Studies on the PI3K/mTOR pathway as cytostatic treatment target in pituitary adenomas

Dissertation

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ABBREVIATIONS

ACRO	Acromegaly-associated pituitary adenomas
ACTH	Adrenocorticotrophic hormone
Cdk	Cell cycle dependent kinase
CRH	Corticotropin releasing hormone
CUSH	Cushing's associated pituitary adenomas
DR	Dopamine receptor
FACS	Fluorescence-activated cell sorting
FSH	Follicle stimulating hormone
GH	Growth hormone
GHRH	Growth hormone releasing hormone
GnRH	Gonadotropin releasing hormone
GSK-3β	Glycogen synthase kinase 3 beta
[^{3}H]-TdR	Thymidine incorporation assay
IGF-1	Insulin-like growth factor 1
IRS-1	Insulin receptor substrate 1
MAPK	Mitogen-activated protein kinase
mTOR	Mammalian target of rapamycin
NFPA	Non-functioning pituitary adenoma
PARP	Poly (ADP-Ribose) Polymerase
PDK-1	Phosphoinositide depend kinase 1
PI(3,4,5)P2	Phosphatidylinositole 3,4,5 trisphosphate
PI(4,5)P2	Phosphatidylinositole 4, 5 bisphosphate
PI3K	Phosphatidylinositole 3-kinase
PRL	Prolactin
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
Rb	Retinoblastoma
RIA	Radioimmunoassay
RLA	Relative luciferase activity
SHP-1	Src homology 2 domain-containing protein tyrosine-phosphatase 1
SSA	Somatostatin analogs
Sstr	Somatostatin receptor
VEGF	Vascular endothelial growth factor

SUMMARY

Pituitary adenomas are benign neoplasms accounting for 15% of all intracranial tumors. They are associated with significant clinical syndromes due to the hormonal excess they produce or to visual/cranial disturbances because of their considerable intracranial mass. Surgery is the primary means for the management of pituitary tumor mass, but it comes with considerable side effects to the patients and their quality of life. Tumor shrinkage by pharmacological agents currently used in neuroenocrinology, such as, somatostatin analogs (SSA) is not observed in a large fraction of pituitary adenomas. Therefore, efforts are taken to investigate how to overcome the resistance to existing treatments and to identify new cytostatic therapeutic agents.

The PI3K/Akt/mTOR signaling pathway is frequently overactivated in a variety of tumors rendering them resistant to chemo- and radiotherapy. The present study shows that Akt overactivation confers resistance to the antiproliferative action of the SSA octreotide in pituitary tumor cells. Blocking the Akt pathway downstream with rapamycin rendered those cells sensitive to octeotide's antiproliferative action. However, the efficacy of the combined treatment was not due to the antiproliferative action of rapamycin, since most of the tumors did not respond to this pharmacological agent. Rapamycin and its analogs (rapalogs) have a cytostatic effect in various tumors. However, resistance to rapalog treatment is reported with increasing frequency due to the elimination of the negative feedback loop exerted by the mTOR substrate p70/S6K on IRS-1. Rapamycin, by inhibiting mTOR and p70/S6K, decreases the inhibitory IRS-1 serine phosphorylation, activates IRS-1 and increases Akt-Ser^{473} phosphorylation. The present study shows for the first time that activating the G protein-coupled receptor Sstr2 with octreotide blocks the rapamycin-induced IRS-1 activation by increasing its inhibitory serine and decreasing its stimulatory tyrosine phosphorylation. This leads to decreased Akt-Ser^{473} phosphorylation in a mechanism involving the phosphotyrosine phosphatase SHP-1. Both octreotide and rapamycin are cytostatic agents blocking the G1/S cell cycle transition and herein it is seen that their potent antiproliferative action depends on the more potent upregulation of the Cdk2 inhibitor p27/Kip1.

A novel drug able to co-target the PI3K pathway up- and downstream is the dual class PI3K/mTOR inhibitor, NVP-BEZ235, which has shown high antiproliferative efficacy in tumors with the overactivated PI3K pathway. In the present study NVP-BEZ235 treatment dramatically decreased cell viability by suppressing the cell cycle activators cyclin E and Cdk2 and upregulating the cell cycle progression inhibitor p27/Kip1. The remarkable sensitivity of

pituitary adenomas to NVP-BEZ235 highlights the importance of the Akt dysregulation in their tumor maintenance.

Altogether, these data provide new therapeutic cytostatic schemes that could prove beneficial for the management of pituitary macroadenomas. In addition they provide the biochemical basis for combating resistance to rapalog treatment also in other tumor types by concomitant administration of biologicals able to inhibit the PI3K pathway upstream.

ZUSAMMENFASSUNG

Hypophysenadenome sind gutartige Tumoren der Hirnanhangdrüse, die etwa 15% aller intrakraniellen Tumoren repräsentieren. Sie verursachen klinische Symptome zum einen durch die erhöhte Hormonproduktion, zum anderen führt die Raumforderung der Adenome zu visuellen (Gesichtsfeldverlust) und allgemeinen intrakraniellen Störungen (z.B. Kopfschmerzen). Die etablierte pharmakologische Therapie mit Octreotid – einem der in der Neuroendokrinologie meistverwendeten Somatostatinanaloga - zur Reduktion der Tumormasse, hat sich in vielen Fällen als unwirksam erwiesen. Die dadurch notwendige operative Tumorresektion kann mit beträchtlichen Nebenwirkungen verbunden sein und damit die Lebensqualität der Patienten beeinträchtigen. Aktuelle Forschungskonzepte bei Hypophysenadenomen haben daher die Überwindung der Resistenz gegenüber vorhandenen Therapeutika und die Identifizierung neuer Wirksubstanzen zum Ziel.

Der PI3K/Akt/mTOR Signalweg ist in vielen Tumorarten überaktiviert und dort ursächlich an der Chemo- und Strahlentherapieresistenz beteiligt. In der vorliegenden Arbeit wurde gezeigt, dass Octreotid-sensitive Hypophysentumorzellen nach Überexpression von Akt auf die wachstumsinhibierende Wirkung von Octreotid nicht mehr ansprechen. Die Behandlung mit dem mTOR-Inhibitor Rapamycin konnte die Resistenz gegenüber Octreotid aufheben und den wachstumsinhibierenden Effekt der kombinierten Octreotid/Rapamycin-Behandlung steigern. Die besondere Wirksamkeit der Kombinationsbehandlung war dabei nicht auf die antiproliferative Wirkung von Rapamycin zurückzuführen, da die meisten Tumore keine Reaktion auf eine alleinige Rapamycin-Behandlung zeigten. Rapamycin und seine Analoga (Rapaloga) besitzen eine nachgewiesene zytostatische Wirkung auf verschiedene Tumorarten. Dennoch belegen neuere Untersuchungen das vermehrte Auftreten von Resistenzen gegenüber Rapamycin, die durch die Beseitigung des negativen Rückkopplungsmechanismus auf IRS-1 durch das mTOR-Substrat p70/S6K verursacht werden. Rapamycin inhibiert sowohl mTOR als auch p70/S6K und reduziert dadurch die inhibierende IRS-1 Serinphosphorylierung, aktiviert IRS-1 und steigert die Akt-Ser^{473} Phosphorylierung. In der vorliegenden Arbeit wird zum ersten Mal gezeigt, dass die Octreotid-induzierte Aktivierung des G-Protein-gekoppelten Sstr2 Rezeptors durch die Erhöhung der inhibierenden IRS-1 Serinphosphorylation und die Reduktion der stimulierenden IRS-1 Tyrosinphosphorylation die Rapamycin-induzierte IRS-1 Aktivierung hemmt. Dies führt zur Reduktion der Akt-Ser^{473} Phosphorylierung unter Mitwirkung der Phosphotyrosin-Phosphatase SHP-1. Sowohl Octreotid als auch Rapamycin wirken zytostatisch

und hemmen den Zellzyklus am G1/S-Übergang. Daher konnte in der vorliegenden Arbeit gezeigt werden, dass die antiproliferative Wirkung der beiden Substanzen zusätzlich mit einer starken Erhöhung des Cdk2 Inhibitors (p27/Kip1) zusammenhängt.

Eine weitere Möglichkeit zur Manipulation des PI3K-Signalwegs bietet der duale PI3K/mTOR Inhibitor NVP-BEZ2335, der eine starke antiproliferative Wirkung in Tumoren mit überaktiviertem PI3K-Signalweg aufweist. In der vorliegenden Arbeit erzielte NVP-BEZ235 eine dramatische Senkung der Zellviabilität durch die Reduktion der Zellzyklus-Aktivatoren Zyklin E und Cdk2. Die starke Reaktion der Hypophysenadenome auf NVP-BEZ235 unterstreicht die Bedeutung von überaktiviertem Akt für die Erhaltung der tumorigenen Eigenschaften.

Zusammengefasst zeigen die in der vorliegenden Arbeit gewonnenen Erkenntnisse neue Möglichkeiten der zytostatischen Tumortherapie auf, welche sich bei der Behandlung großer Hypophysenadenome als hilfreich erweisen können. Es wird gezeigt, über welche biochemischen Mechanismen Rapaloga die Resistenz von Hypophysentumoren gegenüber Octreotid und umgekehrt Octreotid die Rapalog-Resistenz überwinden können, was möglicherweise von Bedeutung auch für andere Tumortypen sein könnte. Dementsprechend könnten von einer effektiven Kombinationstherapie mit Rapaloga und anderen Inhibitoren des PI3K-Signalweges viele Tumorarten profitieren.

1 INTRODUCTION

1.1 The pituitary gland

The pituitary gland, or hypophysis, is often called the “master” gland of the endocrine system due to the irreplaceable role in its regulation. In turn, the pituitary is controlled in large part by the hypothalamus, a region of the brain placed just above the pituitary. It is no larger than a pea, about 1-1.5 cm in diameter and approximately 0.6g (the average weight in human male adult). It resides within a bony depression of the sphenoid bone, the sella turcica, which sits below the optic chiasm, the area where optic nerves cross and enter the brain. The pituitary gland is divided into two main portions: the larger anterior pituitary (adenohypophysis) and the smaller posterior pituitary (neurohypophysis; Fig. 1). A third lobe, pars intermedia, is a prominent and functionally significant feature in rodent pituitary, but rudimentary in humans.

The pituitary gland develops from two embryologically different parts: an invagination of the oral ectoderm, known as Rathke´s pouch and the infundibulum from the neuroectoderm of the floor of the third ventricle. The cells of the anterior wall of Rathke´s pouch differentiate and rapidly proliferate under the influence of certain transcription factors to form the adenohypophysis, while the posterior wall gives rise to the pars intermedia. The infundibulum gives rise to the pituitary stalk and to the neurohypophysis.

The neurohypophysis is composed of modified glial cells, the pituicytes, and nerve endings of the nerve fibres, extending from the hypothalamus. The adenohypophysis is composed of the pars distalis, which is the largest part of the gland containing the hormone producing cells; the pars intermedia, filled with microcysts - rudiments of Rathke´s pouch; and the pars tuberalis/infundibularis, which is an upward extension of the anterior lobe towards and around the pituitary stalk (Asa et al., 1995).

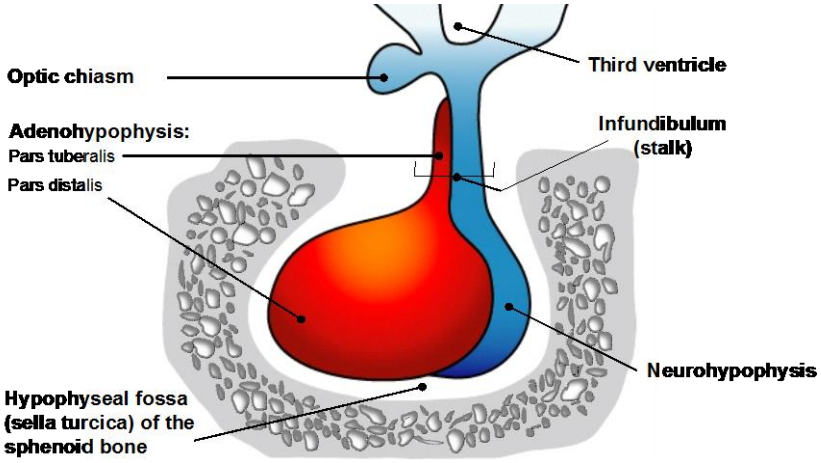

Fig. 1. The hypophysis. The pituitary gland is depicted residing in the invagination of the sphenoid bone, the sella turcica, under the 3rd ventricle. The two lobes, anterior (adenohypophysis; in red) and posterior (neurohypophysis) are indicated.

The pituitary gland secretes hormones regulating a wide variety of bodily activities, including trophic hormones that stimulate other endocrine glands. The adenohypophysis is under the control of releasing hormones originating from the hypothalamus and received via a portal vein system. Neurohypophysis, being a projection of the hypothalamus, does not produce but only stores and releases the hormones.

Six main hormones are produced by the adenohypophysis: growth hormone (GH), prolactin (PRL), adrenocorticotrophic hormone (ACTH), follicle stimulating hormone (FSH), luteinizing hormone (LH), and thyroid stimulating hormone (TSH). GH promotes growth of the skeleton and soft tissues and has important metabolic effects. Its effects are mediated directly through GH receptors or indirectly by inducing insulin-like growth factor (IGF-1) synthesis in the liver. PRL has important role in the initiation and maintenance of lactation. ACTH stimulates glucocorticoid production from the adrenal cortex. It is split product of proopiomelanocortin (POMC), together with β-lipotropic hormone (β-LPH), endorphins, encephalin, corticotrophin-like immunoreactive peptide (CLIP), and α melanocyte stimulating hormone (α-MSH). FSH and LH are collectively referred to as gonadotrophins. In males, LH causes the testes to produce testosterone, while in females it stimulates the ovaries to produce estrogen. Furthermore, LH has a critical role in maintaining the pregnancy. FSH promotes follicular growth in the ovaries and spermatogenesis in the testes. TSH is important for the physiological growth and function of the thyroid gland.

A non-endocrine cell type which comprises 3-5% of all adenohypophyseal cells is the star-shaped folliculostellate cell (Allaerts et al., 1990). Named due to their morphology, they are extended between surrounding endocrine cells. They are source of growth factors and cytokines,

therefore suggesting an important role in the paracrine regulation of hormone secretion (Schwartz & Cherny, 1992; Renner et al., 1996).

1.2 Pituitary adenomas

Most pituitary adenomas are non-metastasizing neoplasms that develop in the adenohypophysis and account for about 15% of all intracranial tumors. Although usually benign, they are associated with significant clinical syndromes due to the hormonal excess they produce, or to visual/cranial disturbances because of their considerable intracranial mass (Melmed et al., 2003; Table 1). Although they are not classified as malignant, pituitary tumors are associated with significant morbidity and mortality.

Pituitary adenomas are classified according to: the clinical presentation based on their hormonal activity *in vivo* (functional classification), tumor size and degree of local invasion (anatomical classification), and specific histological and cytological features (histological classification) (Kovacs et al., 2001).

Anatomical classification results from the neuroradiological examinations (CT-Scan, NMR-Imaging) providing information about the tumor size and extent of local invasion. Adenomas are classified in four grades (Hardy´s classification; Hardy, J., 1979): grade I refers to microadenomas, defined as tumors contained within the pituitary fossa and <10mm in diameter, grade II refers to macroadenomas (>10mm in diameter) that may exhibit suprasellar extension but no invasion to the surrounding bony structures, grade III adenomas are locally invasive tumors, grade IV refers to large invasive tumors that can involve, except the bone, the hypothalamus and the cavernous sinuses.

Histological classification groups the pituitary adenomas according to their reaction to staining into acidophilic, basophilic and chromophobic tumors: GH and/or PRL-producing tumors are acidophilic and ACTH, TSH and FSH/LH secreting are basophilic. Chromophobic adenomas are assumed to be hormonally inactive.

Functional classification groups the pituitary adenomas into clinically functioning or non-functioning, depending on whether the adenoma development leads to an endocrine syndrome.

Prolactinomas are the most common type of all functioning adenomas and are usually microprolactinomas. Due to the PRL overexpression, patients harboring a prolactinoma suffer from amenorrhea, infertility and galactorrhea in females, infertility or impotence in males.

In acromegaly associated pituitary adenomas (ACRO), over-secretion of GH leads to the acromegalic syndrome in adults and gigantism in children. Clinical presentation of acromegaly is characterized by acral and facial changes, hyperhidrosis (abnormally increased perspiration),

headaches, sexual dysfunction and soft tissue enlargement over years. All this progressive disfigurements are combined with comorbidities like hypertension, megalocardia and diabetes mellitus. Occurrence of the cardiovascular, cerebrovascular, metabolic and respiratory comorbidities is associated with average 10-year reduction in life expectancy with at least a doubling of standardized mortality rates in acromegaly patients as it was shown in a recent study (Holdaway et al., 2008).

Corticotrophinomas present with hyperfunction rather than mass effects and they are mostly microadenomas. ACTH hypersecretion and adrenal steroid overstimulation lead to hypercortisolism, which is responsible for the symptoms of the Cushing`s disease. The specific fat arrangement that accumulates in the face giving a typical moon face, as well as buffalo hump and truncal obesity are the one accounting for the most prominent symptoms of the disease. Additionally, the skin gets thinner caused by the loss of the epidermis layer and the tension over accumulated fat produces big and purple striae. Muscle wasting is frequently observed as well hirsutism in women. Due to the high blood pressure serious cardiovascular diseases occur, leading to increased morbidity and mortality rate. Clinical presentation of Cushing's disease also includes psychiatric disorders such as depression and psychosis (Bertagna et al., 2009).

TSH-secreting adenomas or thyrotrophinomas are rare and present with a mild increase in thyroxin levels with inappropriate TSH levels.

Pure gonadotrophinomas secreting intact FSH and LH are very rare. They may cause sexual dysfunction and hypogonadism. Beside one case of increase in testicular size due to FSH overproduction (Heseltine et al., 1989), only few cases of precocious puberty were reported (Tashiro et al., 2000). Increased serum testosterone levels were found in men when present with excess LH production (Klibanski et al., 1987). Nevertheless, gonadotrophinomas are usually not characterised by hormonal overproduction.

Non-functioning pituitary adenomas (NFPA) comprise approximately 25% of all pituitary adenomas. They are clinically silent and mostly present with macroadenomas. Clinical presentation reflects symptoms of an intracranial mass, such as headache, visual field disturbances, pituitary apoplexy and pituitary hormone hyposecretion. Arising hypopituitarism shows different clinical presentation depending on the degree of adenohypophyseal tissue damage. Low impact of the damage combined with significant pituitary stalk compression, leads to weak hyperprolactinemia. Intensive pressure of the tumor mass on the normal pituitary tissue causes a strong tissue destruction and hypopituitarism (Asa&Ezzat, 2009). All such cases show loss of GH production, 96% of the patients have hypogonadism, 81% show central hypothyroidism, and 61% lack ACTH (Arafah et al., 1986).

Table 1. Hypothalamic-pituitary regulation, hormones and pituitary adenomas

Specific Hypothalamic input	Pituitary cell type	Trophic hormone	Hormone function	Clinical syndrome	Comorbidities
Dopamine	Lactotroph	PRL	Lactation	Prolactinoma	Amenorrhea Galactorrhea Hypogonadism Infertility
GHRH	Somatotroph	GH	Bone and muscle growth; Production of IGF-1 in the liver	Acromegaly	Acral enlargement Soft tissue swelling Cardiac hypertrophy Hypertension Hyperglicemia
CRH	Corticotroph	ACTH and other POMC derivatives	Adrenal cortex; regulation of glucocorticoid synthesis and secretion	Cushing's desease	Truncal obesity Hypercortisolism Hyperglicemia Osteoporosis Hirsutism
TRH	Thyrotroph	TSH	Thyroid gland and thyroid hormone regulation	Thyrotrophinoma	Thyroid goiter Hyperthyroxinemia
GnRH	Gonadotroph	LH, FSH	Gonadal and germ-cell regulation; sex steroid hormones	Gonadotrophinoma Non-functioning adenoma	Mass effects Hypogonadism Hypergonadism Clinically silent

1.3 Current treatment for pituitary adenomas

Surgical therapy, pharmacological therapy and radiotherapy represent the three treatment options for pituitary adenomas. The aim of these different strategies is to normalize excess pituitary hormone secretion, alleviate the symptoms caused by hormonal hypersecretion, shrink or remove tumor mass with respective care of the normal pituitary function. The transsphenoidal surgical approach has been the preferred procedure for removal of tumors. Surgery is indicated if there is evidence of tumor enlargement, especially when growth is accompanied by compression of the optic chiasm, cavernous sinus invasion, or the development of pituitary hormone deficiencies. When patients are treated in specialized centres, 70-80% of those with small, well-defined pituitary tumors that are under 1 cm in diameter are cured. In case of macroadenomas, however, 60% recur within 5 years after surgery. For this reason, conventional radiotherapy is usually added adjunctively to prevent tumor regrowth.

Surgery for pituitary tumors is associated with significant post-operative side effects (Melmed et al., 2003). Depending on the tumor aggressiveness and how difficult it is for the surgeon to reach and completely remove the tumor, patients may show different degrees of hypopituitarism and need pituitary hormone replacement. Hypopituitarism is also a common side effect of radiotherapy.

Pharmacological management of pituitary adenomas is limited to agents with antisecretory properties. The drugs currently used to inhibit excess hormone secretion fall into two categories: the dopamine agonists and somatostatin analogs. Both drug categories act by binding to receptors of the G protein-coupled receptor superfamily. G proteins are heterotrimeric membrane-anchored peptides, composed of three subunits α, β, and γ that play a central role in transmitting signals from the cell surface ligand-receptor complexes to downstream effectors (Simon et al., 1991). The family of G proteins includes, among other, the stimulatory Gs, which is involved in the growth hormone- and corticotropin-releasing hormone pathways, the Gq, in the thyrotropin- and gonadotropin-releasing hormone signaling, and the inhibitory Gi, in the somatostatin and dopamine pathways. In endocrine pituitary cells, hormones acting on G protein-coupled receptors regulate intrapituitary hormone synthesis and cell function.

1.3.1 Dopamine agonists

In the normal pituitary, dopamine mediates the tonic inhibitory control of PRL synthesis and secretion, as well as lactotroph cell proliferation induced by hypothalamic dopamine. Dopamine mediates its effects through the dopamine receptor (DR) family which consists of five different receptor subtypes. These are divided into two groups by their molecular, biochemical, and pharmacological characteristics: D1-like, including D1R and D5R, and D2-like, including D2R, D3R and D4R. D2R exists as two isoforms generated by alternative splicing: the long ($D2_lR$) and the short ($D2_sR$). $D2_lR$ includes an insertion of 29 nucleotides in the third cytoplasmatic loop, a region involved in G-protein receptor coupling, enabling the two isoforms to bind different G proteins or to elicit different effects when bound to the same G-protein (Missale et. al, 1998). *D2r* knockout mice, that are not responsive to dopamine, develop lactotroph hyperplasia and prolactinomas (Schuff et al., 2002).

