GH resistance - that is, low IGF-I secretion despite high concentrations of GH (55). IGF-I is classified as an anabolic and mitogenic hormone, it stimulates protein and glycogen synthesis, increases DNA synthesis, stimulates cell cycle progression and inhibits apoptosis (56). Mice carrying null mutations of the IGF-I gene are born small and grow poorly postnatally (57). Conditional liver knockout of the IGF-I gene in mice results in significantly suppressed serum levels of IGF-I, but without any corresponding decrease in growth (58). This suggests that paracrine/autocrine IGF-I effects might be more important than circulating IGF-I 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-I levels were further reduced. In the circulation, ALS forms a complex with IGF-I and IGFBP-3 (see below). This double knockout animal exhibits growth retardation, suggesting that circulating IGF-I contributes to growth, but that local production of IGF also is of importance (59). Interestingly, these studies revealed that IGF-I has dual effects on bone development; circulating IGF-I plays a major role for bone density, whereas both circulating and local IGF-I are involved in linear bone growth.

In addition to IGF-I, there is also an insulin-like growth factor II (IGF-II). IGF-II is important for fetal growth and development, while postnatal growth, at least in rodents, is IGF-II independent. The cellular actions of IGF-II are propagated by the IGF-I receptor while the IGF-II/mannose-6-phosphate receptor is responsible for internalization and degradation of IGF-II. Unlike IGF-I, the expression of the IGF-II gene is not regulated by GH but by other hormones and tissue specific growth factors (60). IGF-II seem to be involved in the progression of tumors by an autocrine mechanism, but expression of IGF-II alone is not sufficient to induce full malignant transformation (61).

IGF-I receptor

The biological actions of IGF-I are mediated by the IGF-I receptor (IGF-IR), which also binds IGF-II. IGF-IR knockout results in more severe growth failure than deletion of IGF-I

and the IGF-IR null mice die of respiratory failure at birth (62). IGF-IR is a transmembrane tyrosine kinase formed as a tetramer consisting of two identical α-subunits and two identical β-subunits (63). Virtually every tissue and cell type express IGF-IR mRNA, although it is a low-abundant transcript. In the adult rat, the highest levels of IGF-I mRNA are observed in the central nervous system, with intermediate levels found in kidney, stomach, testes, lung, heart and liver (63, 64). 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. In contrast, IGF-1R expression is inhibited by tumor suppressors, such as p53, Wilms' tumor-1 and BRCA-1, furthermore the expression declines with ageing and is subject to negative feedback regulation by high IGF-I levels (63).

Binding of IGF-I to IGF-IR activates the intrinsic receptor tyrosine kinase and initiates several signal transduction pathways including the Ras-Raf-MAPK pathway and the PI3K pathway. Due to the homology between IGF-I and insulin, and the homology between IGF-IR and the insulin receptor (IR), IGF-I can interact with the IR although with a lower affinity than that of insulin (65). In plasma IGF-I circulates in up to thousand-fold higher concentrations compared to insulin. Despite the fact that the affinity of IGF-I for the IR is only 5 percent of insulin's affinity for the IR, IGF-I could in theory have a huge impact on glucose levels because of its abundance (66). Some studies do point to a direct role of circulating IGF-I in maintaining glucose homeostasis (67). Circulating IGF-I indirectly confers important metabolic effects by controlling GH secretion and thereby modifying the biological role of insulin in maintaining normal metabolic homeostasis (68). However, the effect of IGF-I on IR is limited since the activity of IGF-I is inhibited by binding to specific binding proteins (66).

