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The Effects of Temozolomide on Normal and Tumoral Pituitary Cells

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1. Abbreviations

ACRO	Somatotroph adenoma
ACTH	Adrenocorticotroph hormone
ADH	Antidiuretic hormone
BER	Base excision repair
BRG1	Brahma related gene 1
CDKN2A	Cyclin dependent kinase inhibitor 2A
$COCl_2$	Cobalt-II-chloride
CREB	c-AMP response binding element
CRH	Corticotropin-releasing-hormone
DMEM	Dulbecco's modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Desoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGF-R	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
EMA	European Medicines Agency
FDA	U.S. Food and Drug Administration
FCS	Fetal calf serum
FGF-2	Fibroblast growth factor 2
FGF-4	Fibroblast growth factor 4
FGFR4	Fibroblast growth factor receptor 4
FSH	Follicle-stimulating hormone
fT3	Free triiodothyronine
fT4	Free thyroxine
GADD45	Growth arrest and DNA damage gene 45
GH	Growth hormone
GHRH	Growth hormone releasing hormone

GnRH	Gonadotropin releasing hormone
GSα	G-protein subunit α
Hif-1α	Hypoxia inducible factor 1α
HMGA2	High mobility group AT-hook 2
HRP	Horseradish peroxidase
IGF-1	Insulin like growth factor 1
KIP1	Kinesin like protein 1
LH	Luteinizing hormone
MEG3	Maternally expressed gene 3
MGMT	Methyl guanine methyl transferase
MRI	Magnetic resonance imaging
MSH6	Mutator S homolog 6
MSH2	Mutator S homolog 2
MTIC	Monomethyl triazenoimidazole carboxamide
NFPA	Non-functioning pituitary adenoma
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PRL	Prolactin
PTAG	Pituitary tumor apoptosis gene
PTTG	Pituitary tumor transforming gene
ras	Rat sarcoma protein
Rb	Retinoblastoma
RIA	Radioimmunoassay
RIPA	Radioimmunoprecipitation assay
rpm	revolutions per minute
SDS	Sodium Dodecyl Sulfate
T3	Triiodothyronine
T4	Thyroxine
TEMED	Tetramethylethylenediamine
TGF-alpha	Transforming growth factor
TMZ	Temozolomide
Tp53	Tumor protein p53
TRH	Thyrotropin-releasing-hormone
Tris	Tris(hydroxymethyl)aminomethane
TSH	Thyroid-stimulating hormone
VEGF	Vascular endothelial growth factor

WST-1	Water soluble tetrazolium 1
ZAC	Zink activated channel

2. Background

2.1. The Pituitary Gland

2.1.1. Physiology of the Pituitary

The pituitary is a pea sized endocrine gland which is located in the sella turcica, a small cavity in the base of the scull. It produces a variety of hormones that act as key regulators for several important physiological functions. The secretion is regulated by the hypothalamus to which the pituitary is connected through the pituitary stalk. The gland is divided into two major parts: the anterior lobe and the posterior lobe. The anterior lobe is regulated by different hypothalamic hormones such as CRH, TRH, GHRH and GnRH, which reach the gland via a system of portal vessels. These hormones prompt the pituitary gland to secrete the respective hormones ACTH, TSH, GH, PRL, LH and FSH. TSH regulates the hormone secretion of the thyroid gland and ACTH stimulates the adrenal glands to produce steroids. GH acts as a universal growth factor in the total body, mainly through the production of insulin like growth factor 1 in the liver. PRL, LH and FSH regulate reproduction in both males and females. Figure 2.1 highlights the regulation of the anterior pituitary hormone secretion and the effects of the respective hormones. The posterior lobe, the neurohypophysis, stores and releases the hormones oxytocin and antidiuretic hormone. These two neuropeptides are synthesized by neurons in the hypothalamus and transported to the posterior lobe along their axons [1]. Upon stimulation by action potentials, the hormone-containing varicosities in the posterior lobe release their content via exocytosis [2]. Oxytocin plays a role in social behavior and it facilitates parturition and milk ejection in women [3]. Antidiuretic hormone leads to vasoconstriction and retention of water in the kidney. Additionally it is also enforcing the effect of CRH on corticotroph anterior pituitary cells [1]. In addition to the two major lobes, the pituitary gland also contains the rudimentary intermediate lobe, whose function in humans has not yet been completely understood [4].