The potent antisecretory action of dopamine led to the establishment of dopamine agonists for the treatment of prolactinomas. Excessive PRL secretion and tumor size are usually reduced and in most cases normalized by long-acting and potent dopamine agonists such as bromocriptine, pergolide and quinagolide. These agents have been used since the early 1970s and were shown to shrink tumor mass by more than 50% in about 80% of patients (Kleinberg et al., 1983; Vance et al., 1990). Like other D2R agonists, bromocriptine lowers serum PRL levels, and reduces

lactotroph proliferation and tumor growth. However, five to ten percent of patients are not responsive to bromocriptine treatment due to changes in D2R expression and additional 10% are intolerant to the side effects (Pellegrini et al., 1989; Kovacs et al., 1995; Autelitano et al, 1995).
D2Rs are also expressed in non-PRL-secreting pituitary tumors (Bression et al., 1982; Bevan et al., 1986; Muhr et al., 1991). However, the results of bromocriptine treatment in D2R-positive GH-secreting and ACTH-secreting pituitary tumors are controversial (Lamberts et al., 1980; Bevan et al., 1992; Miller et al., 1993; Colao et al., 1997). Similar observations have been made in case of the NFPA, where the D2R isoform specific expression seemed to play a role in different growth inhibition response to bromocriptine. Namely, the presence of $D2_sR$ over $D2_lR$ isoform favored the growth suppressive effect of bromocriptine *in vitro* (Renner et al., 1998). A recent study showed that dopamine agonist treatment can prevent postoperative remnant enlargement in NFPA (Greenman et al., 2005). The outcome of the study showed that dopamine agonist therapy was more potent when the treatment was instituted before tumor remnant growth is detected.
A long-acting, high-affinity, selective D2R agonist, cabergoline, has been approved for the treatment of prolactinomas as well as for other tumor types (Bevan et al., 1994; Abs et al., 1998; Cozzi et al., 1998). Cabergoline is as effective as bromocriptine in lowering serum PRL levels and reducing tumor size in prolactinomas (Colao et al., 1997; Lohmann et al., 2001). Lower incidence of reported side effects and weekly *versus* daily administration, gives an advantage to cabergoline over earlier D2R agonists. In corticotrophinomas cabergoline suppressed ACTH secretion *in vitro* and in patients *in vivo* (Pivonello et al., 2004 and 2009). In contrast, in NFPA cabergoline treatment, as in the case with bromocriptine, did not suppress tumor growth in the majority of the cases (Pivonello et al., 2004).

1.3.2 Somatostatin analogs

Somatostatin (SS), also known as somatotropin release-inhibiting factor, is produced by normal endocrine, gastrointestinal, immune and neuronal cells. It has diverse biological effects throughout the body, mainly inhibitory on the hormone secretion (e.g. growth hormone, insulin), cell proliferation and survival. Somatostatin exists as two biologically active isoforms, SS-14 and SS-28, which derive from prosomatostatin by proteolytic processing. There are five somatostatin receptors Sstr1-5, with Sstr2 having two isoforms: Sstr2A and B, generated by alternate splicing (Patel et al., 1993). All Sstrs belong to the G protein-coupled receptors (GPCR), are mainly coupled to the Gi protein and inhibit adenylate cyclase and cAMP accumulation (Patel et al., 1999).

Pituitary adenomas express all Sstr subtypes except the Sstr4. ACRO preferentially express Sstr2 and Sstr5, prolactinomas and corticotrophinomas express Sstr5 more than Sstr2 (Greenman & Melmed, 1994a; Greenman & Melmed, 1994b; Shimon et al., 1997; Jaquet et al., 2000; Nielsen et al., 2001), while NFPA expresses predominantly Sstr3 and lower levels of Sstr2 (Nielsen et al., 2001; Taboada et al., 2007).

Native somatostatin is metabolically unstable and has short half-life. Synthetic somatostatin analogs (SSA) overcoming these limitations, such as octreotide and lanreotide, were approved for clinical use and especially for the treatment of acromegaly (Lamberts et al., 1985 and 1992). Treatment with these two SSA normalizes circulating GH- and IGF-1 levels in approximately 60-70% of acromegalic patients (Vance et al., 1991; Ezzat et al., 1992; Newman et al., 1995). A third SSA, RC-160 (vapreotide), is mostly used in preclinical studies.

Synthetic SSA bind mainly to Sstr2 and with lower affinity to Sstr5. Analogs specific for the other Sstr subtypes were also developed (Shimon et al., 1997), as well as bi-specific analog with equal affinity for Sstr2 and 5 (BIM23244), which was found to suppress not only GH but also PRL release, making it useful for the treatment of mixed GH/PRL secreting pituitary adenomas (Saveanu et al., 2001). The trend of creating multi-specific SSA picked at the development of the multiligand SSA pasireotide SOM230, which has high affinity for Sstr1, 2, 3, and 5 (Bruns et al., 2002). In acromegalic patients, SOM230 was able to suppress GH secretion in all patients even in those cases that were resistant to the standard octreotide treatment (van der Hoek et al., 2004). In patients with Cushing's disease, SOM230 decreased urinary free cortisol and ACTH levels in approximately 80% of cases, indicating that it would be effective for the management of this disease (Boscaro et al., 2009). Finally, taking into account the cross-talks between receptors of the same family, somatostatin-dopamine hybrid molecules were synthesized with high affinity to Sstr2 and D2R (BIM23A387) or Sstr2, Sstr5 and D2R (BIM23A760 and BIM23A761).

Although the use of SSA has been based on their antisecretory action, these agents are also characterized by antiproliferative properties (Reubi & Laissue, 1995; Susini & Buscail, 2006; Florio et al., 2008). In immortalized pituitary tumor cells SSA were shown to limit cell growth through cytostatic or apoptotic mechanisms (Cheung & Boyages, 1995; Srikant et al., 1995).

1.3.3 Antiproliferative somatostatin receptor signaling

The most commonly used SSA, octreotide and lanreotide, mainly target Sstr2, therefore most preclinical studies have focused in elucidating the mechanisms of action of this receptor subtype. Interestingly Sstr2 was found to act as a tumor suppressor in pancreatic cancer (Benali et al., 2000) highlighting the importance of this receptor subtype in cell growth control.

The antiproliferative action of somatostatin and its analogs was attributed to their inhibitory action on the growth factor receptors (Tsuzaki & Moses, 1990; Lee et al., 1991; Florio et al., 1996). Sstr2 antiproliferative action begins with the activation of phosphotyrosine phosphatases (PTP), which are proteins able to remove the phosphate group from tyrosine residues and subsequently alter protein function. These PTP dephosphorylate and inhibit tyrosine kinase receptors and their downstream mitogenic signaling cascades (Pan et al., 1992). PTP activity was found to be increased after somatostatin treatment in many cell systems (Buscail et al., 1994; Florio et al., 1994 and 1996; Reardon et al., 1996) and in human pituitary tumors in primary cell culture (Florio et al., 1999 and 2003). Sstrs were found to associate with members of the Class I Cys-based PTP, and specifically with the cytosolic src homology 2 (SH2) domain containing SHP-1 (PTP1C) and SHP-2 (PTP1D), and the membrane anchored PTPη (DEP1). Sstr2 was found to be constitutively associated with SHP-1 through Gαi3 (Buscail et al., 1994; Lopez et al., 1997). Treatment with somatostatin or octreotide activates SHP-1 which dephosphorylates tyrosine kinase receptors (e.g. epidermal growth factor receptor, insulin receptor) and its substrates (e.g. insulin receptor substrate 1 (IRS-1); Bousquet et al., 1998), and subsequently inhibits growth factor receptor signaling pathways.

1.4 Mitogen-activated protein kinase (MAPK) pathway

The MAPK pathway mediates the mitogenic action of growth factors, hormones and cytokines. In certain cases, depending on the cell system and extracellular environment, the MAPK (or ERK1/2) signaling is able to arrest cell growth in order to promote cell differentiation.

The pathway is initiated through activation of the cytoplasmic tyrosine kinase domain of the growth factor receptor (Cano et al., 1995; Davis et al., 1995; Fig. 2). Docking proteins such as Grb-2 and Sos bind to the phosphotyrosine residues of the activated receptor. Activated Sos promotes the removal of GDP from the Ras family of small GTPases, which can then bind GTP and become active. GTP-bound activated Ras associate with, bring to the membrane and activate the Raf family of kinases (A-Raf, B-Raf and c-Raf/Raf-1). Raf kinases (MAPK kinase kinases) phosphorylate and activate mitogen activated ERK kinase MEK1/2 (MAPK kinases) which then phosphorylate and activate the p44 and p42 MAPK (ERK1/2). Raf-1 can also be activated by the src family of tyrosine kinases.

1.4.1 Sstr2 & the MAPK pathway

Sstr2 was shown to inhibit growth factor induced MAPK phosphorylation and activation in neuroblastoma and glioma cells, but this effect was not always accompanied by a strong antiproliferative action (Cattaneo et al., 2000; Held-Feindt et al., 2000). In contrast, Sstr2

overexpressed in CHO cells activated ERK1/2, which together with activated p38-MAPK led to decreased cell proliferation (Sellers et al., 2000), through Ras, B-Raf, and the small GTPase Rap1 (Lahlou et al., 2003).

1.5 Phosphatidylinositol 3-kinase (PI3K) pathway

The PI3K/Akt pathway is the most common signaling pathway dysregulated in cancer (Fig.2). PI3K is a member of the Class I PI3K family that consists of two subgroups, IA and IB, which transmit signals from tyrosine kinase and G protein-coupled receptors. Class I PI3Ks catalyse the phosphorylation of a membrane lipid phosphatidylinositol 4, 5-bisphosphate (PtdIns(4,5)P_2) at the D-3 position of the inositol ring, to generate PtdIns(3,4,5)P_3, a potent second messenger required for survival signaling, cell growth, cell cycle entry and insulin action (Leslie et al. 2001). PI3K exists as a heterodimeric complex composed of a p85 kDa regulatory subunit and a p110 (α, β, γ and δ) kDa catalytic subunit. p85 is directly associated with many active tyrosine kinases through the physical interaction of its SH2 domain with phosphotyrosine residues on the kinase. In some cases, this interaction is not direct, but mediated through intermediate phosphoproteins, such as the insulin receptor substrates IRS-1 and IRS-2 (White et al.1998).

PtdIns(3,4,5)P_3 recruits a subset of signaling proteins containing pleckstrin homology (PH) domains to the membrane where they are activated. These proteins include protein serine-threonine kinases (e.g. Akt and PDK1), protein tyrosine kinases (e.g. Tec family) and exchange factors for GTP-binding proteins (e.g. Grp1 and Rac). Activation of Akt occurs through two crucial phosphorylation events, the first on Thr^{308} by PDK1, the second at Ser^{473} by the mTORC2 complex. Through the phosphorylation of a diverse set of substrates, Akt regulates cell survival, progression, growth and metabolism. In the case of the apoptosis inducing factor Bad, Akt phosphorylation prevents Bad from binding to Bcl-2 and Bcl-XL, thus inducing a cell survival response (Datta et al., 1997). The FKHR/FoxO family of forkhead transcription factors is directly phosphorylated and inactivated by Akt. FoxO mediates the transcription of tumor suppressor p27/Kip1 that is involved in cell cycle progression and control (Medema et al., 2000). Akt phosphorylation of FoxO proteins results in their nuclear exclusion and accumulation in the cytoplasm. In this way Akt is negatively regulating the transcription of pro-apoptotic genes normally stimulated by FoxO.

Another target of Akt is glycogen synthase kinase 3 beta (GSK-3β). This protein kinase is constitutively active in unstimulated cells and phosphorylates many proteins, such as, glycogen synthase, c-myc, and cyclin D1, in order to keep them in inactive states or to promote their degradation (Diehl et al., 1998; Ferkey & Kimelman, 2000). GSK-3β phosphorylation by Akt

turns off the catalytic activity of this enzyme, resulting in the activation of the proliferative pathways that are normally repressed by GSK-3β (van Weeren et al., 1998).

Another important Akt target is the mammalian target of rapamycin (mTOR). The TOR/mTOR proteins play a fundamental role in coupling nutrient availability with cell growth and this function of TOR is conserved throughout eukaryotic evolution. mTOR is a part of two distinct complexes: mTORC1 and mTORC2 (Wullschleger et al., 2006; Sabatini et al., 2006). Akt regulates mTOR through the tuberous sclerosis complex (TSC) which is composed of TSC1 (hamartin) and TSC2 (tuberin) (Manning et al., 2002). TCS2 and its binding partner TSC1 are mutated in a familial tumor syndrome called tuberous sclerosis, which presents with widespread benign tumors (hamartomas) in kidney, lung, brain and skin. TSC2 negatively regulates mTOR signaling through its ability to act as a GTP-ase activating protein for the GTP-ase Rheb, by exchanging a GTP for a GDP and switching the signal off (Zhang et al., 2003). The activity of the TSC1/TSC2 complex is in turn negatively regulated by Akt-mediated phosphorylation (Inoki et al., 2002; Potter et al., 2002). The downstream events that follow the inactivation of mTOR result in blockage of G1/S transition point of the cell cycle and abrogation of the protein synthesis. Two critical downstream targets of mTOR signaling underlie this mode of action: eIF4E binding protein 1 (4EBP1) and p70/S6K1 protein kinase. 4EBP1 binds and inhibits the activity of the translation factor eIF4E. eIF4E recognises the 5'-7-Me-GTP cap of mRNAs and is required for cap-dependent translation. Stimulation of cells with growth factors or high amino acid levels induce the multi-site phosphorylation of 4EBP1, resulting in the release of eIF4E and the activation of protein synthesis. Importantly, several 4EBP1 phosphorylation sites are directly phosphorylated by mTOR. p70/S6K1 was one of the earliest characterized protein kinases and its activity is strongly stimulated by growth factors, such as, insulin and platelet derived growth factor (PDGF), and nutrients, such as, amino acids. p70/S6K phosphorylates the S6 protein of the 40S ribosomal subunit. S6 activation dramatically increases the translation of eEF-2 elongation factor for protein synthesis (Jefferies et al., 1997). In addition, p70/S6K is responsible for switching off PI3K signaling through IRS-1. Namely, p70/S6K phosphorylates IRS-1 at serine residues leading to its dissociation from tyrosine kinase receptors and its inactivation (Paz et al., 1999; Harrington et al., 2004).

The other way of termination of the PI3K signaling occurs by degradation of PtdIns(3,4,5)P_3. This is mediated by at least two different types of phosphatases; PtdIns(3,4,5)P_3 can be converted to PtdIns(3,4)P_2 through the action of the SH2-containing inositol phosphatases (SHIP1 and SHIP2) or back to PtdIns(4,5)P_2 via the action of the lipid phosphatase termed phosphatase and tensin homologue deleted on chromosome ten (PTEN). Loss of PTEN protein

or function was found in a large fraction of advanced human cancers (Dahia et al., 1997; Wang et al., 1997; Cairns et al., 1998; Kohno et al., 1998; Aveyard et al., 1999) pointing to a role for PTEN in metastatic cancer progression.

1.5.1 Sstr2 & the PI3K pathway

Sstr2 was shown to activate PI3K signaling, when overexpressed in CHO cells, in a mechanism involving Gβγ and SHP-2 (Stetak et al., 2001; Lahlou et al., 2003). In contrast, activation of overexpressed or endogenous Sstr2 inhibited the PI3K pathway in other cell systems (Bousquet et al., 2006; Theodoropoulou et al., 2006). Sstr2 was shown to directly bind p85 in CHO/sst2 and pancreatic tumor AR4-2J cells and this is a unique feature of Sstr2, not shared by another member of the Sstr family (Sstr3) (Bousquet et al., 2006). Sstr2 activation disrupted its association with p85 and decreased p85 tyrosine phosphorylation levels. In pituitary tumor cells p85 was found to physically associate with SHP-1 and Sstr2. Activation with octreotide led to decrease in p85 tyrosine phosphorylation which was SHP-1 dependent (Theodoropoulou et al., 2006). In pituitary tumor cells Sstr2 inhibited PI3K and subsequently decreased PDK1, Akt and GSK-3β phosphorylation, leading to GSK-3β activation and subsequent cell cycle arrest.

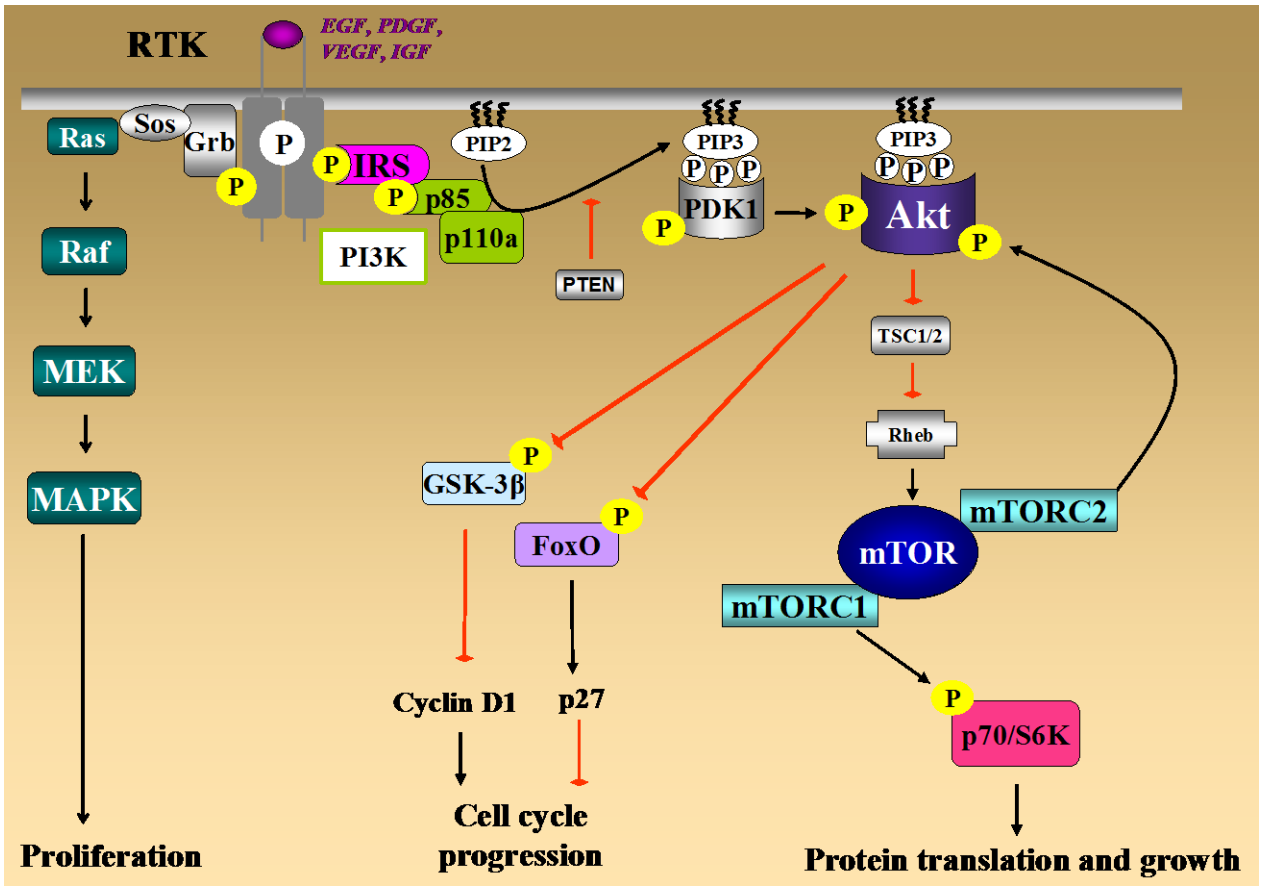

Fig. 2. Shematic depiction of the MAPK and PI3K/Akt/mTOR pathways (see text for details).

1.6 Cell cycle

The progression of the eukaryotic cell through the cell cycle is strictly controlled at the different crucial points (restriction points), the G1/S and G2/M. The proper flow of the cell in and out of the restriction point is primarily guided by the activation of cyclin-dependent kinases (Cdks) by their respective cyclin partners. Growth factor stimulation results in a rapid expression of the D-type cyclins (D1, D2 and D3) in the early G1 phase of the cell cycle. Growth factor-induced D-type cyclins form catalytically active kinase complexes with Cdk4 and Cdk6 and initiate phosphorylation and inactivation of the tumor suppressor retinoblastoma (Rb) (Sherr et al., 1999; Fig. 3). Partial phosphorylation of Rb (pRb) activates specific transcription factors which allow transcription and expression of cyclin E and A. Cyclin E complexes with its associated kinase Cdk2, completing the phosphorylation and inactivation of pRb and leading to irreversible entry into the S-phase of the cell cycle (Koff et al., 1992). This in turn dissolves complexes of pRb with members of the E2F family of transcription factors and associated chromatin-modifying enzymes, allowing transcription of genes required for the S-phase (Schwarz et al., 1993).

The activity of cyclin D/Cdk4/6 complexes is inhibited by members of the INK4 (Inhibitor of Cdk4) group of Cdk inhibitors of which p16/INK4a is a founding member. This tumour suppressor gene responds to conditions of cellular stress to shut off CDK4/6 activity, and thus lock Rb in its active, antiproliferative state (Deshpande et al., 2005). In contrast to the INK4 proteins, the Cip/Kip family of inhibitors has broader spectrum of acting, affecting the activities of cyclin D-, E-, and A-dependent kinases and the cyclins themselves. The family includes p21/Cip1, p27/Kip1 and p57/Kip2. p27/Kip1 is the primary regulator of cyclin E/Cdk2 complex, since by sequestering Cdk2 it prevents the complex formation.

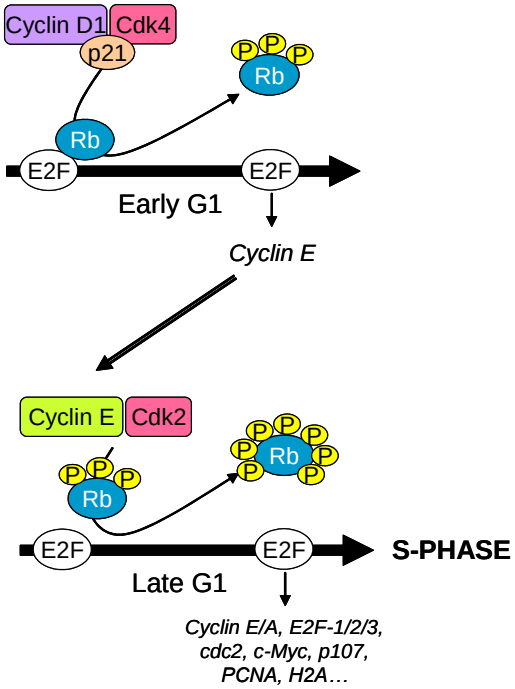

Fig. 3. Cell cycle components involved in G1/S transition point of the cell cycle.
In response to mitogenic signals, the machinery of the cell cycle progression starts with the synthesis of the D-type cyclins which form active complexes with their associated cyclin dependent kinases Cdk4 and 6 (Sherr et al., 2000). The G1 to S transition is primarily governed by cyclin E and its associated kinase Cdk2, which hyperphosphorylates retinoblastoma (Rb) (Koff et al., 1992). This in turn dissolves complexes of pRb with members of the E2F family of transcription factors and associated chromatin-modifying enzymes, allowing transcription of genes required for S-phase (Schwarz et al., 1993). Cyclin/Cdk complexes are inhibited by cyclin kinase inhibitors, such as, p21/Cip1 and p27/Kip1. p27/Kip1 is the primary regulator of cyclin E/Cdk2

complex, since by sequestering Cdk2 it prevents the complex formation.

1.6.1 Sstr2 & cell cycle

Somatostatin is cytostatic in several tumor cell types, causing G1/S cell cycle arrest. Sstr2 was found to upregulate p21/Cip1 after stimulating both ERK1/2 and p38-MAPK in CHO cells (Sellers et al., 2000). The most important mediator of Sstr2-induced G1 cell cycle arrest was found to be p27/Kip1; activated Sstr2 upregulates p27/Kip1 in a mechanism involving SHP-1 (Pagès et al., 1999; Lopez et al., 2001).