IGF binding proteins

IGF-I is transported in plasma bound to a family of specific high-affinity IGF binding proteins (IGFBP-1 -6) (56). These circulating IGFBPs protect IGF-I from proteases and prolong the half-life of IGF-I in plasma. The IGFBPs can also sequester IGF-I away from the IGF-IR, thereby inhibiting mitogenesis, differentiation, survival and other IGF-I

stimulated effects. Proteolysis of IGFBPs can reverse this inhibition or generate IGFBP fragments with novel bioactivity. In addition, IGFBP-interaction with cell surface components may concentrate IGF-I near its receptor, enhancing IGF-I activity. IGF-I receptor-independent actions of the IGFBPs are also increasingly recognized (69). The majority of IGF-I (about 75 percent) in serum is transported in a 150 kDa complex with IGFBP-3 and the acid labile subunit (ALS) (70). This trimeric complex forms a reservoir of IGF-I in circulation by extending the half-life from about 10 minutes when in free form, to 30 - 90 minutes in binary complexes, to more than 12 hours when bound in the ternary complex and also by preventing passage of the complex across the vascular endothelial barrier (71). ALS is expressed predominantly in the parenchymal liver cells, and its expression is tightly regulated by GH (72). 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 (73) or indirectly via IGF-I production in adjacent hepatocytes (74, 75).

Most studies on the IGF independent actions of IGFBPs have focused on IGFBP-3 and IGFBP-5. Growth inhibitory and proapoptotic IGF-independent effects of IGFBP-3 have been reported both *in vitro* and *in vivo* (76, 77). Low levels of serum IGFBP-3 together with high levels of IGF-I have in several epidemiological studies indicated increased risk for developing cancers in tissues such as colon, breast, lung and prostate (78, 79).

IGFBP-5 shares considerable structural homology with IGFBP-3 and is also involved in trimeric complexes with IGF-I and ALS (80). IGFBP-5 has been reported to have both IGF-I stimulatory and inhibitory effects. It binds to extracellular matrix proteins, stimulates cell migration, and enhances bone formation and osteoblast proliferation (81). 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 to translocate to the nucleus via an importin β-dependent pathway (82), though the cellular consequences of these actions are yet unknown.

Animal experiments have shown that insulin reduces hepatic secretion of IGFBP-1 (83). Also in humans, elevated IGFBP-1 occurs universally in conditions associated with decreased insulin levels, including fasting, exercise, and insulin-dependent diabetes (84-86). IGFBP-1 has been shown to stimulate cell migration by engaging $\alpha_5\beta_1$ integrin, the fibronectin receptor (87). After integrin-binding, IGFBP-1 is reported to exert IGF-I independent, proapoptotic effects in breast cancer cells (88).

Several studies describe IGFBP-4 as a potent inhibitor of the anabolic effects of IGF-I by regulating IGF-I bioavailability. However, specific proteases can cleave IGFBP-4 into fragments with low affinity for IGF-I thereby increasing the anabolic IGF-I effects (89).

GH/IGF-I pathophysiology

Abnormal secretion of GH 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. Hypersecretion of GH in adults is usually caused by a pituitary somatotroph tumor and results in acromegaly. This leads to elevated levels of IGF-I and 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. Acromegaly has been associated with a two- to three-fold increased mortality, most commonly due to cardiovascular, cerebrovascular, and respiratory disease (90). The development of cardiovascular disease in acromegaly is in some respect paradoxical since GH is beneficial in models of heart failure (91). Also, patients with heart failure have improved cardiac function when treated with GH (92) and the cardiac abnormalities associated with GH deficiency are corrected after GH therapy (93). Administration of supraphysiological doses of GH to healthy subjects is associated with ventricular hypertrophy and increased cardiac output (94). Acromegalics frequently develop cardiomyopathy, but why and how this turns in to a maladaptive process is yet incompletely revealed (95). Hypertension caused by GH/IGF-I-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 (96). 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 (97).

Acromegaly and elevated GH levels are reported to increase the risk of developing malignancies, particularly in the colon, breast and prostate (98-101). Elevated GH levels have further been correlated with diabetic complications and increased renal and glomerular growth (102, 103). Thus, there are several scenarios in which elevated GH levels are implicated in physiological disorders.

GHR antagonists

Current treatment modalities for acromegaly consist of surgery, radiation therapy, and medical treatment with dopamine agonists (suppress growth hormone secretion in acromegalics), somatostatin analogs and the recently developed GH antagonist Pegvisomant (Pfizer). A significant proportion of acromegalics require long-term medical therapy for control of their GH/IGF-I levels (104). Pegvisomant is a genetically engineered analog of hGH that in contrast to other treatments for acromegaly inhibits GH action instead of GH secretion (105). Clinical studies report reduced serum IGF-I into the age-related reference range in over 90 percent of the patients treated with Pegvisomant (106).