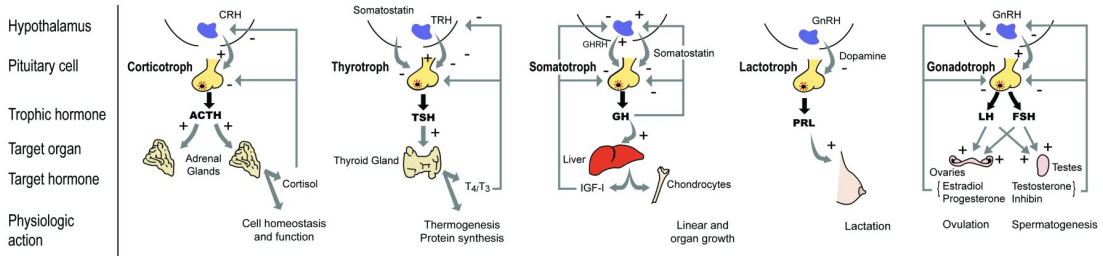

Figure 2.1. **Physiologic functions of the pituitary gland.**
Figure modified after [5]. Stimulation by the hypothalamic hormone CRH leads to secretion of ACTH, which stimulates the adrenal glands to produce cortisol, which in turn inhibits further CRH and ACTH release and plays an important role in regulating among other things the immunosystem, electrolyte balance and glucose metabolism. The hypothalamic hormone TRH stimulates the pituitary gland to secrete TSH, which promotes the secretion of T3 and T4 in the thyroid gland. T3 and T4 both regulate metabolism and cell growth and inhibit TRH and TSH secretion in a negative feedback mechanism. The hypothalamic hormone GHRH leads to pituitary secretion of GH. GH stimulates growth and IGF-1 production. High IGF-1 levels reduce GH and GHRH production as a negative feedback. PRL secretion is constantly inhibited by dopamine secretion and regulates lactation in women. The hypothalamic hormone GnRH stimulates LH and FSH secretion of the pituitary gland, which modulate the function of ovaries and testes.

2.1.2. Tumors of the Anterior Pituitary

Pituitary tumors account for approximately 13 % of all brain tumors [8]. The prevalence of pituitary tumors, determined by both radiographic and autopsy studies, has been estimated to be 16.7 % [9]. Data from the Swedish cancer registry suggest that the incidence of pituitary neoplasms has increased over an observation period of 33 years [10]. Typically, pituitary adenomas are benign tumors that don't metastasize and that may even be silent and detected only incidentally. However, there are some tumors that can lead to death through hormonal excess and there are some that demonstrate local aggressive behavior. The following sections will give an overview of these different tumors.

2.1.2.1. Tumorigenesis

Currently, there are two conflicting theories about the underlying mechanisms of pathogenesis of pituitary tumors. The hypothalamic hypothesis attributes tumor development to a prolonged stimulation by hypothalamic hormones or respectively the loss of hypothalamic inhibition on pituitary cells. This hypothesis is strengthened by the fact that hypothalamic hormones have been shown to enhance pituitary cell growth and to cause hyperplasia in animal models [11, 12]. However, it was shown that the tissue sur-

rounding the tumors is free of hyperplasia. Furthermore, recurrence rates after complete tumor removal are low [13]. These two observations seem to contradict the hypothalamic hypothesis. The second hypothesis for pituitary tumorigenesis suggests that adenomas derive from a single mutated cell, independent of hypothalamic stimulation. Monoclonality has already been demonstrated in a large number of pituitary adenomas by gene expression analyses [14, 15]. If hypothalamic stimulation was the cause for tumor development, one would expect the tumor to be polyclonal since the stimulation affects the entire gland. Besides that, mutations in genes like p53, Rb or Ras that are commonly found in other cancer types occur rarely in pituitary adenomas [16, 17, 18]. Hence, the details of tumorigenesis remain to be unvealed and other studies suggest a mechanism such as a multistep-wise progression where genetic alterations arise more likely in a fertile environment [19].

The occurrence of certain allelic deletions and genetic mutations in pituitary cells contributes to tumorigenesis. Allelic deletions have been shown to correlate with tumor invasiveness. 75 % of the deletions are seen in 1p, 10q, 11q13 and 13q12–14 [20]. Overexpressed oncogenes and silenced tumor suppressor genes associated with pituitary tumors are shown in table 2.1. The Pituitary tumor transforming gene (PTTG) is one of these factors and seems to play an important role in tumorigenesis. It was found to be overexpressed in pituitary tumors compared to normal pituitary tissue and it has been shown to correlate with invasiveness [21].