Although p27/Kip1 is an important downstream target of somatostatin's antiproliferative signaling, cells like the rat pituitary tumor GH3, that lack p27/Kip1 expression (Qian et al., 1996), also responded to SSA treatment by decreasing cell proliferation. In these cells, Sstr2 was shown to induce the expression of the tumor suppressor Zac1, in a mechanism involving Gαi, SHP-1, GSK-3β and the Zac1 activator p53 (Theodoropoulou et al., 2006). RNA interference experiments in pituitary tumor cells revealed that Zac1 is essential for octreotide's antiproliferative action (Theodoropoulou et al., 2006). A retrospective immunohistochemical analysis on archival paraffin embedded tumoral tissue from acromegalic patients treated with SSA pre-operatively, revealed a strong positive correlation between treatment response and ZAC1 immunoreactivity. In this set of patients strong ZAC1 immunoreactivity positively correlated with IGF-I normalization and tumor shrinkage after SSA treatment (Theodoropoulou et al., 2009). These data suggest ZAC1 as marker and possibly a predictor of successful SSA treatment.

Altogether these *in vitro* studies reveal a potential antiproliferative and cytostatic action for SSA, which contrasts with their inability to arrest tumor growth in a significant percentage of pituitary adenomas *in vivo*. To understand the intrinsic defects that render pituitary tumors in general and NFPA in particular resistant to the antiproliferative action of SSA and to identify targets for novel cytostatic treatments, one has to go back to the mechanisms of pituitary tumorigenesis.

1.7 Pituitary tumorigenesis

There are two prevailing theories for pituitary tumorigenesis: the hormonal stimulation theory or hypothalamic hypothesis and the theory of an intrinsic pituitary defect or monoclonal expansion model. Nowadays it is widely accepted that pituitary adenomas are monoclonal in origin, i.e. they derive from a single transformed cell (Alexander et al., 1990).

A single mutation is not enough to convert a typical healthy cell into a cancer cell that proliferates without restraint. Cancerogenesis requires that several independent rare incidences

occur together in one cell. Its transformation and abnormal proliferation results from a disruption in one or more regulators of cell cycle progression and apoptosis. This is mostly related to an activation of a cell growth promoter (proto-oncogene) or an inactivation of a cell growth blocker/inducer of apoptosis (tumor suppressor gene; TSG).

Proto-oncogenes are the cellular counterparts of the oncogenes, which when overexpressed cause tumor formation. Proto-oncogene products are involved in mediating signals from the cell surface to the nucleus, like protein tyrosine kinases (e.g. src), serine/threonine kinases (e.g. Raf) and G proteins (e.g. Ras, gsp), cell surface receptors (e.g. c-erb/*neu*), transcription factors (e.g. c-myc, c-fos, c-myb), and cell cycle regulators (e.g. cyclin D). Proto-oncogenes can be transformed to oncogenes and lead to tumour formation by an activating mutation or gene overexpression.

The other mechanism that can lead to cancer development is functional inactivation of a TSG. Unlike proto-oncogenes, which require that only one copy of the gene be mutated to disrupt gene function, both copies of a particular tumor suppressor gene must be altered to inactivate gene function. Accordingly, an additional alteration in the remaining allele, a second hit, is necessary for the TSG inactivation to occur (Knudson's two-hit hypothesis; Knudson, Jr., 1975). In the classical Knudson's model the 2nd hit is loss of heterozygosity (LOH), i.e. loss of the wild-type, non-mutated allele. The prototype example of this model is the retinoblastoma gene (*Rb1*) in both familial and sporadic forms of retinoblastoma (Knudson, Jr., 1971). Homozygous deletion of both alleles of a gene may also lead to LOH of a TSG. A good example is *p16/CDKN2A* in head and neck cancers, in which 70% of primary tumors lose the gene through this mechanism (Reed et al., 1996). The third possibility for LOH or reduced expression of TSG may occur through hypermethylation. Methylation is an epigenetic modification of the genome, in which a methyl group is added to the 5-position of cytosine within the context of the cytosine-guanine dinucleotide clusters, referred to as "CpG islands". In normal mammalian cells, DNA methylation is responsible for the silencing of the inactive X chromosome (Lee & Jaenisch, 1997) but approximately 60% of genes have CpG islands in their promoters which are normally unmethylated.

Accordingly the initial event transforming a normal pituitary cell into a tumor cell can be an activating mutation in a proto-oncogene or an inhibiting mutation in a TSG. Somatic mutations frequently found in other malignancies involving Ras, c-erbB2/*neu*, protein kinase C (PKC), p53, and Rb are usually absent in the majority of pituitary tumors. However, approximately 40% of ACRO contain mutations in the gsp gene encoding for the stimulatory Gs protein that substitute at arginine 201 or glutamine 227 (Landis et al., 1989). This affects two critical

domains of the Gsα that are involved in GTP hydrolysis and are therefore maintaining the mutant Gsα in constitutive active form. These mutations were also detected in 10% of NFPA (Tordjman et al., 1993; Williamson et al., 1994).

Another candidate gene is provided in multiple endocrine neoplasia type 1 (*MEN1*).This autosomal dominant disorder is characterised by neuroendocrine tumors of the pituitary, parathyroid, pancreas and duodenum (Marx et al., 1999; Pannett & Thakker, 1999). Germline mutations, leading to loss of function, have been identified in both familial and sporadic MEN1 patients (Agarwal et al., 1998; Farell et al., 1999). In addition, *MEN1* is on the 11q13, in a chromosomal region that is found deleted in 5-20% of pituitary adenomas (Boggild et al., 1994). Although *MEN1* mutations are found in 30% of sporadic pancreatic tumors (Zhuang et al., 1997) and 20% of sporadic parathyroid tumors (Heppner et al., 1997), they appear extremely rarely, if at all, in sporadic pituitary adenomas (Zhuang et al., 1997; Prezant et al., 1998; Schmidt et al., 1999). *MEN1* gene expression is not downregulated in the pituitary tumors (Asa & Ezzat, 1998; Farell et al., 1999), but levels of the *MEN1* gene product menin are reduced in 80% of pituitary adenomas, indicating dysregulation at protein level (Theodoropolou et al., 2004).

Another gene residing at the 11q13 that was shown to be mutated in familial isolated pituitary adenomas (FIPA) is the aryl hydrocarbon interacting protein (AIP). AIP germline mutations were identified in two Finnish families presenting with prolactinomas and somatotrophinomas, and in some sporadic patients with acromegaly (Vierimaa et al., 2006). AIP is associated with the aryl hydrocarbon receptor (AHR), a ligand-inducible transcription factor that mediates the cellular response to xenobiotic compounds including various environmental pollutants (Carver & Bradfield, 1997). Heterozygous germline AIP mutations are found in 15% of FIPA, 50% of familial acromegaly and in few sporadic acromegalic patients (Daly et al., 2007). Pituitary tumors harboring AIP mutations are almost exclusively somatotrophinomas (87.5%) or, more rarely, prolactinomas (9.4%; Buchbinder et al., 2008). Patients with AIP mutations are mostly male, suffer macroadenomas and are younger at diagnosis than unselected patients with pituitary adenomas (Cazabat et al., 2007).

Many oncogenes or TSGs that are expected to be involved in pituitary tumorigenesis are not mutated but dysregulated at transcriptional or translational level. Cyclin D1 is not found in the normal pituitary, but is overexpressed in 67% of the NFPA and 37% of somatotrophinomas (Hibberts et al., 1999). RB1 was suspected as a candidate TSG in pituitary tumor formation, when mice heterozygous for *Rb1* mutation were shown to have a complete predisposition to pituitary tumors derived from the intermediate lobe (Jacks et al., 1992; Hu et al., 1994).

Although LOH studies revealed loss of the *RB1* locus only in invasive pituitary adenomas and in the rare cases of pituitary carcinomas, in the vast majority of the pituitary adenomas the *RB1* locus seems to be intact (Zhu et al., 1994; Pei et al., 1995; Bates et al., 1997). However, no RB1 protein product was detected in somatotrophinomas and NFPA (Simpson et al., 1999). In these tumors RB1 gene harbours no mutations but its promoter was found to be hypermethylated. p27/Kip1 gene (*CDKN1B)* is another gene that when deleted in mice, leads to the development of pituitary tumors of the intermediate lobe (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al. 1996). In human pituitary tumors no p27 gene mutations were found and the levels of p27 gene transcript were shown to be comparable to that of the normal pituitary gland (Ikeda et al., 1997; Jin et al., 1997; Dahia et al., 1998; Takeuchi et al., 1998), but p27/Kip1 protein levels were found to be reduced in pituitary tumors and especially in the corticotrophinomas and metastatic pituitary adenomas (Lidhar et al., 1999).

p16/INK4 (*CDKN2A)* expression was found to be lost in majority of pituitary adenomas, a finding that was not associated with gene mutation or gene loss (Woloschak et al., 1996), but with hypermethylation of the gene promoter (Farrell et al., 1997). A subsequent study has demonstrated a significant prevalence of hypermethylated p16 gene in NFPA (70%) versus somatotrophinomas (9.5%; Simpson et al., 1999).

Another TSG that plays an important role in pituitary tumorigenesis is the zinc-finger protein ZAC1/LOT1/PLAGL1. *ZAC1* (zinc finger protein inducing apoptosis and cell cycle arrest) resides on chromosome 6q24-25, which is frequently lost in solid cancers (Thrash-Bingham et al., 1995; Fujii et al., 1996; Theile et al., 1996). In parallel it was identified as a gene lost in transformed rat ovarian epithelial cells (Abdollahi et al., 1997). Its ability to induce apoptosis and G1 arrest, established this candidate TSG as the first gene structurally unrelated to *p53* that shares its apoptotic and cell cycle blocking properties (Spengler et al., 1997). ZAC1/Zac1 is highly expressed in the pituitary gland (Varrault et al., 1998), primarily in the anterior lobe of the mouse pituitary (Pagotto et al., 1999). Knocking down Zac1 in murine tumor pituitary cell lines enhanced DNA synthesis, demonstrating a role in pituitary cell proliferation (Pagotto 1999). Studies on ZAC1 gene status in pituitary adenomas revealed LOH in 8 out of 18 pituitary adenomas, but no mutation in the ZAC1 coding region. However, ZAC1 mRNA and protein were reduced in most pituitary adenomas and dramatically reduced or absent in NFPA (Pagotto et al., 2000), suggesting a role for ZAC1 in the pathogenesis of this type of pituitary tumors.

1.7.1 Growth Factors & Pituitary Tumorigenesis

Growth factors are polypeptides with critical functions in mitogenesis, angiogenesis, and gene transcription. The pituitary gland was shown to be the site of synthesis and action of several

growth factors, which act in autocrine/paracrine way to regulate pituitary cell growth and hormone secretion (Renner et al., 1996). Alterations in the expression of growth factors and/or their receptors may significantly contribute to the formation and growth of tumors. Several pituitary-driven growth factors were shown to induce pituitary hyperplasia with or without ultimate adenoma development when expressed in transgenic mice (Borrelli et al., 1992; McAndrew et al., 1995; Heaney et al., 1999; Melmed et al., 2003).

The epidermal growth factor (EGF) family and its receptors have been implicated in tumorigenesis in a number of neoplasms. The pituitary gland expresses transforming growth factor (TGF)-α and EGF, which alter pituitary hormone secretion and induce cell proliferation (White & Bancroft, 1983; Childs et al., 1995, Ezzat et al., 2001). TGF-α was found to mediate estrogen-induced lactotroph proliferation (Oomizu et al., 2000) and TGF-α overexpression under the control of the pituitary prolactin promoter results in lactotroph adenomas in transgenic mice (McAndrew et al., 1995). EGF is expressed in all types of pituitary adenomas (LeRiche et al., 1996). EGF and TGF-α bind to a tyrosine kinase receptor (EGFR), which is the cellular homologue of the *v-erb*B oncogene product (Downward et al., 1984). EGFR is overexpressed in several types of tumors, such as breast, ovarian cancer and glial cell tumors (Xu et al., 1984; Klijn et al., 1992). In the pituitary, expression of this receptor correlated with tumor aggressiveness, with the highest levels detected in ACRO (LeRiche et al., 1996) and NFPA (Chaidarun et al., 1994). In another study EGFR was found to be predominant in corticotrophinomas (Theodoropoulou et al., 2004).

TGF-β family members affect pituitary cell growth. At least three different forms are represented in the pituitary: inhibin A, inhibin B, activin, activin A and activin B. Inhibin subunits are expressed by pituitary gonadotroph adenomas (Haddad et al., 1994) and activin stimulates hormone secretion by these tumors (Alexander et al., 1991). Activin effects are mediated by activin receptors and follistatin (Ying et al., 1988), which binds activin and suppresses its activity. While activin receptors are well expressed in gonadotroph adenomas, the expression of follistatin is reduced or absent (Penabad et al., 1996), implicating enhanced activin signaling as a pathogenetic mechanism.

Bone morphogenic proteins (BMPs) are also members of the TGF-β family. BMP-2 and BMP-4 have been shown to play a role in the initial steps of the development of the anterior pituitary (Scully & Rosenfeld, 2002). BMPs exert their effects through binding to the tyrosine kinase receptors and subsequently transduce the signals through Smad proteins, which act as transcription factors in the nucleus. Smad4, which functions as a signal cotransducer, in turn regulates c-myc, a protooncogene that controls cell cycle and mediates the effects of TGF-β on

cell proliferation (Chen et al., 2001). The overexpression of the BMP inhibitor, noggin, or a dominant-negative BMP-receptor (BMPR1A) in the anterior pituitary leads to an arrest in the development of PRL-secreting cells (Scully & Rosenfeld, 2002). In prolactinomas derived from dopamine D2-receptor-deficient mice noggin was found to be downregulated while BMP-4 was overexpressed in the same prolactinoma model as well as in estradiol-induced rat prolactinomas and human prolactinomas (Paez-Pereda et al., 2003). In contrast, BMP-4 inhibited ACTH secretion and cell proliferation and its expression was found to be reduced in corticotrophinomas in comparison to the normal pituitary (Giacomini et al., 2006).

Vascular endothelial growth factor (VEGF) is one of the most important angiogenic factors since it increases endothelial cell proliferation and migration (Ferrara & Bunting, 1996) and vessels' permeability, by inducing pores and fenestration in the endothelium (Esser et al., 1998). The expression of VEGF and its receptors (VEGFR-1,2,3) correlates with the degree of vascularisation in many experimental and clinical tumors (Plate et al., 1992; Brown et al., 1995). VEGF is secreted by normal and adenomatous pituitary (Lloyd et al., 1999; Ochoa et al., 2000; Lohrer et al., 2000; Onofri et al., 2004). The anterior pituitary endocrine cells that showed the highest percentage of VEGF immunostaining were somatotrophs, corticotrophs and folliculostellate cells (Vidal et al., 1999). All VEGFRs are found to be expressed in normal and tumoral pituitary gland, whereas VEGFR-1 and -2 are predominantly expressed in gonadotrophinomas, corticotrophinomas and NFPA (McCabe et al., 2002; Lloyd et al., 2003). Recent study revealed VEGFR-1 expression in endocrine pituitary tumor cells and VEGFR-2 in vascular endothelial cells (Onofri et al., 2004). Stimulation of the VEGFR-1-positive MtT-S rat pituitary tumor cell line increased cell proliferation involving the PI3K signaling pathway, demonstrating a role not only in angiogenesis, but also in controlling tumor cell growth (Onofri et al., 2004).

1.7.2 Growth factor signaling cascades & Pituitary tumorigenesis

The above growth factors contribute to tumor progression by overactivating downstream survival and mitogenic pathways. In pituitary adenomas, Akt isoforms were found to be significantly overexpressed compared to the normal pituitaries, while PTEN protein levels were lower (Musat et al., 2005). Within the individual pituitary tumor types, NFPA showed the highest level of the activated pAkt-Ser^{473} indicating that Akt may be overactivated in these tumors.

Another study underscored the importance of the activated PI3K/Akt pathway in pituitary tumors. A knock-in mutant mouse harbouring a mutation in the thyroid receptor-β gene, served as a TSH animal model for the evaluation of the role of PI3K/Akt signaling pathway in the

pathogenesis of TSH-secreting pituitary tumors (Lu et al., 2008). Akt, mTOR and p70/S6K were found to be activated in mutated mice, contributing to the increased cell proliferation and pituitary growth. Additionally, activated Akt decreased apoptosis by inhibiting key apoptotic regulators such as proapoptotic proteins Bad and FoxO3a.

Pituitary tumors were also shown to have high levels of phosphorylated MEK1/2 and ERK1/2 and the c-myc phosphorylation at Thr^{58}/Ser^{62}, which is the site phosphorylated by Akt, was decreased in all tumor types (Dworakowska et al., 2009).

Altogether, these data show that components of the growth factor signaling cascades and especially of the survival PI3K pathway are dysregulated in pituitary adenomas and may contribute to their pathogenesis and treatment response.

1.8 Pharmacological targeting of the PI3K pathway

1.8.1 Rapamycin

Rapamycin (sirolimus) is a macrolide fungicide that was first isolated from the soil bacteria *Streptomyces hygroscopicus* in the early 1970s (Sehgal et al., 1975). Initially developed clinically for its immunosuppressant properties, it was soon shown that it has antiproliferative properties and its potential for cancer treatment was explored.

Rapamycin demonstrated activity against several murine tumors, such as melanocarcinoma, mammary and colon solid tumors (Douros et al., 1981). It is also a potent inhibitor of antigen-induced proliferation of T cells, B cells and antibody production (Dumont et al., 1990; Kay et al., 1991; Kahan et al., 1991). Rapamycin forms a complex with the ubiquitous intracellular protein, FKBP12, which specifically binds mTORC1 inhibiting downstream signaling events. The downstream events that follow the inactivation of mTOR result in the cell cycle arrest in the G1/S phase and blockage of the protein synthesis.

mTOR mediates Akt induced cell proliferation and tumor growth. Tumors bearing PTEN mutations and/or Akt overactivation are targeted for treatment with mTOR inhibitors. Indeed, in an experimental animal model of Akt-induced prostate intraepithelial neoplasia, treatment with rapamycin analog RAD001 (everolimus) reversed the neoplastic phenotype in the prostate of these animals (Majumder et al., 2004). RAD001 is an orally available derivative of rapamycin that apart from immunosuppressive properties demonstrates potent antiproliferative action against a variety of tumor cell lines *in vitro* or in animal models (Beuvink et al., 2001; Lane et al., 2003).

Despite the potent antiproliferative action of rapamycin and its analogs *in vitro* or *in viv*o in animal models, their efficacy in clinical trials was modest (Huang et al., 2003). Analysis of

breast cancer biopsies derived from patients treated with RAD001 revealed increased Akt phosphorylation (O'Reilly et al., 2006). The reason for this lies in the elimination of the negative feedback exerted by the mTOR target p70/S6K on the PI3K/Akt pathway.

Rapamycin- and rapalog-induced Akt phosphorylation could be overcome by cotreatment with an agent that targets the PI3K pathway upstream to decrease Akt phosphorylation and improve the antiproliferative action of rapalog treatment. Indeed, addition of small molecule inhibitors against PI3K (Sun et al., 2005) or IGF-IR (O'Reilly et al., 2006), abolished the rapalog-induced Akt phosphorylation and improved its efficacy.

1.8.2 Dual PI3K/mTOR inhibitor, NVP-BEZ235

To target the PI3K/Akt/mTOR pathway both up- and down-stream, small molecules able to concomitantly inhibit the p110 catalytic subunit of PI3K and mTOR kinase were synthesized. The pan-PI3K/mTOR inhibitor, NVP-BEZ235 (hereafter BEZ235), is an orally available synthetic compound that potently inhibits Class I PI3Ks in an ATP-competitive manner. It exhibits significant antitumor activities in a broad range of experimental tumors by inducing G1/S arrest and/or apoptosis (Maira et al., 2008; Brachmann et al., 2009). It also affects tumor vasculature by inhibiting the VEGF-induced angiogenesis in rat mammary carcinoma model (Schnell et al., 2008). Furthermore, tumor growth suppression was shown in PI3K- mutated cell lines (mutation in *PIK3CA* gene encoding for the p110α catalytic subunit) and xenograft models of human cancer (Maira et al., 2008; Serra et al., 2008; Cao et al., 2008). In contrast, BEZ235 did not inhibit tumor growth in a mouse model of lung cancer bearing a *Kras* mutation (Engelman et al., 2008). Such observations raise the awareness that single targeting of one pathway may not be sufficient for treatment of certain cancers. Thus, careful consideration regarding the molecular pathology of the particular cancer will be an important factor in the further development of these inhibitors.