AIMS

A GH antagonist offers the possibility of higher normalization rates and greater specificity of effect compared to other current therapies for acromegaly (106). Although Pegvisomant effectively normalizes IGF-I in most acromegalics, a small molecule, nonpeptidyl drug that could be administered orally would further improve patient acceptability and compliance. Such a compound could also be effective for treatment of other disorders related to a disturbed GH/IGF-I axis including diabetic complications and certain cancers.

The specific aims of this study were:

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

To study the effects of one selected GH antagonist on different markers of the GH/IGF-I axis *in vivo* in hypophysectomized (HX) GH-substituted rats.

COMMENTS ON METHODOLOGY

mRNA quantification

Quantitative mRNA analysis or gene expression analysis aims to measure the concentrations (or copy numbers) of specific RNA sequences. The different methods available for RNA quantification are usually developed for different purposes and also differ a lot in throughput. Traditionally, hybridization methods such as dot blots, northern blot, RNase protection assays (RPA) and semiquantitative reverse transcriptase PCR have been used for quantifying steady state levels of mRNA. However, the requirement of large samples, the relative insensitivity, and the lack of sufficient throughput make these techniques unsuitable for screening of a large number of samples.

Today, real-time PCR (RT-PCR) is recognized as a reproducible, highly sensitive and specific technique for mRNA detection (107, 108). It is commonly used when the starting material is limited and when high sensitivity and accuracy is needed. However, RT-PCR requires special and quite expensive instruments and reagents and optimization of the reactions can be time consuming. The RT-PCR method is most suitable when only a few genes are to be analyzed. When the sensitivity of RNA quantification assays increases, methods for normalization become increasingly important. Which genes chosen for normalization can have significant impact on the results and there is no consensus on which genes to use. Variations in expression of the commonly used housekeeping genes after different treatments and in different states of cellular proliferation are reported (109, 110). No single gene or parameter has been identified as being invariable in every case. In addition, an internal standard is most suited when expressed at levels comparable to that of

the target mRNA. A suitable standard must therefore be determined independently for each experimental system.

Primary cells

Since the liver is rich in GHR and many of the markers of GHR activity are produced in the liver, hepatic cells are valuable for studies on GH induced gene expression. GH responsive liver-derived cell lines have been difficult to identify. However, cultured primary rat hepatocytes can maintain the differentiated functions of normal adult liver and have been shown to produce IGF-I in response to GH stimulation (111, 112).

***In vivo* animal models of acromegaly**

The hypophysectomized rat has often been used for studies of GH deficiency since these animals display growth arrest and reduced serum levels of IGF-I that GH replacement effectively restores (113). This model has the advantages of adjustable GH levels and is also devoid of feedback inhibition on the hypothalamus-pituitary axis. Transgenic mice over-expressing the human GH-releasing hormone (hGHRH) gene have also been used as an animal model for acromegaly (114). The overproduction of hGHRH results in a dramatic increase in GH and IGF-I secretion. However, only one rare type of acromegaly results from ectopic over-expression of GHRH, and GHRH stimulates pulsatile GH secretion in contrast to the continuously high levels seen in acromegalics. GH transgenic rats over-expressing either bovine or human GH (115), or rats implanted with GH-producing GC cells (116), have the advantage of providing continuously high GH levels, although differences between individuals are difficult to avoid, and the levels of GH cannot be adjusted.

Serum IGF-I measurements

Analysis of IGF-1 serum concentrations is common practice for diagnosis and monitoring of treatment efficiency in patients with GH-secretory disorders, deficiency or excess (66). Total IGF-I is generally measured by means of immunoassays with IGF-I antibodies, either competitive radioimmunoassay (RIA) or immunoradiometric assays (IRMA) (117). When using these assays, the interference from IGFBPs in the sample has to be eliminated.