Oncogenes	**Tumor suppressor genes**
CREB	GADD45
Cyclin D1	Menin
EGF/TGF-alpha/EGF-R	p16/CDKN2A
FGF-2, FGF-4	p18
GSα	p27/KIP1
PTTG	Rb
FGFR4	Tp53
ras	MEG3
HMGA2	BRG1
	ZAC
	DAP kinase
	PTAG

Table 2.1. **Factors involved in pituitary tumorigenesis.**
Table modified after [6, 7] Listing is not exhaustive.

2.1.2.2. Subtypes of Pituitary Tumors

Pituitary adenomas can be classified into different subtypes according to their endocrine activity or immunohistochemical properties [22]. The prevalence of the adenoma subtypes as classified by immunohistochemistry was studied in a large series of post mortem pituitaries by Buurman et al. They found prolactinomas in 39,5 % of pituitary adenomas, corticotroph adenomas in 13,8 %, gonadotroph adenomas in 6,6 %, somatotroph adenomas in 2% and thyrotroph adenomas

in 0,6%. 22,5% were non-functioning adenomas, which do not stain for pituitary hormones [23]. In addition to that, many adenomas that secrete more than one hormone were reported. Most of them co-secrete growth hormone and prolactin. Anyway, when elevated prolactin levels are found, it does not always have to be due to a prolactin producing tumor. Instead, hyperprolactinemia is frequently found in patients with large pituitary adenomas. Compression of the pituitary stalk by large adenomas prevents the hypothalamus from inhibiting prolactin production, therefore leading to moderately elevated blood levels of prolactin. This phenomenon is called the "stalk effect". Recent research indicates that there must even be other factors than the "stalk effect" that influence the elevated prolactin levels in large non-prolactinomas. For example lactotroph stimulating factors that have not yet been identified are supposed to be secreted by tumors deriving from the "pars tuberalis" and mimick a "stalk effect" [24, 25].

Next to the specific effects of hormone secretion, all pituitary tumors have so called "mass effects" due to their size: The obstruction of blood flow to the pituitary gland often leads to hypopituitarism, which is observed in approximately 48% [26]. Besides that, pituitary neoplasms can cause considerable complications through the compression of the optic chiasm, which causes bilateral hemianopsia. An increase in intrasellar pressure and stretching of the meninges can furthermore lead to headaches [26]. The adenomas that cause these mass effects are usually greater than 10 mm in diameter and referred to as macroadenomas while smaller ones are named microadenomas.

In addition to the majority of the sporadic forms of adenomas, pituitary adenomas can also originate from genetic syndromes such as multiple endocrine neoplasia type 1 (MEN1), Mc Cune Albright Syndrome, Familial isolated pituitary adenomas and Carney complex [27].

Somatotroph Adenomas

Somatotroph adenomas are characterized by excessive secretion of growth hormone, which leads to the typical clinical picture of acromegaly in adults and very rarely to gigantism in children [28]. Acromegalic patients develop a characteristic facial appearance with a prominent forehead, prognathism and nasal bone hypertrophy. Often, patients observe an increase in ring or shoe size due to acral enlargement. Additionally, acromegaly is connected to a variety of other clinical features which are summarized in table 2.2. Because symptoms are so diverse and often unspecific, diagnosis is often delayed for many years [29]. A recent meta-analysis demonstrated that the mortality rate of acromegalic patients is increased by 72% compared to the general population [30].

System	Clinical feature
Bone and joint	Acral changes, gigantism, prognathism, arthritis, osteopenia, carpal tunnel syndrome
Heart	Cardiomyopathy, hypertension, arrhythmias, valvulopathy, heart failure
Thyroid	Goiter
Skin	Tags, excessive oily perspiration
Pancreas	Glucose intolerance, diabetes
Lung	Obstructive sleep apnea
Kidney	Antinatriuresis, fluid retention, increased aldosterone, renal failure
Gonads	Hypogonadism
Muscle	Proximal myopathy
Colon	Polyps
Fat	Lipolysis
Visceromegaly	Tongue, thyroid, salivary gland, liver, spleen, kidney, prostate

Table 2.2. **Clinical manifestations of acromegaly.**
Table modified after [31]. Listing is not exhaustive.

Lactotroph Adenomas

Adenomas that secrete prolactine, so called prolactinomas, represent the majority of pituitary neoplasms [23]. The most frequently encountered clinical manifestations include galactorrhea, amenorrhea, infertility and sexual dysfunction. Prolactinomas occur significantly more often in women than in men [32, 33]

Adrenocorticotroph Adenomas

Oversecretion of ACTH and consequently high levels of cortisol result in a variety of clinical symptoms, which are generally referred to as Cushing's disease. Patients have a characteristical fat distribution pattern. Fat pads arise particularly centripetal, such as in the abdominal region as well as dorsocervical and supraclavicular, causing the typical buffalo hump and a moon face [34]. Constantly elevated cortisol levels also cause numerous other symptoms that are shown in table 2.3.