2 MATERIALS AND METHODS

Reagents

PRODUCT	COMPANY
Acetic acid	MERCK (Darmstadt, Germany)
Acridine orange	Sigma (St.Luis. MO, USA)
Ammonium persulfate	Sigma (St.Luis. MO, USA)
Amphotericin B	Biochrom (Berlin, Germany)
Agar	Life Technologies (Paisley, Scotland, UK)
Beta-mercaptoethanol	MERCK (Darmstadt, Germany)
Bovine serum albumin (BSA)	Invitrogen Corp.(Paisley, Scotland, UK)
Chloroform	Sigma (St.Luis. MO, USA)
Collagenase	Worthington Biochemical Corp. (Lakewood, NJ, USA)
Developer solution	Kodak (Stuttgart, Germany)
Diethyl-pyrocarbonate (DAPC)	Sigma (St.Luis. MO, USA)
Dimethyl sulfoxide (DMSO)	Sigma (St.Luis. MO, USA)
DNAse I	Invitrogen Corp.(Paisley, Scotland, UK)
dNTP Mix	MBI Fermentas (Vilnius, Lithouania)
Dulbecco's modified Eagle medium (DMEM)	Invitrogen Corp.(Paisley, Scotland, UK)
D-valine-DMEM	Invitrogen Corp.(Paisley, Scotland, UK)
Ethidium bromide	Sigma (St.Luis. MO, USA)
Fetal calf serum	Gibco (Karlsruhe, Germany)
Fixer solution	Kodak (Stuttgart, Germany)
Formamide	Sigma (St.Luis. MO, USA)
Glucose	MERCK (Darmstadt, Germany)
Hepes	Sigma (St.Luis. MO, USA)
Hexanucleotide Mix	Roche (Mannheim, Germany)
Hyaluronidase	Sigma (St.Luis. MO, USA)
Isoamylalcohol	MERCK (Darmstadt, Germany)
Isopropanol	Sigma (St.Luis. MO, USA)
L-Glutamine	Biochrom AG (Berlin,Germany)
Lithium chloride	MERCK (Darmstadt, Germany)
Lumi-Light Western Blotting Substrate	Roche (Mannheim, Germany)
Luciferine	Roche (Mannheim, Germany)
Magnesium chloride hexahydrate	MERCK (Darmstadt, Germany)
Marker 1kb Plus	Life Technologies (Paisley, Scotland, UK)
MEM-Vitamins	Biochrom AG (Berlin, Germany)
[methyl-^{3}H]-Thymidine	Amersham Biosciences (Uppsala, Sweden)
Milk powder	Roth (Karlsruhe, Germany)

Nitrocellulose membrane Hybond-ECL	Amersham Biosciences (Uppsala, Sweden)
Nonidet P-40 (NP-40)	Sigma (St. Luis, MO, USA)
NVP-BEZ235	Novartis (Basel, Switzerland)
Octreotide	American Peptide Company (Sunnyvale, CA, USA)
ONPG	Sigma (St. Luis, MO, USA)
Penicillin+Streptavidine mix	Biochrom AG (Berlin, Germany)
Phenol	Roth (Karlsruhe, Germany)
Plasmid preparation Kit	QIAGEN (Hilden, Germany)
Phosphatase inhibitor coctail	Roche (Mannheim, Germany)
Phosphate based buffer (PBS)	Life Technologies (Paisley, Scotland, UK)
Polyacrylamide	Invitrogen Corp.(Paisley, Scotland, UK)
Potassium chloride (KCl)	MERCK (Darmstadt, Germany)
Propidium iodide	Sigma (St. Luis, MO, USA)
Protease inhibitor coctail	Sigma (St. Luis, MO, USA)
Rapamycin	Sigma (St. Luis, MO, USA)
Reporter lysis buffer	Promega Corp. (Madison, WI, USA)
Protein A/G Sepharose	Amersham Biosciences (Uppsala, Sweden)
RNase A	Roche (Mannheim, Germany)
RNAsin (RNAse inhibitor)	Promega Corp. (Madison, WI, USA)
Reverse transcriptase (Super Script II^{TM})	Invitrogen Corp.(Paisley, Scotland, UK)
RIA reagent kit	Siemens Health Care Diagnostics (Deerfield, USA)
SB415286	MERCK (Darmstadt, Germany)
Sodium deoxycholate	MERCK (Darmstadt, Germany)
Sodium dodecyl sulfate (SDS)	MERCK (Darmstadt, Germany)
Sodium chloride (NaCl)	Roth (Karlsruhe, Germany)
Sodium hydrogen phosphate dihydrate	MERCK (Darmstadt, Germany)
Sodium dihydrogen phosphate monohydrate	MERCK (Darmstadt, Germany)
Sodium peroxide (NaOH)	MERCK (Darmstadt, Germany)
SuperFect®	QIAGEN (Hilden, Germany)
Taq DNA polymerase	MBI Fermentas (Vilnius, Lithouania)
TEMED	Sigma (St.Luis. MO, USA)
Transferrin	Sigma (St.Luis. MO, USA)
Trichloracetic acid	Roth (Karlsruhe, Germany)
Tris pure	ICN Pharmaceuticals (Aurora, OH, USA)
Triton X-100	Roth (Karlsruhe, Germany)
Trizol	Invitrogen Corp.(Paisley, Scotland, UK)
Trypsin	Sigma (St.Luis. MO, USA)
Tween 20	Sigma (St.Luis. MO, USA)
Ultima Gold Scintillation Solution	Packard Bioscience (Gromingen, Netherlands)
WST-1 assay	Roche (Mannheim, Germany)

Solutions

Collagenase mix	1000 U/ml Collagenase : 400 mg/ 100 ml solution Trypsin inhibitor: 10 mg/ 100 ml solution Hyaluronidase : 100 mg/ 100 ml solution BSA : 400 mg/ 100 ml solution DNAse : 500µl/ 100 ml solution
DEPC water	200µl DEPC/l deionized water Leave under the fume hood overnight, autoclave
HDB buffer	Glucose : 18mg/ 100 ml solution Penicillin/Streptavidin: 1ml/ 100 ml solution Amphotericin B : 1 ml/ 100 ml solution
ONPG buffer	1M Na_2HPO_4: 55.3 ml 1M NaH_2PO_4: 20.3 ml Destillated water: 339.2 ml $MgCl_2 \cdot 6H_20$: 154.5 mg ONPG: 500,0 mg
LB medium	Peptone :10 g/l Yeast extract: 5 g/l NaCl : 5 g/l NaOH 1M : 2 mL/L Adjust to pH 7.0
RIPA lysis buffer	Tris-HCl: 50 mM NaCl: 150 mM NP-40: 1% Sodium deoxycholate: 0.5% SDS: 0.1%
Running buffer for Western blotting	10x Tris pure: 30.3 g/l Glycin : 144.2 g/l SDS : 10 g/l Adjust to pH 8.6
Transfer buffer for Western blotting	1x Running buffer: 700 ml Destillated water: 100 ml Methanol: 200 ml
Tris buffer	Tris pure: 12.114 g/l Adjust to pH 7.6
Tris based buffer (TBS)	1x Tris pure: 2.42 g/l NaCl : 8 g/l Adjust to pH 7.6
Tris-HCl	1M Tris pure: 121,14 g/l Add 25% HCl to a pH 8.2

2.1 Reagents

Cell culture materials were purchased from Life Technologies (Karlsruhe, Germany), Nunc (Wiesbaden, Germany) and Sigma (St. Luis, MO). Octreotide was provided by the American Peptide Company (Sunnyvale, CA), MG132 and rapamycin from Sigma, and BEZ235 from Novartis (Basel, Switzerland). Lithium chloride and SB415286 were from Calbiochem (Merck, Darmstadt, Germany). Octreotide was dissolved in 0.001 mol/l acetic acid and rapamycin, SB415286, and BEZ235 in DMSO.

2.2 Human pituitary adenomas

This study was performed after approval of the ethics committee of the Max Planck Institute and after receiving informed consent from each patient or from their relatives. All together, 69 NFPA and 5 ACRO were included in the study (Table 2). The tumors were removed through transsphenoidal surgery and diagnosed by clinical, biochemical, radiological and surgical findings.

Table 2. Gender and mean age of the pituitary adenomas involved in the study

Tumor type	Non-functioning adenoma	Acromegaly
n	69	5
Gender (m/f)	44 / 25	2 / 3
Mean age (m/f)	60±14 / 61±15	36±4 / 63±6

2.3 Pituitary adenomas in primary cell culture

Post-surgical specimens were washed with HDB buffer, which contains 15 mmol/l Hepes, pH 7.4, 137 mmol NaCl, 5 mmol/l KCl, 0.7 mmol/l Na_2HPO_4, 10 mmol/l glucose, 2.5 mg/ml amphotericin B and 10^5 U/l penicillin/streptomycin. Sliced fragments were enzymatically dispersed in a buffer containing 4 g/l collagenase, 10 mg/l DNAse II, 0.1 g/l soybean trypsin inhibitor, and 1 g/l hyaluronidase (37°C, approximately 45 minutes). Dispersed cells were centrifuged and resuspended in D-valine Dulbecco´s Modified Eagle´s Medium (DMEM) supplemented with 2 mM essential vitamins, 40 U/l insulin, 20 mg/l natrium selenate, 5 mg/l transferrin, 30 pM triiodthyronine (T_3), 10% fetal calf serum, 2 mmol/l L-glutamine, 2.5 mg/l amphotericin B and 10^5 U/ml penicillin/streptomycin. D-valine-DMEM was used to suppress the growth of contaminating fibroblasts. Cell viability was determinated with fluorescence

microscope by acridine orange/ethidium bromide staining. Acridine orange, but not ethidium bromide enters a cell with intact cell membrane, a living cell, yielding green fluorescence. If the cell is dead and therefore the membrane disrupted, ethidium bromide can enter and intercalate into the DNA staining the cell red. The cell viability was calculated as the percentage of green cells in the total number of cells (counted in a Neubauer chamber). Cells were seeded in 48-well tissue culture plates (100.000 cells per well) and incubated at 37°C for 48 hours prior the stimulation.

2.4 Immortalized pituitary tumor cell lines

The immortalized cell lines used in this study were the mouse corticotrophinoma cell line At-T20 (American Type Culture Collection, Manassas, VA) and rat mammosomatotroph cell line GH3. Cells were cultured in DMEM supplemented with 10% FCS, 2 nmol/l glutamine, 0.5 mg/l partricin, and 10^5 U/l penicillin-streptomycin at 37°C and 5% CO_2. When confluent, cells were washed with PBS, trypsinized, centrifuged at 1200g for 4 minutes, and platted according to the demands of each experiment.

2.5 Proliferation assays: [^{3}H]-thymidine incorporation and WST-1

Cell proliferation in primary cell culture was measured using [^{3}H]-thymidine incorporation assay ([^{3}H]-TdR) and was performed in 10% FCS-DMEM supplemented with D-valine. Cells were treated with octreotide (0.01, 0.1, and 1nM), rapamycin (1nM), and BEZ235 (100, 10 and 1nM) for 24 hours before adding [^{3}H]-thymidine (0.5 µCi/mL). For the proliferation studies in pituitary tumor cell line AtT-20, [^{3}H]-TdR assay was performed in 10% FCS-DMEM. Cells were treated with 1nM octreotide and 0.01, 0.1 and 1nM rapamycin for 24 hours before adding [^{3}H]-thymidine (0.5 µCi/mL). Cells treated with the carriers in which octreotide, rapamycin, and BEZ235 were dissolved were used as control. After a total incubation period of 48 hours, the supernatants were removed and cells were precipitated with 10% ice-cold trichloroacetic acid and washed with cold PBS. DNA was hydrolyzed using 0.5mol/l NaOH and 0.1% Triton X-100. Incorporated [^{3}H]-thymidine was determined with a liquid scintillation counter. All treatments were carried out in quadruplicates.

Cell proliferation was also assessed using the nonradioactive colorimetric WST-1 assay (Roche Molecular Biochemicals, Basel, Switzerland) according to kit instructions. Immortalized pituitary tumor cells were seeded in 96-well tissue culture plates (10.000 cells per well) and incubated at 37°C. After being left overnight to attach, cells were treated with 1nM octreotide,

0.001, 0.01, 0.1, 1nM rapamycin and 1, 10, and 100nM BEZ235 dissolved in 10% DMEM for 24 hours. The carriers in which the substances were dissolved were used as controls.

The WST-1 compound, a tetrazolium salt, is cleaved by the mitochondrial respiratory chain into the product dye that directly correlates to the number of viable cells in the culture. The reaction product was measured in an enzyme-linked immunosorbent assay (ELISA) plate reader at 450 nm. All treatments were carried out in quadruplicates.

2.6 Hormone measurement by radioimmunoassay (RIA)

Before hormone measurements, the human acromegalic primary cell culture was stimulated for 24 h with 1nM rapamycin in D-valine serum-free medium and afterwards the supernatants were collected for RIA analysis.

Radioimmunoassay (RIA) is a highly sensitive technique used for the quantitative measurement of substances such as enzymes, proteins and hormones that normally exist in very low concentrations. In this study, RIA has been used to measure the concentration of human GH (hGH) secreted in the medium by human acromegalic tumors.

RIA uses radiolabeled antigens to detect antigen-antibody (Ag-Ab) reactions. The procedure follows the basic principle of radioimmunoassay where there is competition between a radioactive and a non-radioactive antigen for a fixed number of specific antibody binding sites. The antigens are labeled with the iodine-125 (I^{125}) isotope, and the presence of Ag-Ab complexes is detected using a gamma counter.

Initial step in performing RIA is the development of the highly specific antibody for the hormone of interest. The human GH antibodies were included in the specific RIA reagent kit provided by IMMULITE®2500, containing specific antigens, antiserums and standards. A small amount of antibody is mixed with a certain amount of the sample (cell culture supernatant) containing the hormone to be measured. At the same time, a certain amount of tracer (standard antigen labeled with the radioactive isotope I^{125}) is added to the mixture. The samples are incubated 1 hour at 37°C, allowing the time for the hormone to bind to the antibody. The mixture is prepared in such quantities that there is not enough antibody to bind both, the tracer and the hormone to be measured, so the natural competition for binding the antibody is created. The quantity of each hormone bound is proportional to their concentration and the amount of tracer bound to specific antibody is inversely proportional to the concentration of the natural hormone. After the system has reached equilibrium, the quantity of radioactive hormone bound to the antibody is measured in a gamma counter. Summarized, the amount of radioactivity measured in the sample is inversely proportional to the amount of hormone in the sample.

Quantification of the unknown free hormone in the sample is achieved by comparing its activity with a standard curve prepared by using increasing amounts of known concentrations of the hormone.

2.7 Western immunoblotting

Principle

Western blotting (formally called protein immunoblot) is a technique for identifying specific proteins in a given sample such as tissue or cell extracts, serum, liquor or cell culture supernatants and simultaneously determining its molecular weight. It uses gel electrophoresis to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/ non-denaturing condition). The proteins are then transferred to a membrane (typically nitrocellulose or PVDF), where they are detected using antibodies specific to the target protein. The procedure involves primary and secondary antibodies as well as different ways of visualization of the signal.

For the needs of Western blotting experiments, $1x10^{-6}$ At-T20 and GH3 cells were seeded in a 10 cm-diameter dish using DMEM supplemented with 10% FCS. If cell cycle components were studied, the cells were synchronized in G1 phase by serum deprivation overnight. Cell lysates were prepared by washing the cells one time with cold PBS, adding 1.5 ml of PBS and scraping on ice. The lysates were transferred into Eppendorf tubes and briefly centrifuged at 4000g for 5 minutes. The supernatants were removed and 100-200 µl of protease inhibitor cocktail diluted 1:100 and phosphatase inhibitor cocktail diluted 1:10 in RIPA lyses buffer was added per tube, depending on the size of the pallet. Finally the proteins were extracted lysing the cells by sonication (SONIFIER® Cell disruptor B15) and the protein concentration was determined with Bradford dye assay (Bradford, M., 1976). The samples were separated by polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane overnight.

Blocking of non-specific binding sites on the nitrocellulose membrane was achieved with 5% non-fat dry milk dissolved in 1xTBS solution containing 0.1% Tween 20 for 1 hour with agitation. The incubation with the primary antibody (diluted in 1x TBS containing 2.5% non-fat dry milk and 0.1%Tween) was performed over night at 4°C with agitation (Table 3).

Table 3. List of the primary antibodies used in the Western blotting studies

Antibody	Source	Host	Dilution
Phospho-Akt (Ser^{473})	Cell Signaling Tech.,Inc.,Danvers.,MA	rabbit	1:500
Akt	Cell Signaling Tech.,Inc.,Danvers.,MA	rabbit	1:1000
Phospho-Serine	Zymed, South San Francisco, CA	rabbit	1:500
Phospho-Tyrosine (4G10)	Upstate, Charlottesville,Virginia	mouse	1:1000
Phospho-IRS-1($Ser^{636/639}$)	Cell Signaling Tech.,Inc.,Danvers.,MA	rabbit	1:1000
IRS-1	Upstate, Charlottesville,Virginia	rabbit	1:1000
Phospho-p70 (Ser^{389})	Cell Signaling Tech.,Inc.,Danvers.,MA	rabbit	1:500
p70/S6K	Cell Signaling Tech.,Inc.,Danvers.,MA	rabbit	1:1000
Phospho-mTOR (Ser^{2448})	Cell Signaling Tech.,Inc.,Danvers.,MA	rabbit	1:1000
Phospho-mTOR (Ser^{2481})	Cell Signaling Tech.,Inc.,Danvers.,MA	rabbit	1:1000
mTOR	Cell Signaling Tech.,Inc.,Danvers.,MA	rabbit	1:1000
Cyclin D1	Cell Signaling Tech.,Inc.,Danvers.,MA	mouse	1:500
Cyclin D3	Cell Signaling Tech.,Inc.,Danvers.,MA	mouse	1:1000
Cyclin E	Santa Cruz Biotech.,Santa CruzCA,USA	mouse	1:1000
Cdk6	Cell Signaling Tech.,Inc.,Danvers.,MA	mouse	1:1000
Cdk4	Cell Signaling Tech.,Inc.,Danvers.,MA	mouse	1:1000
Cdk2	Santa Cruz Biotech.,Santa Cruz,CA,USA	mouse	1:1000
p27/Kip1	Cell Signaling Tech.,Inc.,Danvers.,MA	rabbit	1:500
p21/Cip1	Cell Signaling Tech.,Inc.,Danvers.,MA	mouse	1:1000
Phospho-Rb (Ser^{780})	Cell Signaling Tech.,Inc.,Danvers.,MA	rabbit	1:500
Cleaved PARP	Cell Signaling Tech.,Inc.,Danvers.,MA	rabbit	1:500
PARP	Cell Signaling Tech.,Inc.,Danvers.,MA	rabbit	1:1000
Actin	Millipore, Temecula, CA	mouse	1:10000

Horseradish peroxidase-conjugated secondary antibodies in the concentration range from 1:1000 to 1:5000 were used against mouse and rabbit (Cell Signaling). Each experiment was performed in duplicates.

After the treatment with the first antibody, the membrane is rinsed 3 x 10 minutes each in 1xTBS 0.1% Tween before the secondary antibody is applied. The membrane was incubated with the secondary antibody diluted 1:2000 in 2.5% non-fat dry milk 1xTBS 0.1% Tween solution for 90 minutes at room temperature.

Three further washes in 1xTBS 0.1% Tween, 10 min each, were then performed before incubating the membrane in the Lumi-light Western Blotting Substrate solution, prepared according to the manufacturer's instructions. An x-ray film was exposed to the membrane in an autoradiography cassette, to detect the light given off by the enzymatic reaction. Depending on the strength of the signal, varying from just a few seconds to 1h, the film was removed and developed to visualize the immunoreactivity bands. The bands were present wherever there was a protein - primary antibody - secondary antibody - enzyme complex.

2.8 Coimmunoprecipitation

Principle

Immunoprecipitation (IP) is the method where an antigen is precipitated out of the solution by using an antibody that specifically binds to that antigen. Coimmunoprecipitation is a specific type of immunoprecipitation used for extraction of intact protein complexes. The technique is based on targeting a specific protein complex from the cell extract by using an antibody that targets a known protein that is believed to be a member of a larger complex of proteins. By targeting this known member with an antibody it may become possible to pull the entire protein complex out of solution and thereby identify unknown members of the complex. Immunoprecipitation requires that the antibody is coupled to a solid substrate (e.g. protein A Sepharose or Agarose) during the procedure. Coimmunoprecipitated proteins are fractionated by SDS-PAGE and are detected by autoradiography and/or by Western blotting with an antibody directed against that protein.

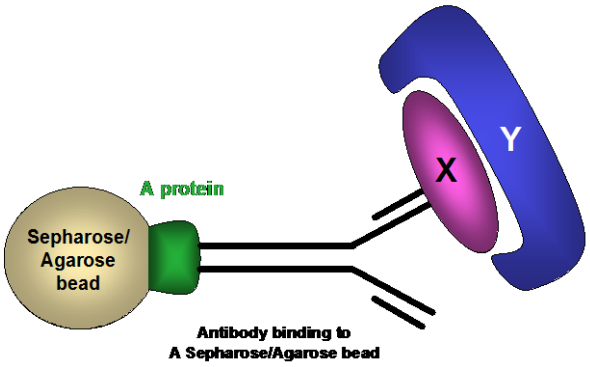

Fig. 4. Schematic depiction of interactions during coimmunoprecipitation. A protein Sepharose/Agarose beads bind anti-X antibody. Protein X complexes with protein Y, which can be detected by Western blotting using anti-Y antibody.

In this study, AtT-20 cells were treated with octreotide, rapamycin and their combination for 5 minutes and collected in ice-cold lysis buffer (100mM NaCl, 20mM Hepes pH7.4, 1mM sodium orthovanadate, 1mM EDTA, 2mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40). 600 µg protein was immunoprecipitated with an antibody against IRS-1 (Upstate) or with a control

rabbit IgG. For capturing the protein-antibody complex protein A sepharose was used (Amersham Pharmacia Biotech; Fig. 4). The immunoprecipitates were extensively washed and protein bound to sepharose was eluted, dissociated and separated by 10% SDS-polyacrylamide gel electrophoresis. Western blotting was performed using the horse-radish peroxidase conjugated antiphosphotyrosine 4G10 (Upstate), anti-phosphoserine (Zymed, South San Francisco, CA) or -IRS-1 antibody (Upstate). Coimmunoprecipitation was performed in two independent experiments and was repeated using protein G Sepharose.

2.9 Transfection studies and RNA interference

Plasmid preparation, cell transfection and reporter assay

The plasmids used in this study were the SHP-1/C453S expression vector, which expresses the dominant negative mutant of SHP-1 (SHP-1dn; Pages et al., 1999), constitutive active, myristoylated Akt expression vector (Myr-Akt; Andjelkovic et al., 1997), the empty vector used as a control, and a vector expressing the luciferase gene under the control of E2F responsive element promoter (E2F-Luc). Akt is activated when it is translocated to the membrane. Thus, myristoylated Akt, which is anchored to the cell membrane, is constitutively activated. In all experiments Rous sarcoma virus-β-galactosidase construct was cotransfected and β-galactosidase activity was measured to correct variations in transfection efficiency.

Competent bacteria, i.e. bacteria pretreated in such way to easily incorporate a plasmid, were transformed, left to grow overnight at 37°C on agar containing ampicilin, and stored at +4°C. A colony was picked from each plate and left in 250ml LB medium containing 50µg/µl ampicilin overnight at 37°C. Plasmid preparation was performed using QIAGEN plasmid purification system.

Cell transfection was performed on confluent AtT-20 cells grown in a 6-well plate, using SuperFect® (Qiagen GmbH, Hilden, Germany). Cells (3×10^5/ well) were transfected for 3 hours with 1µg of SHP-1dn plasmid (or empty plasmid, Myr-Akt or E2F-Luc), left in cell growth medium overnight and treated with DMSO, octreotide alone or in combination with rapamycin. Lysates of cells treated for 3 hours were analyzed for pAkt-Ser^{473} and pIRS-1-$Ser^{636/639}$ by Western blotting and lysates of cells treated for 5 min were immunoprecipitated with IRS-1 as described above. Each transfection experiment was performed in duplicates. To confirm the SHP-1dn and Myr-Akt incorporation, Western blotting was performed for hemagglutinin (Acris, Hiddenhausen, Germany).

To determine the levels of E2F driven luciferase expression after the treatment with octreotide, rapamycin and their combination, a luciferase reporter assay was performed. Luciferase is an

enzyme found in *Photinus pyrallis*, a common firefly. Luciferase emits light in the presence of luciferin and ATP, which can be detected at 563nm wavelength.

The E2F-Luc construct (Mercury^{TM} pathway profiling system, Clontech Laboratories, Inc., Palo Alto, CA) has the E2F responsive element upstream to the TATA box of the herpes simplex virus thymidine kinase promoter and the reporter gene luciferase. Cells transfected with E2F-Luc were treated with octreotide, rapamycin and their combination for 6 hours. Subsequently the cells were washed one time with PBS, lysed with reporter lysis buffer, and scraped and transferred into Eppendorf tubes. The cells were briefly centrifuged and the supernatants were collected to be assayed as follows: 20µl of the supernatant was pipetted into a non-transparent 96-well plate, 50µl luciferin as substrate was automatically added and luciferase activity was measured by a Berthold luminometer. β-galactosidase was assayed as following: 30µl distillated water and 20µl of the supernatant were pipetted into a transparent 96-well plate, 50µl ONPG buffer was added and the light-protected reaction was incubated at 37°C until a yellow shade was obvious. β-galactosidase activity was measured in ELISA plate reader at 420 nm. Data are expressed as the ratio of E2F relative luciferase activity to β-galactosidase activity. Each experiment was performed in triplicates.

RNA interference

The RNA interference pathway is often used to study the function of genes in cell culture and *in vivo* in model organisms. The RNA interference is a cellular process where small, specific double-stranded RNA (dsRNA) molecules interact in a complex with mRNAs to which they are homologous and cause their degradation leading to a decrease in the expression of the corresponding protein. For this purpose synthetic dsRNA that matches the sequence of the gene of interest is introduced into the cell and activates the silencing apparatus. mRNA molecules that correspond to the targeted gene sequence are degraded.

Double-stranded small interfering RNA (siRNA) against mouse p27/Kip1 (*Cdkn1b*) was obtained from Santa Cruz Biotech (sc-29430). One scrambled siRNA (Scramble II; MWG Biotech) was used as a control. AtT-20 cells were transfected with 100nM scrambled or siRNA against p27/Kip1 using SuperFect® (Qiagen) for 3 hours and were left in cell growth medium overnight to recover. The day after, they were split and distributed for proliferation assays and for RNA extraction. Cells for RNA extraction were harvested at the same time with the proliferation assay, i.e. 48 hours after transfection with the siRNA. Each experiment was performed twice.