Extraction with acid ethanol followed by cryopreservation and the use of truncated IGF-I (des (1-3) IGF-I) with reduced IGFBP affinity as radioligand, is a recommended procedure (118, 119). Less than 1 percent of the total circulating IGF-I is free, *i.e.* not in complex with IGFBPs. Since the IGFBPs can alter IGF-bioactivity without changing extractable IGF-I, the bioactivity of IGF-I might be closer related to the free fraction than the total amount of IGF-I. However, since there is not yet a consensus on a standard method for determining free IGF-I, serum total IGF-I remains the prevailing measurement (120, 121).

RESULTS AND DISCUSSION

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

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-I 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-I mRNA response to hGH and potential hGH antagonists.

As previously shown by others (122), 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 RPA to give adequate amount of IGF-I 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-I 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-I 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 (123). 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-I 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.

A rat *in vivo*-model for evaluation of growth hormone receptor antagonists (manuscript)

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-I system and the

hypotalamo-pituitary axis can be avoided. The hypophysectomized (HX) rat is an established model with reduced growth and IGF-I levels that can be restored by GH treatment (124). 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 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-I and hepatic mRNA levels of components of the GH/IGF-I axis. Serum IGF-I 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-I 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-I, IGFBP-3, ALS, IGF-IR 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-I, 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-I (73-75). The kinetics of IGF-I stimulated IGFBP-3 expression appears to follow the same time course as GH induction of IGF-I mRNA. IGFBP-3 mRNA is reported to increase by 3 h treatment with IGF-I, with maximum increases observed between 8–12 h of treatment (125). If as some studies suggest, the induction of IGFBP-3 is secondary to GH stimulation, via IGF-1 (74, 75), 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-I (as well as ALS) mRNA levels probably had time to rise, while the rise in IGFBP-3 had just started. The reduction of IGF-I 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-I in serum. The IGF-I mRNA levels could also have had time to rise after the last dose of antagonist, with the effect on IGF-I protein level not being evident yet. Moreover, the effect on IGF-I 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. We conclude that the GH-treated HX rat can be a useful model for studying effects of compounds that inhibit GH receptor activation.

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 (126), 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-I, also inhibit diabetes-induced glomerusclerosis (103), reduce hypoxia-induced retinal neovascularization (127), and decrease size and progression of dimethyl butyric acid-induced breast tumors (128).

FUTURE PERSPECTIVES AND CONCLUSION

The ability of a GH antagonist to effectively normalize IGF-I 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-I, but also on IGFBP-3 and ALS expression, can as observed in this study cause a substantial decrease in serum levels of IGF-I. 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. GH antagonist treatment in acromegaly could potentially lead to increased tumor volume since the reduced feedback inhibition from IGF-I 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 (129). Nevertheless, attention to this possible side effect is warranted. In addition to treatment of acromegaly, a GH antagonist could potentially be used to halt

progression of diabetic end organ damage, such as proliferative diabetic retinopathy or diabetic nephropathy, and in the treatment of cancers that are GH- or IGF-I dependent.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to everyone who contributed to this thesis and especially to:

My wonderful supervisors Professor Agneta Mode and Vendela Parrow:
Agneta, for being an outstanding academic role model, for sharing your knowledge in molecular endocrinology with me and for your positive attitude, kindness, enthusiasm and never-ending patience at bad hours with Pelle waiting.
Vendela, for being an excellent mentor for me at Biovitrum, for sharing your knowledge in cell- and molecular biology, in science in general and the biotech business, for encouraging me, and for the interesting discussions about everything under the sun.

My wonderful colleagues at Biovitrum; Sonja Gustafsson, Marguerite Luthman, Jan Hörling, Carlo Zaniol, Ulla Källström and everybody else in ADS. For your helpfulness, encouragement, good spirits and all the laughs.

My collaborators and coauthors Karin Fhölenhag, Joanna Chmielewska, Hanna Simko, Ladan Aryan and Pia Axelsson-Lendin, for invaluable technical help, making this thesis possible.

Cissi and Pia in the AM group for sharing your results, reagents and knowledge of various matters and for always being helpful and friendly.

Last but not least, my family; my father Anders, mother Solveig, sisters Sara and Andrea for your encouragement and support. And Fredrik, thank you for being there.

This work was supported by Biovitrum AB and by grants from the Swedish Research Council and Karolinska Institutet.

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