2.10 Cell cycle analysis by fluorescence-activated cell sorting (FACS)

Principle

The DNA content of individual cells provides information about their ploidy (of particular relevance in tumors), and for the distribution of cells across the cell cycle after added stimuli, e.g. drug treatment. Analysis of a population of cells' replication state can be achieved by fluorescence labeling of the nuclei of cells in suspension and then analyzing the fluorescence properties of each cell in the population. In brief, quiescent and G1 cells will have one copy of DNA and will therefore have 1X fluorescence intensity, cells in G2/M phase of the cell cycle will have two copies of DNA and accordingly will have 2X intensity. Since the cells in S phase are synthesizing DNA they will have fluorescence values between the 1X and 2X populations. The resulting histogram consists of three populations: two Gaussian curves (1X and 2X peaks) and the S-phase population (Fig. 5).

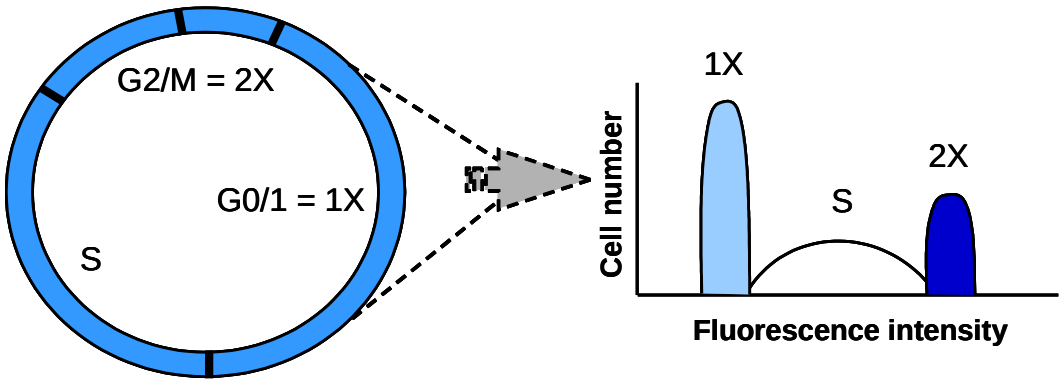

Fig. 5. Shematic illustration of DNA distribution during the cell cycle.

The fluorescent dye that is used to measure the DNA content binds to DNA in a manner that reflects accurately the amount of DNA present. The most widely used dye is propidium iodide (PI), which has red fluorescence and can be excited at 488nm. As PI stains all double stranded nucleic acids, the cells have to be incubated with RNase to remove any double stranded RNA. PI cannot enter intact plasma membranes, therefore the cells have to be fixed or permeabilised before adding the dye.

Cell cycle data were analysed by FACS after PI staining. AtT-20 cells ($1x10^6$) were plated in 10 cm-diameter dishes, synchronized in G1 phase with serum-free medium and subjected to the indicated treatments for 24 hours. Cells were trypsinased, pelleted, washed twice with PBS and permeabilised with 70% ethanol for 60 minutes in 4°C or overnight at -20°C. 1 ml of propidium PI solution (50µg/ml in PBS), containing 100 µg/ml DNase-free RNase A was added to

approximately 2 x 10^6 cells for 30 minutes at 37°C. Analysis was performed by Beckman Coulter EPICS^{TM}XL^{TM} (Beckman Coulter, Inc., Fullerton, CA, USA) flow cytometry apparatus, using EXPO32 Software. AUX parameter *versus* FL2LIN gating was performed to exclude doublets from G2/M region.

Protocol for staining cells with propidium iodide

Fixation

The cells are harvested in the appropriate manner and a single cell suspension is prepared in a buffer (e.g. PBS+2%FBS or PBS+0,1% BSA). The cells are washed 2X with PBS and resuspended at 1-2x10^6 cells/ml in PBS. Fixation of the cells is achieved by adding 3 ml/1ml PBS of -20°C absolute ethanol (slowly, drop by drop while vortexing). The 70% EtOH cell suspension should be kept at least 1h at 4°C prior the measurement. If the measurement doesn't happen immediately after the fixation, the cells can be stored at -20°C in this fixation buffer until ready for analysis (for several weeks).

Staining

Fixed cells are washed 2X with PBS by centrifuging 2000rpm for 5minutes. The pellet is resuspended in 0, 5 ml solution mix of 50µg/ml propidium iodide solution (light sensitive) and 50 µl of 100 µg/ml DNase-free, RNaseA and incubated at 37°C for 30 minutes. After incubation the cells can be analysed by flow cytometry.

2.11 Statistical analysis

Statistical analysis was performed using SPSS version 15.0 (SPSS, Chicago, IL). All the results/differences were assessed by one-way ANOVA in combination with Scheffe's test. $P<0.05$ was considered as significant.

3 AIM OF THE STUDY

The present study aims to identify new pharmacological treatments that will limit pituitary tumor growth and viability by improving the cytostatic action of drugs commonly used for pituitary management, such as SSA, and testing small molecule inhibitors designed to target members of the PI3K/mTOR pathway. The majority of pituitary adenomas included were NFPA, since they present with tumor mass effects and cannot be managed by the existing pharmacological means.

Pituitary adenomas, and especially NFPA, are resistant in their majority to the well documented *in vitro* antiproliferative action of SSA, despite the presence of somatostatin receptors. PI3K pathway overactivation is believed to contribute to chemotherapy and radiation resistance seen in several cancers. This pathway was also found to be overactivated in human pituitary adenomas, hence it was chosen as the focus of the study. Tumors with overactivated PI3K pathway are currently managed with the mTOR inhibitors rapamycin and its analogs. However, the sensitivity to rapamycin is compromised by the elimination of a downstream negative feedback loop eventually leading to increased Akt overactivation. Blocking the PI3K/Akt pathway upstream could suppress this side effect, and for this the SSA octreotide was used, since it was shown to effectively inhibit PI3K and its signaling cascade in pituitary tumor cells. Similarly dual inhibitors able to concomitantly target PI3K and mTOR could provide effective antiproliferative action without the feedback side effect.

The cytostatic agents used in the present study were rapamycin, alone or in combination with octreotide, and the dual PI3K/mTOR small molecule inhibitor BEZ235. Treatments were performed in human pituitary adenomas in primary cell culture and their cytostatic action was determined by measuring DNA synthesis by thymidine incorporation and mitochondrial activity by colorimetric non-radioactive assays. Immortalized pituitary tumor cell lines were used to delineate the signaling cascades employed by the tested compounds and to elucidate the downstream cell cycle targets mediating their cytostatic action. This was of particular importance, since analyzing how each tumor type responds to a particular agent helps to understand the intrinsic mechanisms responsible for its tumorigenessis, and facilitates the development of therapeutic schemes that will be most beneficial.

4 RESULTS

COMBINED OCTREOTIDE-RAPAMYCIN TREATMENT IN IMMORTALIZED PITUITARY TUMOR CELLS AND HUMAN PITUITARY ADENOMAS

Overactivated Akt abolishes antiproliferative effect of octreotide in immortalized pituitary tumor cells

The PI3K/Akt pathway activation has been implicated in chemotherapy resistance in various cancers. To examine the role of Akt overactivation in pituitary tumor cell resistance to the SSA antiproliferative action, constitutive active Akt (Myr-Akt) was overexpressed in immortalized pituitary tumor cell line, AtT-20. In cells transfected with the empty vector, 24 h treatment with 1nM octreotide resulted in 20% cell growth inhibition as determined by the nonradioactive colorimetric WST-1 assay (Fig. 6). Changes over 20% were considered significant. Introduction of constitutive active Akt rendered the cells resistant to octreotide's antiproliferative treatment. Therefore, Akt overactivation is implicated in SSA treatment resistance.

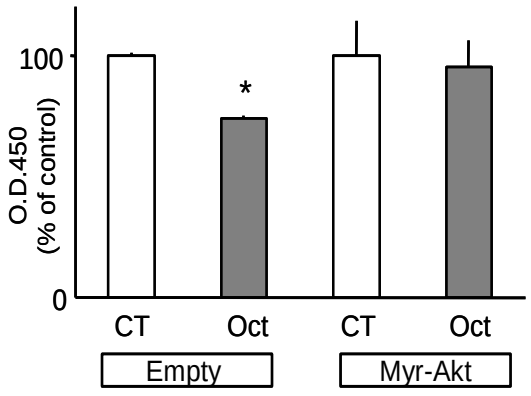

Fig. 6. Effect of constitutive active Akt on octreotide's antiproliferative action in AtT-20 cells; cell proliferation was determined in cells transfected with 0.5ng empty vector or Myr-Akt, treated with 1nM octreotide for 24 hours. *: P<0.05.

Antiproliferative action of the combined octreotide-rapamycin treatment in NFPA in primary cell culture

Treatment of 28 NFPA in primary cell culture with the SSA octreotide did not suppress proliferation in most cases, similar to previous reports (Shomali & Katznelson, 2002; Colao et al., 2003). Seven out of 28 NFPA responded to 1nM octreotide (% mean suppression: 26 ±5). Members of the PI3K/Akt pathway are activated in human pituitary tumors, showing the highest

levels of overexpressed Akt in NFPA (Musat et al., 2005). In Akt overexpressing tumors the mTOR inhibitor rapamycin is used to reverse the resistance to radio- and chemotherapy. Addition of 1nM rapamycin to octreotide inhibited [^{3}H]-TdR uptake by 20% and more in all 28 cases, even in the ones responsive to octreotide (% mean suppression: 40±13; *P*=0.007, Fig. 7A). In eight tumors that provided enough cells for dose response studies, the suppressive action of the combined octreotide-rapamycin treatment (1nM; % mean suppression: 40±16; *P*=0.001) remained after adding concentrations of octreotide as low as 0.1nM and 0.01nM to 1nM rapamycin (% mean suppression 30±19, *P*=0.029 and 22±18, *P*=0.016 respectively; Fig. 7B). Therefore, addition of rapamycin confers sensitivity to octreotide treatment in NFPA *in vitro*.

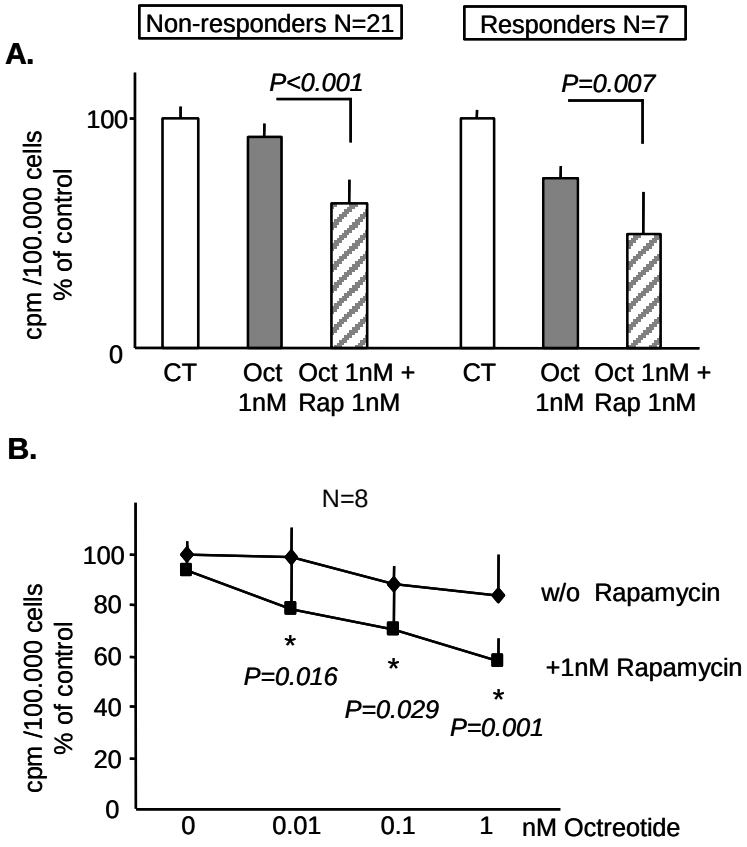

Fig. 7. [^{3}H]-TdR uptake inhibition in human NFPA in primary cell culture treated with 1nM octreotide and 1nM octreotide plus 1nM rapamycin; (A) mean of seven NFPA that were responsive to single octreotide treatment and mean of 21 NFPA non responsive to the antiproliferative action of octreotide. (B) Dose curve study on eight out of 28 NFPA treated with 0.01, 0.1 and 1nM octreotide alone or in combination with 1nM rapamycin. All treatments were performed in 10% FCS-DMEM supplemented with D-Valine. Data were calculated as mean±SEM and are expressed as percentage of vehicle treated control. Each measurement was done in four independent wells.

Antiproliferative action of single rapamycin treatment in NFPA in primary cell culture

The reason for the better antiproliferative effect of the combined treatment was not solely due to the influence of octreotide as it was demonstrated in Fig. 7. Therefore, single rapamycin treatment was assessed in order to see if the better effect can be attributed to this drug alone. In order to test the antiproliferative effect of single rapamycin treatment, 1nM rapamycin was

applied to 28 NFPA in primary cell culture. Only 8 out of 28 tumors responded by decreasing [^{3}H]-TdR incorporation (mean suppression as % of vehicle control: 69±13; P<0.05). Most of the NFPA (20 out of 28) did not respond to 1nM rapamycin displaying resistance to this treatment (Fig. 8). These data suggest that it is the addition of octreotide that is able to reverse rapamycin resistance and moreover improve rapamycin's antiproliferative effect in NFPA *in vitro*.

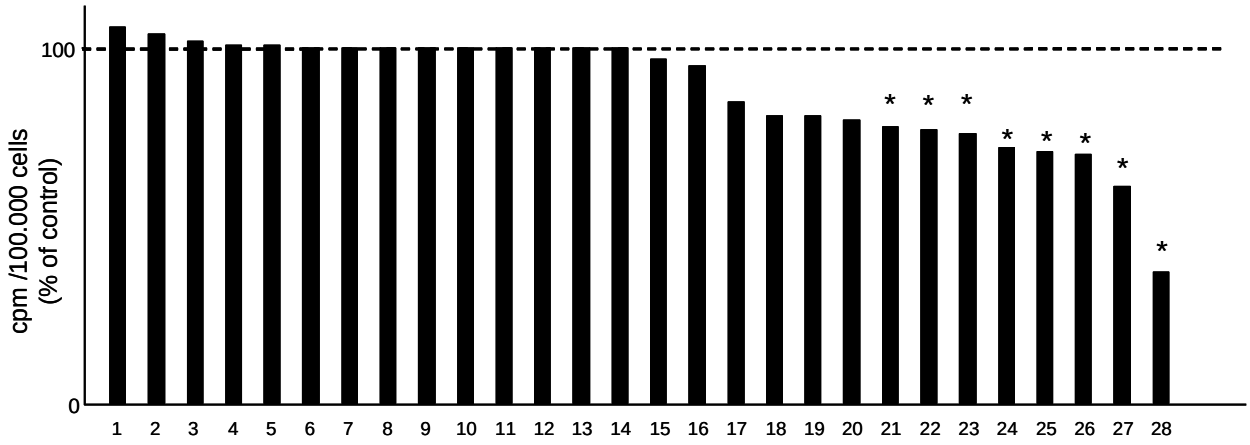

Fig. 8. [^{3}H]-TdR uptake inhibition in human NFPA in primary cell culture treated with 1nM rapamycin. Cell proliferation was determined after 24 hours treatment. All treatments were performed in 10% FCS-DMEM supplemented with D-Valine. Data were calculated as mean±SEM and are expressed as percentage of vehicle treated control. Each measurement was done in four independent wells. *: P<0.05.

Antiproliferative action of single rapamycin treatment in immortalized pituitary tumor cells

To test this hypothesis the efficacy of the single rapamycin treatment was examined in the pituitary tumor cell lines, AtT-20 and GH3. Rapamycin treatment in AtT-20 had small antiproliferative effect at 1nM concentration (25% growth suppression, P<0.05) and no effect at lower doses, similar what was observed in most NFPA (Fig. 9A, 9C). Addition of 1nM octreotide to 1nM rapamycin in AtT-20 cells led to 50% inhibition, (P<0.001 vs. vehicle control and P=0.003 vs. rapamycin alone, Fig. 9B and 9D) as determined by both [^{3}H]-TdR and the WST-1 assay. The antiproliferative effect of the combined treatment remained even at lower rapamycin concentrations (P<0.01). Octreotide alone had a mild antiproliferative effect (20% growth suppression, P<0.01). These data demonstrate resistance to rapamycin treatment in AtT-20 cells and constitute them as a model of rapamycin insensitive cells. In contrast, GH3 cells responded to rapamycin treatment at all dosages except at the lowest (Fig. 9E).

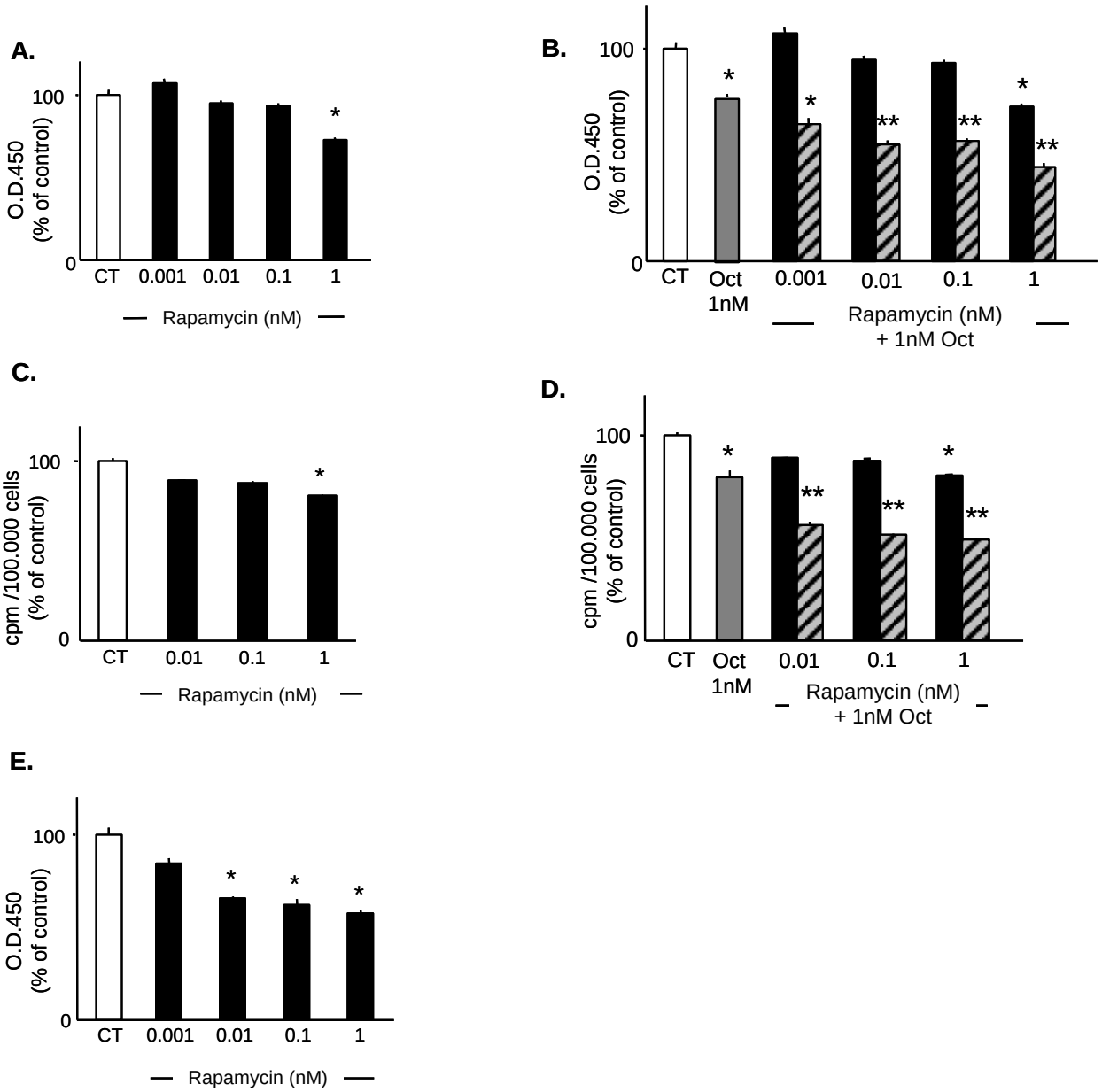

Fig. 9. Antiproliferative effect of 0.001, 0.01, 0.1 and 1nM rapamycin alone or in combination with 1nM octreotide in AtT-20 (A, B, C, D) and GH3 (E) cells. Treatments were performed in 10% FCS-DMEM. Cell proliferation was determined after 24 hours treatment. Data are presented as percentage of each vehicle-treated control. *: P<0.05, **: P<0.001. Each measurement was done in four independent wells.

Octreotide reverses rapamycin-induced increase in Akt phosphorylation

The elimination of rapamycin-induced p70/S6K negative feedback loop leads to increased pAkt-Ser^{473} levels in treated cells and subsequent treatment resistance (O'Reilly et al., 2006). Treatment with 1nM rapamycin showed no changes in pAkt-Ser^{473} in GH3 cells (Fig. 10A), but

increased it in AtT-20 cells (Fig. 10B). In these cells, addition of 1nM octreotide to rapamycin treatment decreased pAkt-Ser^{473} levels, clearly showing that octreotide affects rapamycin's feedback loop on Akt phosphorylation. Octreotide treatment did not affect rapamycin's suppressed p70/S6K or mTOR phosphorylation at Ser^{2448} and Ser^{2481} sites (Fig. 10C).

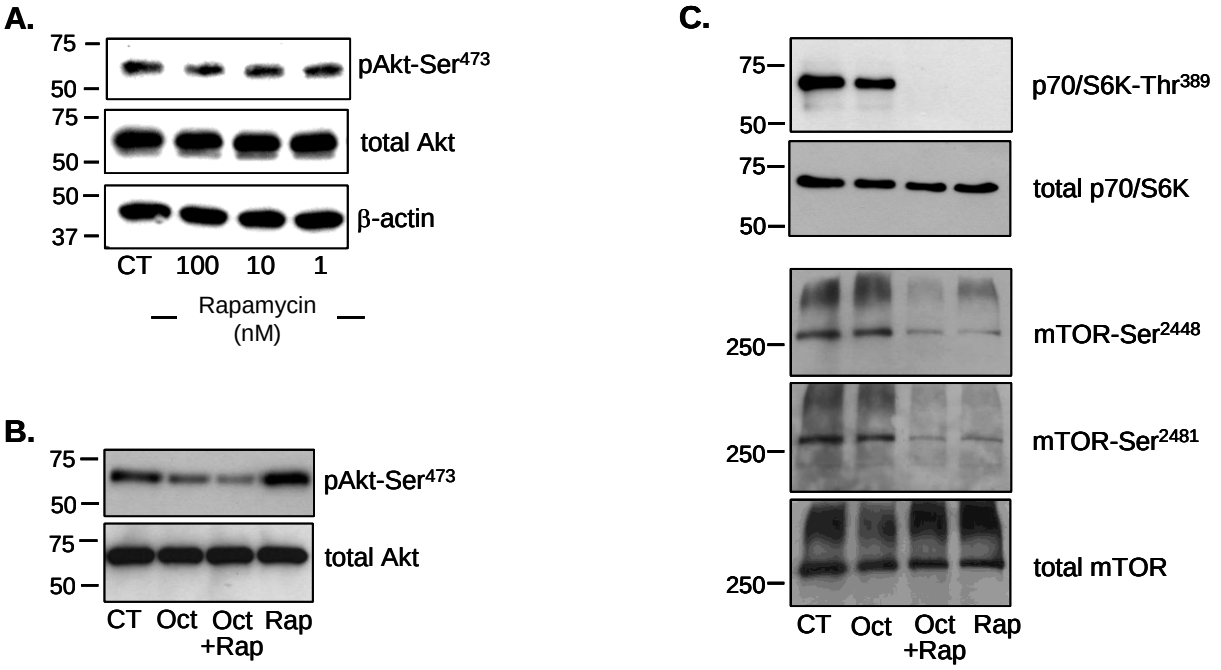

Fig. 10. (A) GH3 cell lysates were treated with 100, 10, and 1nM rapamycin for 24 h. Protein expression levels were examined by Western blotting using anti-pAkt-Ser^{473} and anti-Akt. Equal protein loading was examined by detection of β-actin. Treatments were contacted in the presence of 10% FCS. Representatives of three experiments are shown. (B) AtT-20 cell lysates treated with DMSO (CT), 1nM octreotide plus DMSO (Oct), 1nM rapamycin and 1nM octreotide plus 1nM rapamycin for 3 hours, analyzed by Western blotting using anti-Akt and -pAkt-Ser^{473}. Representatives of three experiments are shown. (C) AtT-20 cell lysates treated with DMSO (CT), 1nM octreotide plus DMSO (Oct), 1nM rapamycin and 1nM octreotide plus 1nM rapamycin analyzed by Western blotting using anti-p70/S6K-Thr^{389}, -total p70/S6K, -mTOR-Ser^{2448}, -mTOR-Ser^{2481}, and -total mTOR.

Octreotide treatment inhibits rapamycin's action on IRS-1

Rapamycin treatment increases pAkt-Ser^{473} by decreasing the inhibitory IRS-1 serine phosphorylation. In AtT-20 cells treatment with octreotide increased the levels of IRS-1 detected with the phospho-serine antibody, which had been suppressed by rapamycin (Fig. 11A). In contrast, octreotide suppressed IRS-1 levels detected with the 4G10 phospho-tyrosine antibody (Fig. 11A), confirming what was shown in Sstr2 overexpressing CHO cells (Bousquet et al., 2006). Furthermore rapamycin was found to decrease pIRS-1-Ser$^{636/639}$ levels, which is the

site primarily phosphorylated by p70/S6 kinase, and addition of octreotide reversed this effect (Fig. 11B). Octreotide was shown to transduce its effects on IRS-1 tyrosine phosphorylation through SHP-1 (Bousquet et al., 2006). Transfection with a catalytically inactive SHP-1 abolished octreotide's mediated increase in pIRS-1-Ser$^{636/639}$ levels (Fig. 11C) and decrease in pAkt-Ser^{473} levels (Fig. 11D).These data show that octreotide increases serine phosphorylated IRS-1 levels and decreases pAkt-Ser^{473}, in a mechanism involving SHP-1, and suggest that it could improve rapamycin's antiproliferative action (Fig. 11E).

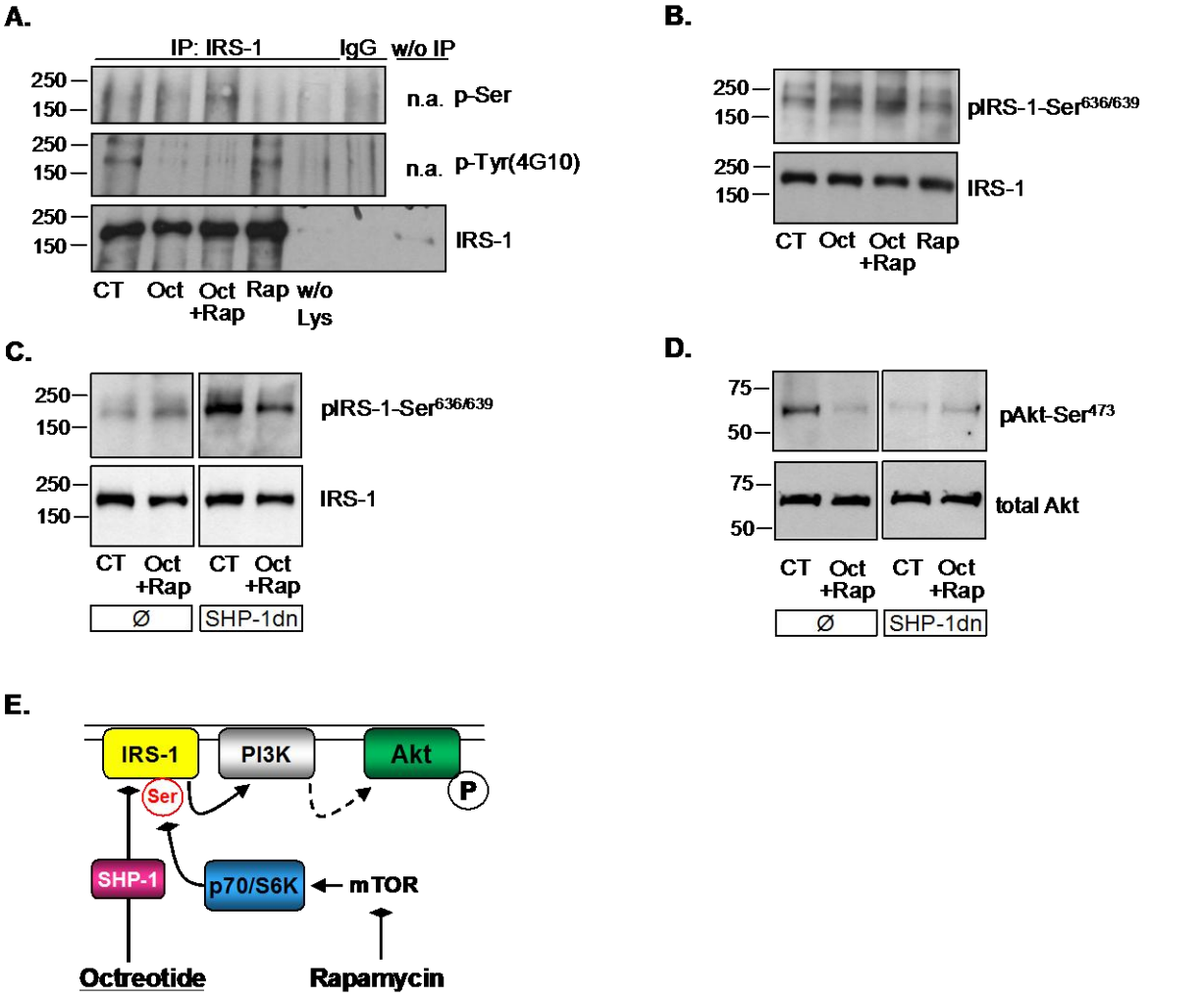

Fig. 11. (A) AtT-20 cell lysates treated with DMSO (CT) and 1nM octreotide plus 1nM rapamycin were immunoprecipitated with Protein A Sepharose and anti-IRS-1 or a control rabbit IgG. The immunoprecipitated fractions were analyzed by Western blotting using antiphosphoserine, -phosphotyrosine 4G10, and -IRS-1 (B) AtT-20 cell lysates treated like in (A), analyzed by Western blotting using anti-pIRS-1-Ser$^{636/639}$ and -IRS-1. (C) Control and SHP-1dn transfected AtT-20 cell lysates treated with DMSO (CT) and 1nM octreotide plus 1nM rapamycin analyzed by Western blotting using anti-pIRS-1-Ser$^{636/639}$ and -IRS-1, (D) anti-Akt and -pAkt-Ser^{473}. Representatives of two independent transfection experiments are shown. (E) Scheme showing how octreotide affects rapamycin's feedback loop on Akt phosphorylation. Diamond-ended arrows: inhibition; solid arrows: direct action; dashed arrows: indirect action. Representatives of two experiments are shown. n.a.: not applicable.

Effect of the combined octreotide-rapamycin treatment on cell cycle components

Both rapamycin and octreotide are cytostatic (Marx et al., 1995; Cheung et al., 1995; Srikant et al., 1995) therefore, the mechanism behind the superior antiproliferative action of combined octreotide-rapamycin treatment was sought among the proteins important for the G1/S cell cycle transition. Indeed, the combined treatment accumulated the cells in the G0/G1 phase (Fig. 12A). Cell cycle progression starts with the activation of D-type cyclins and their associated kinases Cdk4 and 6 (Sherr et al., 2000). Octreotide did not affect cyclin D1, cyclin D3, Cdk4 and Cdk6 levels (Fig. 12B). Rapamycin alone or in combination with octreotide decreased cyclin D1, cyclin D3, Cdk4 and Cdk6 (Fig. 12B), while octreotide did not potentiate rapamycin's effect, suggesting that the potent antiproliferative action of the combined treatment is not due to a more potent suppression of D-type cyclins.

The G1 to S transition is primarily governed by the cyclin E/Cdk2 complex (Koff et al., 1992). Single and combined rapamycin treatment decreased Cdk2 protein levels, (Fig. 12B). Cyclin E/Cdk2 complexes act by hyperphosphorylating and inhibiting retinoblastoma (Rb). The resulting phosphorylated Rb dissociates from E2F transcription factors leading to increased E2F-driven gene transcription (Schwarz et al., 1993). Rapamycin decreased Rb phosphorylation levels but addition of octreotide almost eliminated them (Fig. 12C). Furthermore the combined octreotide-rapamycin treatment reduced E2F transcriptional activity more potently than rapamycin alone (P=0.012, Fig. 12D), while octreotide alone had a small effect, which did not reach statistical significance. E2F transcription factors drive the transcription of several genes, whose products are important for the transition to the S-phase, including cyclin E. The combined octreotide-rapamycin treatment decreased cyclin E protein expression more potently than each individual treatment alone (Fig. 12E). Single octreotide treatment decreased cyclin E, similar to what was reported for the somatostatin analog RC-160 in CHO/Sstr2 cells (Pages et al., 1999) and somatostatin in pancreatic acinar cells (Charland et al., 2001). Treatment with the protease inhibitor MG132 did not affect cyclin E suppression after single or combined octreotide and rapamycin treatment suggesting that octreotide is affecting cyclin E transcription rather than protein stability (Fig. 12F).

These results suggest that the combined treatment decreases E2F transcriptional activity and cyclin E transcription prohibiting the tumor cells from entering the S phase of the cell cycle. However, the rapamycin-induced decrease in Cdk2 levels alone cannot explain the better suppressive effect of the combined octreotide-rapamycin treatment on E2F transcriptional activity and cyclin E transcription.

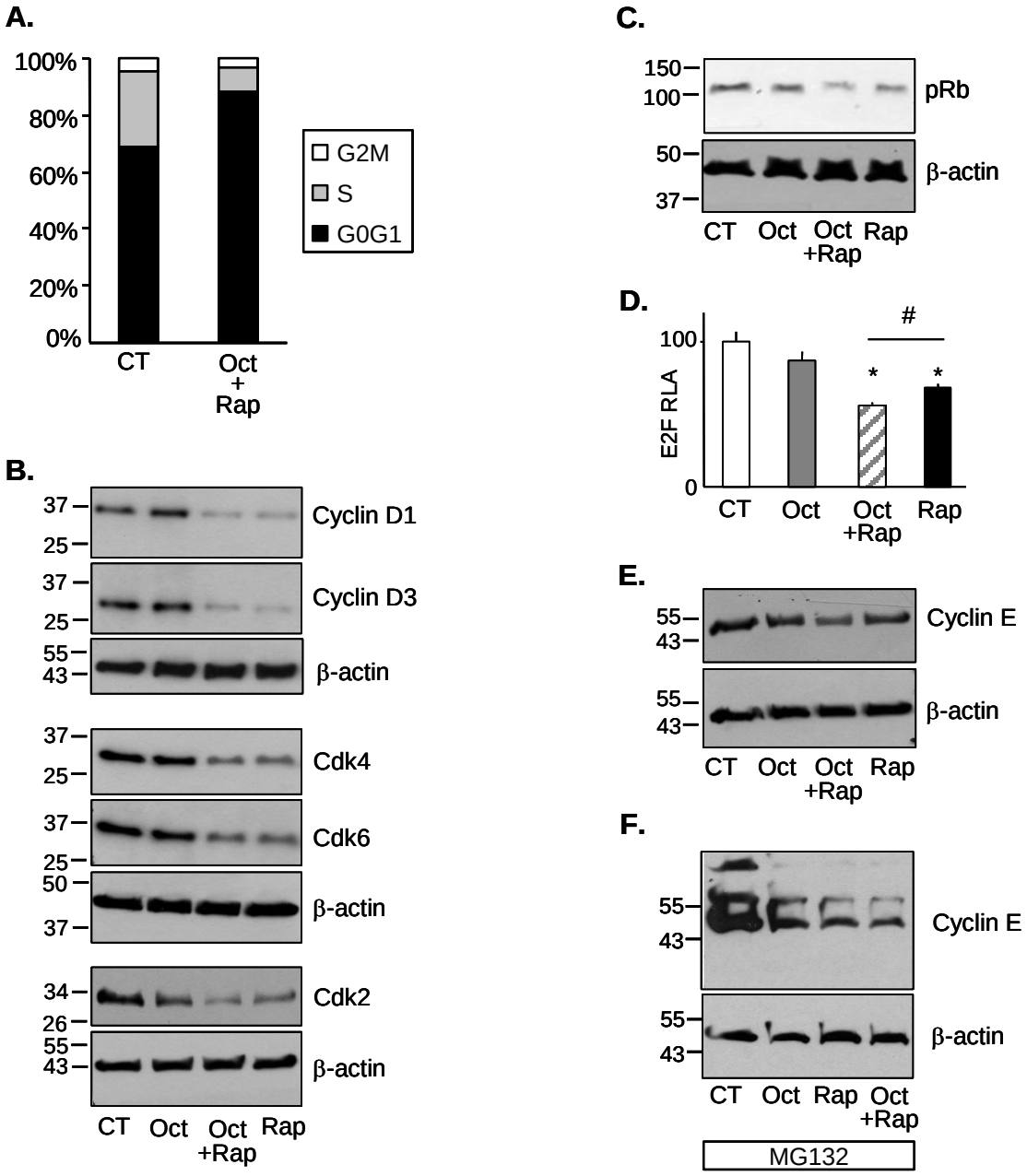

Fig. 12. (A) Cell cycle content of AtT-20 cells incubated with vehicle or 1nM rapamycin plus 1nM octreotide for 24 hours was analysed by FACS. AtT-20 cell lysates treated with DMSO (CT), 1nM octreotide plus DMSO (Oct), 1nM rapamycin and 1nM octreotide plus 1nM rapamycin for 24 hours analyzed by Western blotting using (B) anti-cyclin D1, -cyclin D3, -Cdk4, -Cdk6, - Cdk2; representative β-actin is shown and (C) anti-pRb-Ser^{780}. Cells were serum deprived for 24 hours before treatment. Treatments were contacted in the presence of 10% FCS. Representatives of three experiments are shown. (D) Effect of 6 hours treatment with 1nM octreotide, 1nM rapamycin, and their combination on E2F-mediated transcription in AtT-20 cells transfected with E2FLuc. RLA: relative luciferase activity. Results are shown as E2F-RLA: βGAL ratio. Each experiment was repeated twice. *: $P<0.05$, #: $P=0.012$. (E) Western blotting for cyclin E. The membrane blotted for Cdk2 (shown in (B)) was used after stripping with 100mM Tris-HCl pH2.0. (F) AtT-20 cell lysates treated as in B in the presence of the protease inhibitor MG132 (10μM) for 24 hours analyzed by Western blotting using anti-cyclin E.

The combined octreotide-rapamycin treatment upregulates p27/Kip1

The principal regulator of the cyclin E/Cdk2 complex is the cyclin kinase inhibitor p27/Kip1. Single octreotide and rapamycin treatments increased p27/Kip1 protein levels, similar to what was described in previous studies (Nourse et al., 1994; Pages et al., 1999), but their combination had a stronger effect on p27/Kip1 transcript (Fig. 13A) and protein levels (Fig. 13B). These data suggest that increased p27/Kip1 transcription is an adjuvant mechanism by which the combined treatment exerts its superior antiproliferative action. Indeed knocking down p27/Kip1 abolished the antiproliferative action of the combined octreotide-rapamycin treatment without affecting that of the single rapamycin treatment (Fig. 13C).

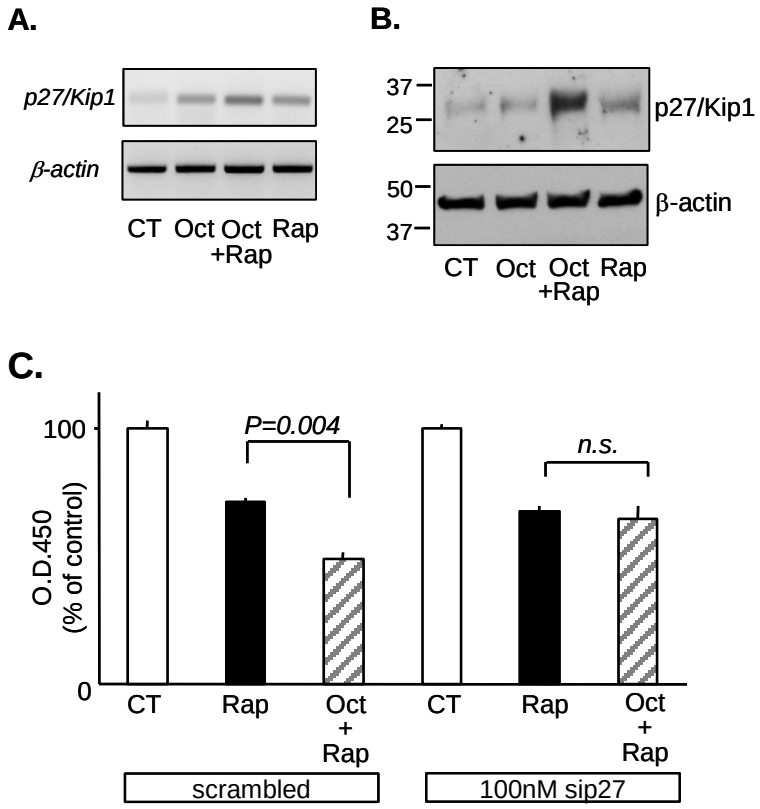

Fig. 13. (A) p27/Kip1 expression in AtT-20 cells after 24 hour treatment with DMSO (CT), 1nM octreotide plus DMSO (Oct), 1nM rapamycin and 1nM octreotide plus 1nM rapamycin as determined by RT-PCR. (B) Western blotting for p27/Kip1 on AtT-20 cell lysates treated with DMSO (CT), 1nM octreotide plus DMSO (Oct), 1nM rapamycin and 1nM octreotide plus 1nM rapamycin for 24 hours. (C) Effect of p27/Kip1 RNA interference on the antiproliferative action of the combined rapamycin-octreotide treatment; cell proliferation was determined in cells transfected with 100nM scrambled or p27/Kip1 siRNA, treated with 1nM rapamycin alone or in combination with 1nM octreotide for 24 hours. n.s.: not significant.

The stronger effect of the combined treatment on p27/Kip1 indicates that the two drugs may induce p27/Kip1 through different pathways. The stimulatory effect of octreotide on basal and rapamycin-induced p27/Kip1 was not observed in AtT-20 cells transfected with a dominant negative SHP-1 mutant (Fig. 14A) or with a constitutively active myristoylated Akt (Fig. 14B), while neither intervention affected rapamycin's effect. Instead rapamycin's stimulatory action on p27/Kip1 was blocked after inhibiting GSK-3β with 20mM lithium chloride (Fig. 14C) or 14μM of the selective inhibitor SB415286 (Fig. 14D). These data indicate that octreotide increases p27/Kip1 downstream to SHP-1 mediated Akt inhibition while rapamycin by activating GSK-3β.

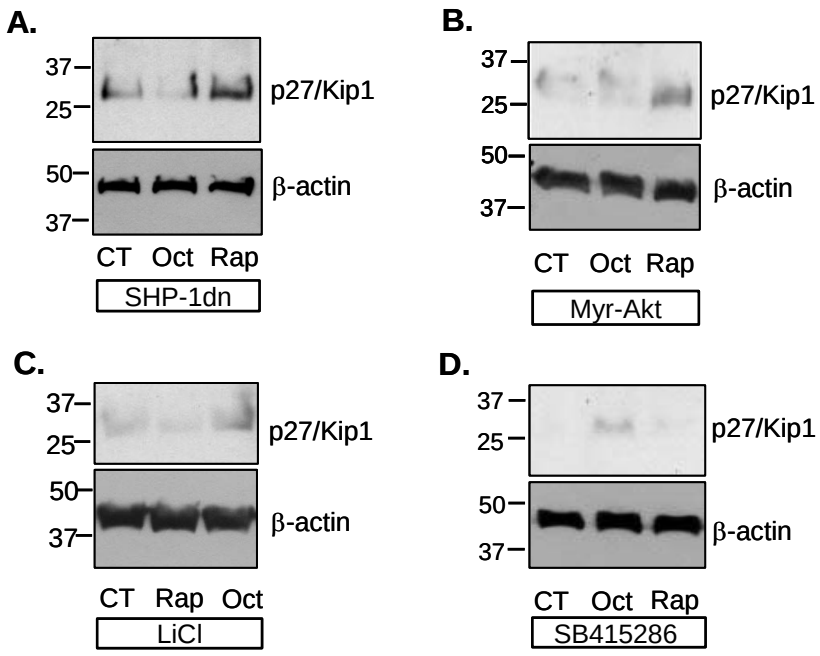

Fig. 14. Western blotting for p27/Kip1 on AtT-20 cell lysates treated with DMSO (CT), 1nM octreotide plus DMSO (Oct), 1nM rapamycin and 1nM octreotide plus 1nM rapamycin for 24 hours; (A) SHP-1dn and (B) Myr-Akt -transfected AtT-20 cell lysates treated with DMSO (CT), 1nM octreotide plus DMSO (Oct), and 1nM rapamycin, and (C) AtT-20 cell lysates treated as in B and C in the presence of the GSK-3β inhibitor lithium chloride (LiCl; 20mM). (D) AtT-20 cell lysates treated as in B and C in the presence of the GSK-3β inhibitor SB415286 (14μM).

SINGLE RAPAMYCIN TREATMENT IN IMMORTALIZED PITUITARY TUMOR CELLS AND HUMAN PITUITARY ADENOMAS

Addition of octreotide does not potentiate rapamycin's action in GH3 cells

Rapamycin had a potent inhibitory effect on GH3 cell proliferation, which was not potentiated by addition of octreotide (Fig. 15).

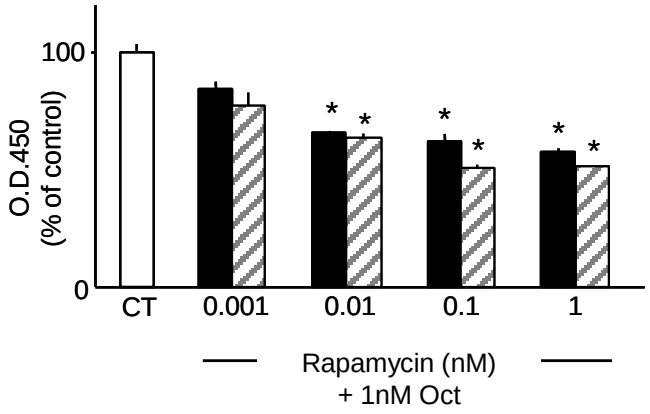

Fig. 15. Antiproliferative effect of 0.001, 0.01, 0.1 and 1nM rapamycin alone or in combination with 1nM octreotide in GH3 cells. Treatments were performed in 10% FCS-DMEM. Cell proliferation was determined after 24 hours treatment. Data are presented as percentage of each vehicle-treated control. Each measurement was done in four independent wells. *: P<0.05.

In these cells, 1nM rapamycin treatment significantly decreased pmTOR-Ser^{2448} and -Ser^{2481} levels, and p70/S6K at Thr^{389} levels, which is the site phosphorylated by mTOR (Fig. 16A and 16B).

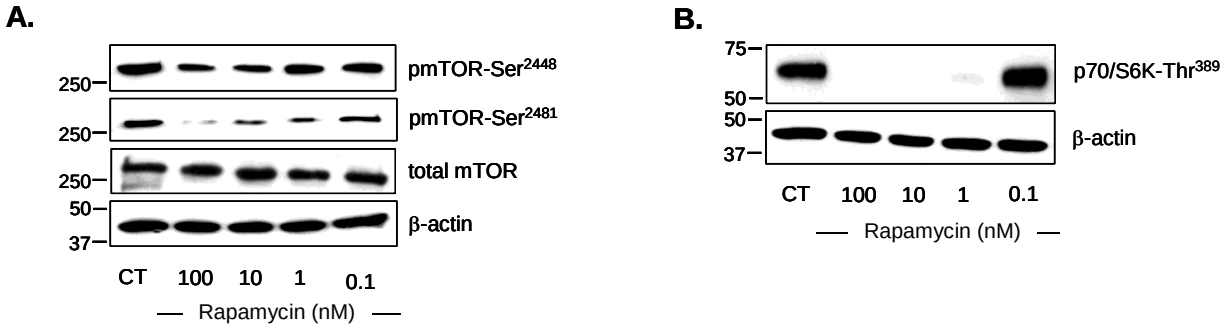

Fig. 16. GH3 cell lysates were treated with 100, 10, 1 and 0.1nM of rapamycin for 24 h. Protein expression levels were examined by Western blotting using anti-p70/S6K-Thr^{389}, -total p70/S6K, -mTOR-Ser^{2448}, -mTOR-Ser^{2481}, and –total mTOR. Equal protein loading was examined by detection of β-actin. Treatments were contacted in the presence of 10% FCS. Representatives of three experiments are shown.

In GH3 cells octreotide treatment also decreased p70/S6K and mTOR protein (Fig. 17). Therefore, the lack of additive/synergic antiproliferative action can be attributed to the fact that the two agents utilize the same pathway.

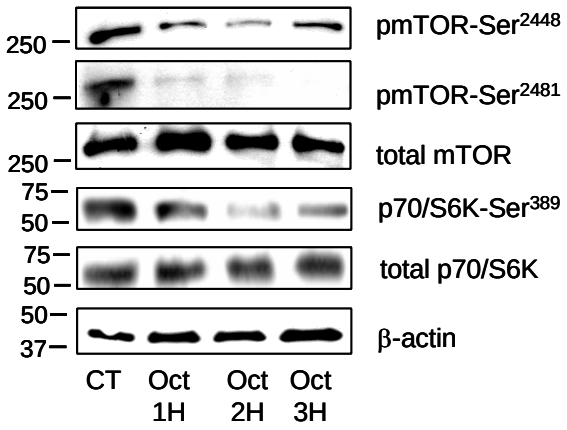

Fig. 17. GH3 cell lysates were treated with 1 nM octreotide for 1, 2 and 3 h. Protein expression levels were examined by Western blotting using anti-p70/S6K-Thr^{389}, -total p70/S6K, -mTOR-Ser^{2448}, -mTOR-Ser^{2481}, and –total mTOR. Equal protein loading was examined by detection of β-actin. Treatments were contacted in the presence of 10% FCS. Representatives of three experiments are shown.

Effect of rapamycin on cell cycle components

The early G1 phase of the cell cycle is marked with the synthesis of D-type cyclins and their association with Cdk4 and Cdk6. Various studies highlighted the reduction in cyclin D1 protein levels as a marker for rapamycin-mediated inhibition of the G1/S cell cycle progression (Law et al., 2006; Grewe et al., 1999; Hashemolhosseini et al., 1998). However, no changes in cyclin D1 protein level following 24 h treatment with rapamycin were observed in GH3 cells, but cyclin D3 expression was decreased (Fig. 18A), implying a role for cyclin D3, but not cyclin D1, in rapamycin-induced cell cycle arrest. In GH3 cells Cdk4 and Cdk6 levels were not affected by rapamycin treatment, but p21/Cip was downregulated (Fig. 18A). Complex formation between Cdk4/Cdk6 and D-type cyclins is stabilized by binding of the p21/Cip1 (LaBaer et al. 1997), therefore rapamycin-induced p21/Cip1 suppression may lead to complex destabilization and Cdk activity inhibition.

Indeed, rapamycin treatment decreased phosphorylated Rb-Ser^{780} levels (Fig. 18A) and E2F transcriptional activity ($P<0.001$, Fig. 18B). Subsequently, the E2F-regulated cyclin E and Cdk2 levels were suppressed (Fig. 18A).

Cyclin E/Cdk2 activity is inhibited by the Cdk inhibitory protein p27/Kip1. p27/Kip1 was reported as an important mediator of rapamycin's induced cell cycle arrest (Luo et al. 1996). However, GH3 cells do not express p27/Kip1 (Qian et al. 1996), but they still respond to rapamycin treatment. These data suggest that the inhibitory action of rapamycin in GH3 cells is

exerted by lowering the availability of cyclin E-Cdk2 complex units rather than interfering with its activity.

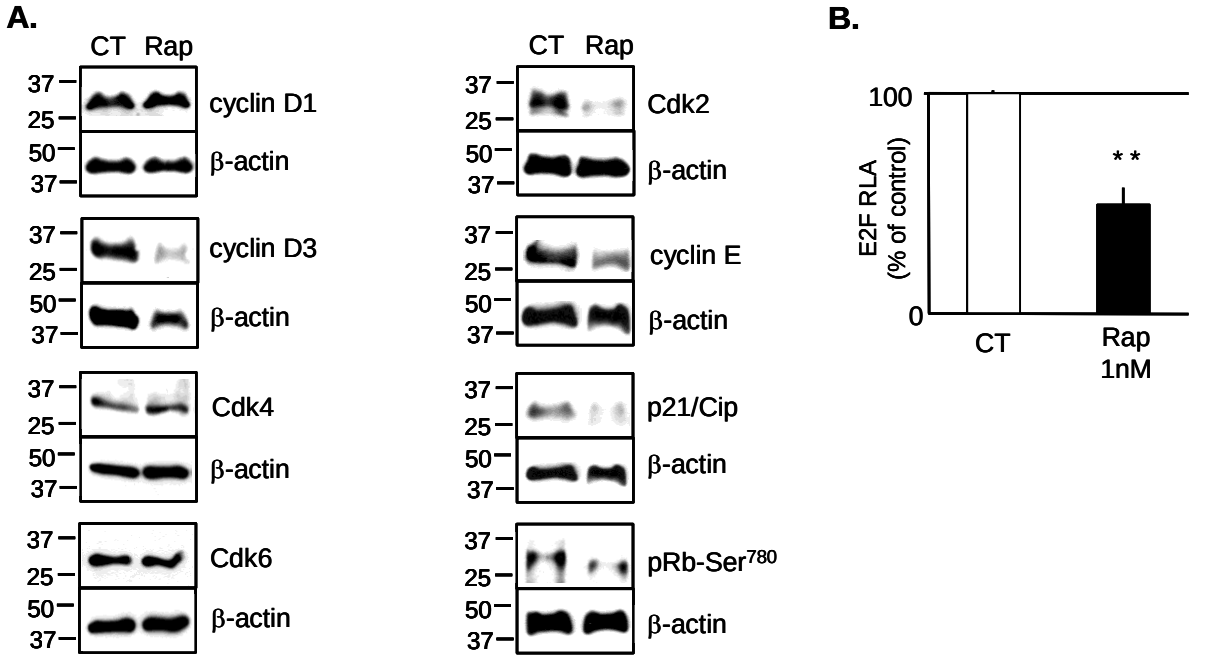

Fig. 18. GH3 cell lysates were treated with 1 nM of rapamycin for 24 h. Protein expression levels were examined by Western blotting using (A) anti-cyclin D1, -cyclin D3, - cyclin E, -Cdk2, -Cdk4 , -Cdk6, -p21/Cip and anti- pRb- Ser^{780}. Equal protein loading was examined by detection of β-actin. Cells were serum deprived for 24 hours before treatment. Treatments were contacted in the presence of 10% FCS. Representatives of three experiments are shown. (B) GH3 cells transfected with E2F-Luc were treated with 1 nM rapamycin for 6 hours. **: P<0.001. RLA: relative luciferase activity. Results are shown as E2F-RLA: βGAL ratio. Each experiment was repeated twice.

Effect of rapamycin in human acromegalic tumors in primary cell culture

GH3 cells are GH-secreting and considered as in vitro model for ACRO. Taking into consideration that they respond well to rapamycin, action of the drug was studied in 5 human acromegalic tumors in primary cell culture. Treatment with 1nM rapamycin for 24 h decreased cell proliferation by more than 20% in all but one tumor samples (% mean suppression: 70±5, Fig. 19A). In four cases where tumor tissue yielded enough cells for GH secretion studies, all cases, apart from one, showed a clear increase in hormone production. These data reveal that in human acromegalic tumors in primary cell culture, rapamycin is able to inhibit tumor cell growth, but fails to interfere and block GH synthesis (Fig. 19B).

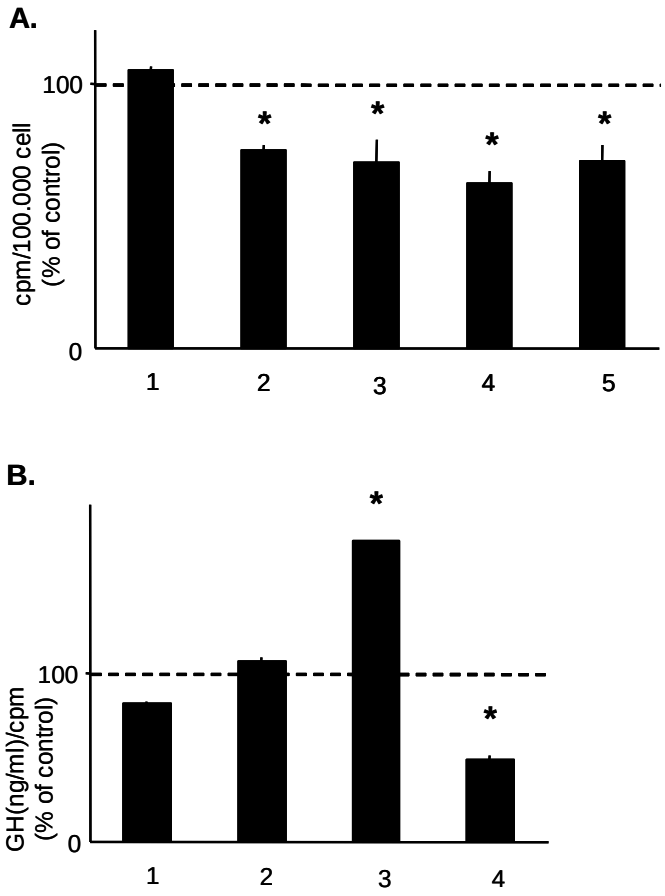

Fig. 19. (A)[^{3}H]-TdR uptake inhibition and (B) GH secretion in human acromegalic tumors in primary cell culture treated with 1nM rapamycin. Treatments were performed in 10% FCS-DMEM supplemented with D-valine. Cell proliferation and GH secretion levels were determined after 24 hours treatment. Data are presented as percentage of each vehicle-treated control. Each measurement was done in four independent wells. *: $P<0.05$

BEZ235 TREATMENT IN IMMORTALIZED PITUITARY TUMOR CELLS AND HUMAN PITUITARY ADENOMAS

Antiproliferative action of BEZ235 treatment in AtT-20 cells

In the first part of the result section resistance to rapamycin treatment in AtT-20 cells was overcome by blocking the PI3K/Akt pathway upstream with octreotide. The pan-PI3K/mTOR inhibitor, BEZ235, inhibits both the PI3K and mTOR kinase activity and blocks the activation of the PI3K/Akt pathway up- and downstream.

In AtT-20 cells BEZ235 treatment reached a significant antiproliferative effect at the doses of 100nM and 10nM (% of suppression: 31 and 25) and had no effect at lower doses of 1nM and 0.1nM (Fig. 20).

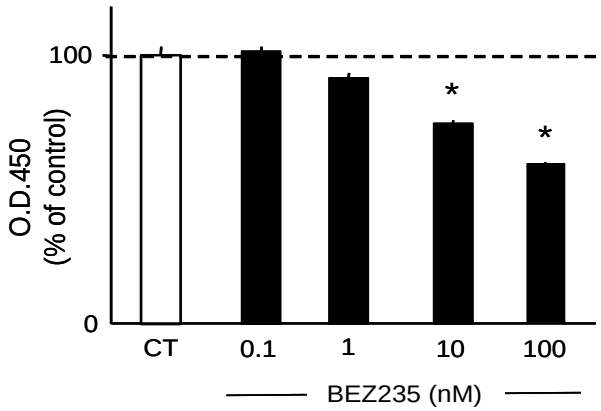

Fig. 20. Antiproliferative effect of 100, 10, 1 and 0.1nM BEZ235 in AtT-20 cells. Treatments were performed in 10% FCS. Cell proliferation was determined after 24 hours treatment. Data are presented as percentage of each vehicle-treated control. Each measurement was done in five independent wells. *: P<0.05.

Effect of BEZ235 on phosphorylation of Akt, mTOR and p70/S6K

BEZ235 inhibited pAkt-Ser, p70/S6K and mTOR, similar to previous reports (Maira et al., 2008; Serra et al., 2008; Marone et al., 2009). The effects were observed at the 100nM and 10nM concentration, reflecting the patern of the BEZ235 antiproliferative action (Fig. 21).

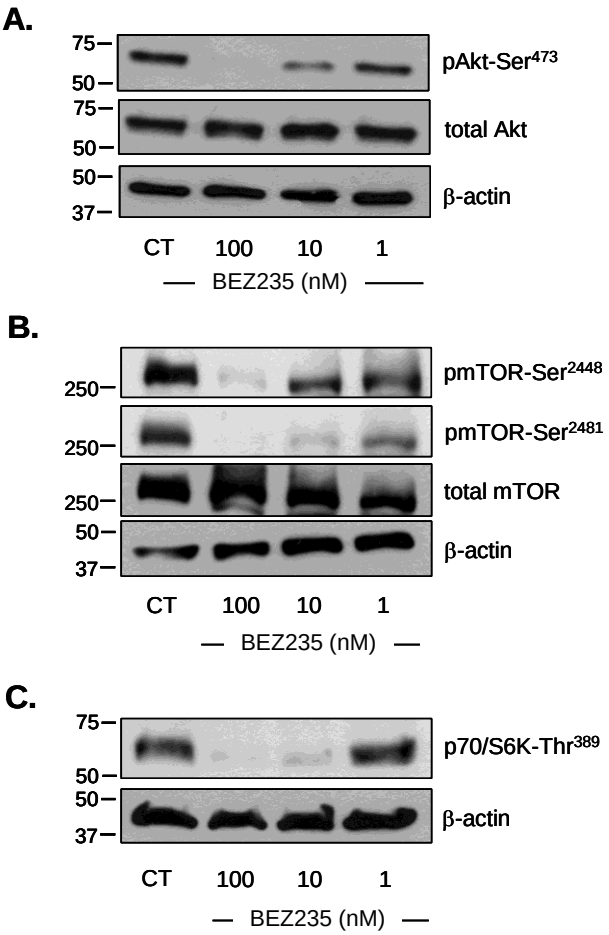

Fig. 21. AtT20 cell lysates were treated with 100, 10, and 1nM of BEZ235 for 24 h. Protein expression levels were examined by Western blotting using (A) anti-pAkt-Ser^{473}, -total Akt, (B) -mTOR-Ser^{2448}, -mTOR-Ser^{2481}, –total mTOR and (C) -p70/S6K-Thr^{389}, -total p70/S6K. Equal protein loading was examined by detection of β-actin. Treatments were contacted in the presence of 10% FCS. Representatives of three experiments are shown.

Effect of BEZ235 on cell cycle components

BEZ235 accumulated AtT-20 cells in G1 phase of the cell cycle (Fig. 22A), similar to what was observed in other tumor cells (Maira et al., 2008; Marone et al., 2009). BEZ235 decreased cyclin D1 without affecting Cdk2 and 4 protein levels. Cyclin D3 expression was reduced only with the highest BEZ235 dose (Fig. 22B). Similarly, p21/Cip1 was downregulated only at the high 100nM dose.

Nevertheless, E2F transcriptional activity was reduced to half after treatment with 10nM BEZ235, reflecting Rb activation and reduced Cdk activity (Fig. 22C). Furthermore, BEZ235 decreased the cyclin E and Cdk2 protein levels downstream to E2F (Fig. 22D). In addition, BEZ235 increased the levels of the Cdk inhibitor p27/Kip1. Therefore BEZ235 may induce G1 arrest by decreasing cyclin D1 and cyclin D3 availability, decreasing E2F-driven cyclin E and Cdk2, while at the same time increasing p27/Kip1 expression.

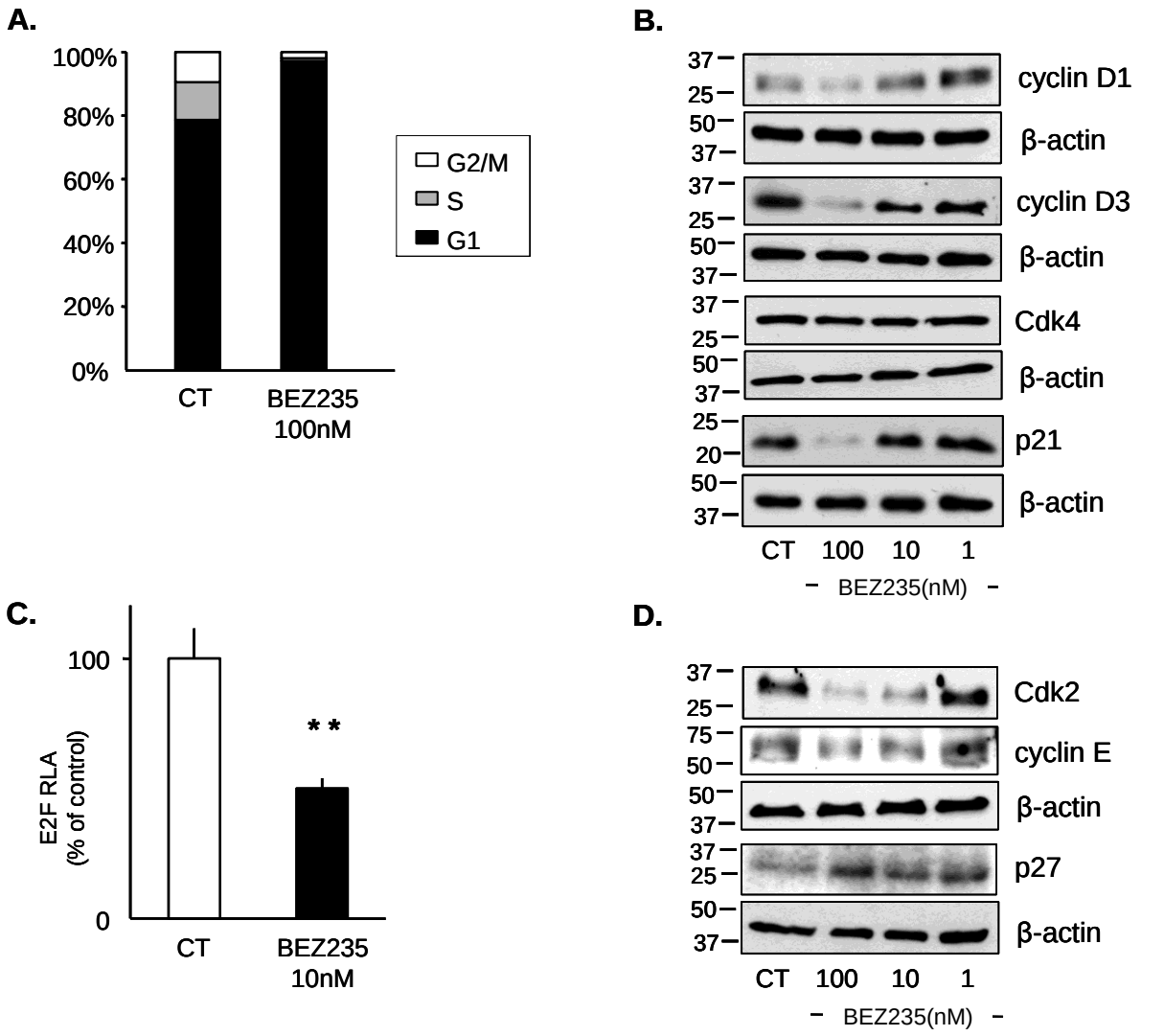

Fig. 22. (A) Cell cycle content of AtT-20 cells incubated with vehicle or 100nM BEZ235 for 24 hours was analysed by FACS. (B) AtT-20 cell lysates were treated with 100, 10 and 1nM of BEZ235 for 24 h. Protein expression levels were examined by Western blotting using anti-cyclin D1, - cyclin D3, - Cdk4, and - p21/Cip1; (C) AtT-20 cells transfected with E2F-Luc were treated with 10nM BEZ235 for 6 hours. **: P<0.001. RLA: relative luciferase activity. Results are shown as E2F-RLA: βGAL ratio. Each experiment was repeated twice. (D) AtT-20 cell lysates were treated like in (B). Protein expression levels were examined by Western blotting using anti-p27/Kip, -Cdk2, and -cyclin E. Equal protein loading was examined by detection of β-actin. Cells were serum deprived for 24 hours before treatment. Treatments were contacted in the presence of 10% FCS. Representatives of three experiments are shown.

Effect of BEZ235 on cell apoptosis

BEZ235 was described to also exert an apoptotic effect (Brachmann et al., 2009). Apoptosis is a so-called programmed cell death in response to biochemical and environmental signals. Apoptosis is mediated by the aspartate-directed proteases caspases (Earnshaw et al., 1999), which act by cleaving the poly (ADP-ribose) polymerase (PARP), an important protein for DNA repair and cell viability (Satoh et al., 1992; Lazebnik et al., 1994; Cohen et al., 1997). Cleaved PARP looses the ability to repair DNA damage and is therefore a marker of cells undergoing apoptosis (Oliver et al., 1998).

Treating AtT-20 cells for 24 h with various BEZ235 concentrations did not change cleaved or total PARP levels (Fig. 23). These data demonstrate that BEZ235 does not induce apoptosis in pituitary tumor cells.

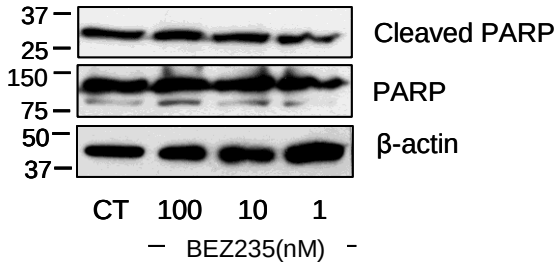

Fig. 23. AtT-20 cell lysates were treated with 100, 10 and 1 nM of BEZ235 for 24 h. Protein expression levels were examined by Western blotting using anti-cleaved PARP and anti-PARP. Equal protein loading was examined by detection of β-actin. Cells were serum deprived for 24 hours before treatment. Treatments were contacted in the presence of 10% FCS. Representatives of three experiments are shown.

Antiproliferative action of BEZ235 treatment in NFPA in primary cell culture

The antiproliferative efficacy of BEZ235 treatment was tested in 41 NFPA in primary cell culture. Summarizing the data, treatment with 1, 10 and 100nM BEZ235 in NFPA decreased proliferation in dose-dependent manner by more than 20% in all but one cases (Fig. 24A). In detail, 37 out of 41 tumors responded to 100nM (% mean suppression: 70±22), 34 out of 41 were responsive to 10nM (% mean suppression: 60±15; Fig. 24B) and 13 out of 41 responded to 1nM (% mean suppression: 19±7). These data show a potent antiproliferative action of BEZ235 in human NFPA in primary cell culture, not only at the high 100nM dose, but also at 10-fold lower concentration of 10nM.

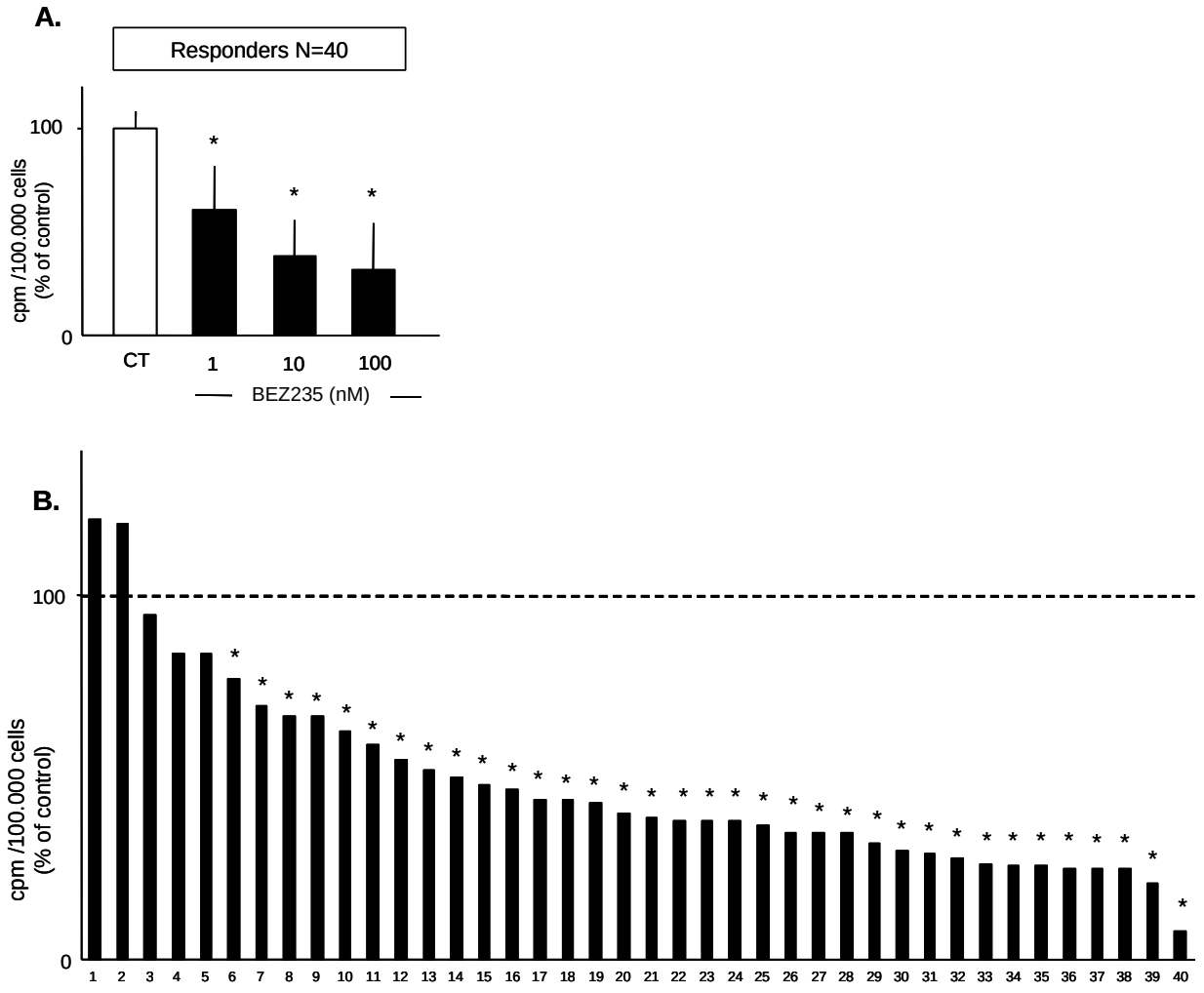

Fig. 24. (A) [^{3}H]-TdR uptake inhibition in human NFPA in primary cell culture treated with 1, 10 and 100nM BEZ235. (B) [^{3}H]-TdR uptake inhibition in human NFPA in primary cell culture treated with 10nM BEZ235 (% mean suppression 52±26). Cell proliferation was determined after 24 hours treatment. All treatments were performed in 10% FCS-DMEM supplemented with D-Valine. Data were calculated as mean±SEM and are expressed as percentage of vehicle treated control. Each measurement was done in four independent wells. *: P<0.05.

5 DISCUSSION

Pituitary adenomas are benign neoplasms that are accompanied by considerable problems due to their size and subsequent intracranial mass effects. The majority of invasive pituitary adenomas are the NFPA. They are clinically silent and often not detected due to the absence of the endocrine syndrome. Up to now there is no successful pharmacological treatment, thus surgery constitutes the primary treatment for NFPA, frequently followed by radiotherapy (Jaffe et al., 2006). Proper pharmacological treatment able to decrease the tumor mass could spare the patients from surgery and its subsequent side effects. Endocrinologically active pituitary adenomas, such as, prolactinomas and acromegaly associated pituitary tumors may also present with tumor mass effects. Prolactinomas are the most frequently occurring pituitary tumor type, while acromegaly is more frequent than previously thought, accounting to 1034 per million according to recent studies (Schneider et al., 2008). Prolactinomas are usually successfully managed with dopamine agonist treatment. Although highly effective in controlling PRL secretion and inducing prolactinoma tumor shrinkage, these agents are not successful in controlling hormone secretion and growth in acromegaly and NFPA. Patients with acromegaly are primarily managed with the SSA octreotide and lanreotide, which achieve biochemical control (i.e. GH and/or IGF-I normalization) in more than 50% of patients (Bevan et al., 2002). SSA also induce tumor shrinkage in 40% of acromegalic patients, indicating a cytostatic action for these agents. However, 60% of acromegalic pituitary adenomas and most NFPA do not show growth impairment after SSA treatment, despite the presence of somatostatin receptors in these tumors (Shomali & Katznelson 2002; Colao et al., 2003; Chanson et al., 2005; Freda et al., 2005; Melmed et al., 2005; Besser et al., 2005; Tichomirowa et al., 2005).

Somatostatin and its analogs bind to five receptor types belonging to the seven transmembrane domain G protein-coupled receptors and limit cell growth by inhibiting the proliferative activity of growth factor receptors (Patel et al., 1999; Hofland & Lamberts, 2004). SSA exert their antiproliferative action by inducing G0/G1 cell cycle arrest and apoptosis (Cheung & Boyages 1995; Srikant et al., 1995). In pituitary tumor cells octreotide suppresses the PI3K/Akt survival pathway (Bousquet et al., 2006; Theodoropoulou et al., 2006). In several cancers the PI3K/Akt pathway is of big importance not only in their oncogenesis and progression, but also in chemotherapy and radiation resistance. Herein it is shown that overexpressing Akt in AtT-20 pituitary tumor cells, that are able to respond to octreotide treatment, rendered these cells resistant to the antiproliferative action of this analog. This observation suggests that

overactivation of the PI3K pathway could also be responsible for SSA resistance in pituitary and other Sstr2-bearing tumor types. Indeed pituitary tumors and especially NFPA were found to display high levels of phosphorylated, i.e. activated, Akt when compared to the normal pituitary (Musat et al., 2005). In oncology, tumors with overactivated PI3K/Akt pathway are sensitive to treatment with the mTOR inhibitor rapamycin and its analogs (rapalogs). Herein, addition of rapamycin to octreotide treatment in NFPA in primary cell culture resulted in significant antiproliferative response in all cases. As predicted, most NFPA did not respond to single octreotide treatment, reflecting previous *in vitro* observations (Florio et al., 1999). Interestingly, the potency of the combined treatment was not due to a strong antiproliferative action of rapamycin since the data derived from single rapamycin treatment revealed poor drug response and subsequent rapamycin resistance. This observation highlights the possibility that octreotide may also sensitize tumor cells to the cytostatic action of rapamycin.

Rapamycin resistance is an increasingly occurring phenomenon also seen in other tumor types. Recent studies have revealed that treatment of patients with breast cancer with the rapamycin analog RAD001 resulted in resistance to this treatment (O´Reilly et al., 2006). Searching for the mechanisms responsible, it was found that tumor biopsies from patients treated with RAD001 have increased phospho-Akt-Ser^{473} immunoreactivity, suggesting that mTOR inhibition results in Akt activation. In fact, subsequent studies revealed that mTOR phosphorylates p70/S6K which in turn phosphorylates and deactivates the IRS-1 laying upstream to PI3K and Akt, providing a negative feedback loop. Hence rapalogs by blocking mTOR release the inhibitory feedback eventually resulting in Akt activation and increased cell survival. Theoretically, inhibition of the PI3K/Akt pathway upstream to mTOR could relieve the rapamycin-induced elimination of the negative feedback loop and enhance rapamycin's action. Indeed, addition of small molecule inhibitors against PI3K (Shi et al., 2005; Sun et al., 2005) or IGF-IR (Um et al., 2004; O'Reilly et al., 2006) reversed the rapamycin-induced increase in Akt phosphorylation and facilitated its efficacy.

As is evident in the present study, co-treatment with octreotide had a similar effect on rapamycin's antiproliferative action. As previously stated rapamycin by inhibiting p70/S6K decreases the IRS-1 inhibitory serine phosphorylation, resulting in IRS-1 activation (Mothe et al., 1996; Paz et al., 1999; Haruta et al., 2000; Um et al., 2005). Activated Sstr2, to which octreotide binds with the highest affinity, was found to suppress IRS-1 tyrosine phosphorylation and inhibit its action in a mechanism involving SHP-1 (Bousquet et al., 2006). Herein it is shown that octreotide in addition to suppressing the stimulatory IRS-1 tyrosine phosphorylation, it also increases the IRS-1 inhibitory serine phosphorylation and especially at the site primarily

phosphorylated by p70/S6K. It is noteworthy that this effect was under the influence of the phosphotyrosine phosphatase SHP-1. Another study showed that related phosphotyrosine phosphatase (PTP2C) binds tyrosine- phosphorylated IRS-1 which in turn gets dephosphorylated and inhibited through this association (Rocchi et al., 1995). Whether SHP-1 regulates IRS-1 in a similar way remains to be explored. Nevertheless the high basal levels of pIRS-Ser$^{636/639}$ in the cells transfected with the catalytically inactive SHP-1 imply a strong interconnection between changes in tyrosine phosphorylation and IRS-1 serine phosphorylation status.

In order to elucidate the mechanism behind the superior antiproliferative action of the combined treatment, cell cycle components were analyzed. The focus was on cyclins, Cdks and their inhibitors that act on the G1 to S transition, since the combined treatment arrested the cells in the G0/G1 phase. Rapamycin alone or in combination with octreotide decreased protein levels of cyclin D1/D3, Cdk4, Cdk6, and Cdk2 with no difference in the extent of the inhibition between the two treatments. However combined treatment suppressed Rb phosphorylation, E2F transcriptional activity and the E2F transcriptional target cyclin E more potently than rapamycin treatment alone. Both octreotide and rapamycin were previously shown to suppress cyclin E (Morice et al., 1993a, 1993b; Charland et al., 2001; Gorshtein et al., 2009), so this finding is not surprising.

Taken together, these results indicate a strong abrogation in Cdk2 activity which cannot be attributed solely to rapamycin-induced decrease in Cdk2. It is known that kinase activity of the cyclin E/Cdk2 complex is inhibited by the cyclin dependent kinase inhibitor p27/Kip1. The protein levels of p27/Kip1 were increased after individual treatments reflecting previous reports (Nourse et al., 1994; Pages et al., 1999). Nevertheless, combined treatment strongly enhanced p27/Kip1 expression, providing with an additional mechanism for its superior antiproliferative action at the level of the cell cycle. Indeed, cells in which p27/Kip1 was knocked down or do not express p27, like the GH3 cells, failed to better respond to the combined octreotide-rapamycin treatment. It is important to note that p27/Kip1 is a tumor suppressor that plays a significant role in pituitary tumorigenesis. p27 knock-out mice present with pituitary tumors (Nakayama et al., 1996) and p27 protein is dysregulated in many human pituitary adenoma types (Lidhar et al., 1999).

The extent of the p27/Kip1 upregulation after the combined octreotide-rapamycin treatment implies a role for this inhibitor as a downstream target of diverse signaling pathways. Octreotide's action on p27/Kip1 was SHP-1-dependend confirming previous studies in pituitary tumor cells (Theodoropoulou et al., 2006). This effect was abolished by Akt overexpression,

indicating that Akt overactivation may block the cytostatic action of octreotide in pituitary tumor cells. On the contrary, neither SHP-1 nor Akt conducted rapamycin's action; it was mediated through GSK-3β, a downstream target of p70/S6K (Zhang et al., 2006), which was previously found to be activated by rapamycin (Dong et al., 2005).

In contrast to NFPA, most acromegalic tumors in primary cell culture and the GH-secreting cell line GH3 responded to single rapamycin treatment. The limited number of cells isolated from these tumors did not allow investigating whether rapamycin plus octreotide may have a stronger antiproliferative action. Further analysis in GH3 cells revealed that rapamycin exerts its antiproliferative effect by inducing G1/S phase arrest. In other tumor cell types, as well as, in NIH 3T3 fibroblasts, rapamycin's cytostatic action was attributed to its suppressive effect on cyclin D1 protein (Hashemolhosseini et al., 1998; Grewe et al., 1999; Law et al., 2006). Actually, this effect on cyclin D1 is so important for rapamycin's action that cyclin D1 overexpression was able to abolish its antiproliferative effect on tumor cells (Law et al., 2006). In contrast, in GH3 cells rapamycin did not change cyclin D1 protein levels but suppressed cyclin D3. Similar action was also observed in other tumor cell lines (Strömberg et al., 2004; Hipp et al., 2005; Garcia-Morales et al., 2006; Wanner et al., 2006), providing an additional regulatory mechanism behind rapamycin's action.

Another factor important for sensitivity to rapamycin is the p27/Kip1 (Luo et al., 1996). However, in the present study the fact that GH3 cells lack endogenous p27/Kip1 (Qian et al., 1996) does not render them resistant to rapamycin treatment, indicating that rapamycin sensitivity is tumor cell type specific. In GH3 cells rapamycin downregulated p21/Cip1 protein levels, similar to what was reported in other studies (LaBaer et al., 1997; Gaben et al., 2004). p21/Cip1, in parallel to its inhibitory action on Cdk, is also acting as a positive regulator of Cdk4/6, by stabilizing its complexes with cyclin D. Therefore, in the p27/Kip1 negative GH3 cells, rapamycin reduces Rb phosphorylation and E2F-mediated transcription by downregulating cyclin D3 and p21/Cip1. These data imply an alternative way of action for rapamycin in p27/Kip1 negative tumor cells.

While the present study was ongoing, the combination of octreotide long-acting release (LAR) and the rapalog RAD001 (everolimus) went in phase II clinical trials for the treatment of advanced neuroendocrine tumors and was shown to be well tolerated with promising antitumor activity (Yao et al., 2008). However, clinical trials are needed to address the efficacy of the combined treatment in patients with NFPA and other neuroendocrine tumors. Interestingly two recent studies in gastroenteropancreatic neuroendocrine tumor cell lines, failed to show

improvement of rapamycin's antiproliferative action after addition of octreotide (Moreno et al., 2008; Grozinsky-Glasberg et al., 2008). The same outcome was observed in the GH3 pituitary tumor cells and was attributed to the lack of p27/Kip1 in these cells (Qian et al., 1996). Intrinsic differences in each individual tumor are suspected to be responsible for these discrepancies and future studies are essential to identify factors that may contribute to resistance to the beneficial effects of the combined treatment.

The inability of GH3 cells to better respond to the combined octreotide-rapamycin treatment suggests that the signaling cascades utilized by octreotide and rapamycin converge. Indeed, in these cells octreotide suppressed the phosphorylation of the rapamycin targets mTOR and p70/S6K. The inhibitory action of octreotide on the mTOR/p70S6K pathway reveals an unexpected mechanism through which this SSA can regulate cell growth. Indeed, SSA can induce tumor shrinkage in a percentage of patients with acromegaly, which is not due to apoptosis or necrosis but rather to decrease in individual cell volume (Melmed et al., 2005; Bevan et al., 2005). The mTOR pathway is the main regulator of cell growth, therefore octreotide and most probably other Sstr2 targeting SSA, may induce tumor shrinkage by suppressing this pathway.

The good response of acromegalic tumors to rapamycin's antiproliferative action prompted the analysis of the drug's effect of GH secretion, since successful acromegaly treatment relies not only on tumor shrinkage but also in complete biochemical control. Rapamycin did not suppress net GH secretion, i.e. normalized with cell viability, in most of the tumors studied. One of the possible explanations for this effect lies in the regulation of GH gene transcription and secretion. Two hypothalamic factors are mainly involved in GH management: growth hormone releasing hormone (GHRH) exerting stimulatory and somatostatin exerting inhibitory effects on GH secretion (Barinaga et al., 1985). Beside the hypothalamic factors, IGF-I plays an important role in GH regulation, being able to suppress basal and GHRH- induced GH gene transcription in primary culture of the rat pituitary cells *in vitro* (Morita et al., 1987). Influence of IGF-I on GH promoter activity was confirmed in a study using the GH-producing pituitary tumor cell line, MtT/S. Interestingly, this negative regulation was abrogated by the broad-spectrum tyrosine kinase inhibitor genistein and the PI3K inhibitor wortmannin, but not by a specific MAPK inhibitor and the mTOR inhibitor rapamycin, indicating involvement of the PI3K, but not of the MAPK or mTOR pathways in GH promoter downregulation (Melmed et al., 1996). Herein, the inability of rapamycin to suppress GH secretion from human GH secreting pituitary tumors in primary cell culture is in agreement with the previously reported *in vitro* observations and further confirms that mTOR is unlikely to contribute to the regulation of GH synthesis.

BEZ235 is a novel synthetic compound that inhibits the kinase activity of the PI3K and mTOR kinase by binding to their ATP pocket, and is therefore able to concomitantly target the PI3K pathway upstream and downstream. The antiproliferative efficacy of BEZ235 is evident in tumors with abnormal PI3K signaling due to loss of PTEN function or gain-of-function PI3K mutations (Maira et al., 2008; Serra et al., 2008; Cao et al., 2009). The overactivation of Akt seen in NFPA suggested that BEZ235 could also be beneficial for the management of this tumor type. Indeed, the present study demonstrates for the first time a potent antiproliferative action of BEZ235 on human NFPA in primary culture and in the immortalized pituitary tumor cell line AtT-20. In these cells no increase in pAkt-Ser^{473} levels was observed despite the abrogation of the p70/S6K phosphorylation, confirming that the negative feedback loop to Akt is not reactivated in BEZ235 treated cells as is the case with rapamycin treatment.

BEZ235 treatment decreased cyclin D1 and D3 protein levels. However, the protein expression of Cdk4, the respective binding kinase, did not change. Interestingly, lack of reduction in Cdk4 protein levels does not seem to be a determining factor in inhibiting the G1 phase at this level, since the p21/Cip1 protein, which was found to play an important role in stabilizing the cyclin D-Cdk4 complexes, was reduced in AtT-20 cells. Upon binding, Cdk4-cyclin D complex becomes active and induces partial phosphorylation of the Rb protein. Hypophosphorylated Rb triggers the release of members of the E2F family of transcription factors which promote the transcription of cyclins involved in progression to S phase of the cell cycle. One of the crucial G1/S transition point cyclins is cyclin E, which complexes with Cdk2. Activation of the cyclin E-Cdk2 complex promotes additional Rb phosphorylation and E2F promoted transcription leading the cells irreversibly into the S phase of the cell cycle. Cdk2 and other related kinases of the G1/S transition point are inhibited by p27/Kip1. Herein it is shown that BEZ235 induced p27/Kip1 was in parallel with the decrease in cyclin E and Cdk2 protein levels. The upregulation of p27/Kip1 upon BEZ235 treatment seen in pituitary tumor cells was also observed in other tumor types (Maira et al., 2008; Serra et al., 2008, Marone et al., 2009), suggesting an important role for this Cdk inhibitor in the drug's cytostatic action.

BEZ235 is cytostatic, but in some breast cancer cell lines it was described to also have apoptotic action (Brachmann et al., 2009). In immortalized pituitary tumor cells no apoptosis was observed at the concentrations used to treat human NFPA in primary cell cultures, as indicated by the absense of changes in cleaved PARP. PARP is important for cell viability and gets cleaved, and therefore deactivated, by death receptor induced caspases (Oliver et al., 1998). Accordingly, the absence of cleaved PARP in BEZ235 treated pituitary tumor cells indicates that the drug decreases cell proliferation without inducing apoptosis in this tumor type. Indeed,

BEZ235 treatment on other cancer types resulted in similar cytostatic but not apoptotic effect (Maira et al., 2008; Serra et al., 2008). In fact, the study reporting BEZ235 induced cell death showed apoptosis only in cell lines bearing PI3K activating mutations and/or HER2 gene amplifications, but not in those with activating EGFR mutations (Brachmann et al., 2009; Faber et al., 2009). Hence the absence of apoptotic events in pituitary tumor cells can be attributed to the fact that these tumors rarely present with PI3K mutations or HER2 gene amplifications (Ezzat et al., 1997; Lin et al., 2009).

The potent antiproliferative activity of the PI3K/mTOR dual inhibitor was also demonstrated *in vivo* on human cancer cells grown as orthotopic xenografts in nude mice (Maira et al., 2008; Cao et al., 2009). It is noteworthy that the tumor cell lines used in these experiments displayed Akt overexpression. Similarly gliomas, which bear PTEN deletions, also responded well to BEZ235 antproliferative treatment (Liu et al., 2009). In the present study almost all human NFPA cultured, responded remarkably well to BEZ235 treatment. It is known that these tumors have Akt overexpression. However, as they also present with hyperphosphorylated MEK1/2 and ERK1/2, as well as, altered expression of various growth factors and their receptors, the role of this Akt overactivation was not stressed. The strong response of NFPA to BEZ235 antiproliferative treatment, similar to that of other tumors with clearly characterized addiction to Akt overactivation, highlights the role of the PI3K/Akt pathway in the tumorigenesis of this type of tumor. Ongoing studies are elucidating the role of the putative Akt addiction in pituitary tumors and the intrinsic mechanisms leading to Akt overactivation. Nevertheless, the herein described results highlight BEZ235 as a promising agent for the better management of NFPA and other macroadenomas. BEZ235 is orally administered and well tolerated and has recently entered phase I/II clinical trial for the treatment of advanced breast cancer. Future clinical trials will show the growth limiting and tumor shrinkage capacity of BEZ235 in patients with pituitary macroadenomas.

In conclusion, targeting the PI3K/mTOR pathway in human pituitary tumors in primary cell culture reversed resistance to the antiproliferative action of agents currently used in neuroendocrine management and potently reduced cell viability. A remarkable exception was the resistance most of NFPA showed to rapamycin and this was managed with the concomitant administration of the SSA octreotide. Indeed, octreotide was found to block one of the mechanisms that are suspected to be responsible for rapamycin resistance by inhibiting IRS-1 and Akt upstream of mTOR. These data led to a novel therapeutic scheme that benefits not only pituitary but also other Sstr2 expressing tumors that are currently resistant to the

antiproliferative action of rapalog treatment. Finally, the present study provides with novel therapeutic agents and treatment combinations that could greatly improve the management of invasive pituitary tumors for which surgery, with all its side effects, is till now the only treatment option.

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PUBLICATIONS

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Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig und ohne unerlaubte Hilfe angefertigt habe. Ich habe weder anderweitig versucht, eine Dissertation oder Teile einer Dissertation einzureichen bzw. einer Prüfungskommission vorzulegen, noch mich einer Doktorprüfung zu unterziehen.

München, 01.03.2010

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