Thyrotroph Adenomas

Thyrotroph adenomas are very rare tumors that produce an excess of TSH and accordingly stimulate growth and hormone production of the thyroid gland. 93% of patients develop a goiter and 70% present with thyroid nodules, but signs of hyperthyroidism are mostly moderate [36, 37]. Frequently, patients are misdiagnosed and 25% are erroneously

thyroidectomied [38].

Gonadotrop Adenomas

Clinical signs of an excess of hormone secretion are only rarely observed in gonadotroph adenomas. Instead, patients suffer from visual loss, symptoms of hypopituitarism and headache as consequences of the mass effects of the usually very large tumors [39]. Gonadotropinomas were reported to secrete functional or nonfunctional FSH or in rare cases LH, leading to different clinical pictures such as ovarian hyperstimulation syndrome in females or hypogonadism in males [40, 41]

Non-functioning Pituitary Adenomas

Clinically non-functioning pituitary adenomas (NFPA) represent an inhomogenous group of tumors. In fact, they are histologically different tumor entities which do not cause symptoms of hormonal oversecretion. Immunohistochemical analyses revealed that 64% of NFPA are silent gonadotroph adenomas, followed by 18% null cell adenomas and 12% silent corticotroph adenomas [42]. Although NFPA are considered clinically 'non-functioning' they often secrete hormones. A study with 22 NFPA showed that 19 were producing gonadotropins or subunits, but apparently this does not come to clinical presentation [43]. Instead, patients with NFPA usually present with symptoms related to compression of local structures. Besides that, NFPA are frequently seen as incidental findings on brain radiographs [26].

System	**Clinical feature**
Bone	Osteoporosis
Heart	Hypertension
Skin	Facial plethora, violaceus striae wider than 1cm, acne, hirsutism, thinning
Pancreas	Glucose intolerance, diabetes
Muscle	Proximal muscle weakness
Fat	Central obesity, sudden weight gain
Gonads	Decreased libido, impotence, menstrual disorders
Immune system	Recurrent opportunistic or bacterial infections
Psyche	Depression, psychosis

Table 2.3. **Clinical manifestations of Cushing's disease.**
Table modified after [35]. Listing is not exhaustive.

Atypical Pituitary Adenomas and Pituitary Carcinomas

In addition to the above mentioned typical pituitary adenomas, numerous tumors that demonstrate highly aggressive behavior are classified as atypical. Up to 55% of pituitary adenomas are reported to be invasive to local structures like the dura, bone and other surrounding tissues [44]. The clinical course of atypical pituitary adenomas is characterized by resistance to current treatment regimens and by frequent recurrence. The 2004 WHO classification defines atypical adenomas by invasive growth, a Ki-67 labeling index $> 3\%$, extensive p53 immunoreactivity and a high mitotic index [6]. While atypical adenomas are seen in even 15% of surgically treated tumors [45], pituitary carcinomas are still exceptional. Table 2.4 shows 179 published cases of pituitary carcinomas. Considering the data from table 2.4, the most prevalent carcinoma subtypes are adrenocorticotroph and lactotroph.

Tumor type	**Colao et al. 2010[46]**	**Since 2010**	**Total**
ACTH	61	11 [47, 48, 49, 50, 51, 52, 53, 54]	72
PRL	53	7 [55, 51, 53]	60
GH	9	1 [56]	10
FSH/LH	8	0	8
TSH	2	1 [57]	3
Non-functioning	17	9 [58, 59, 51, 60, 61]	26
			179

Table 2.4. **Reported cases of pituitary carcinomas**
Table adapted from [46].

For the diagnosis of a pituitary carcinoma, the presence of metastases is strictly required [6]. The sites of metastases are rather systemic than craniospinal [62]. The evolution of pituitary carcinomas is still uncertain. Different theories suggest previous radiotherapy, effects of surgery or transformation out of a benign adenoma to cause progression to malignity. Recent publications presented evidence to suggest the latter hypothesis [46, 44]. This is supported by the fact that the latency period between the presentation with an adenoma until the development of metastases varies from 4 months to 18 years (mean 6.6 years) [62].

The prognosis of metastasized pituitary tumors is poor. 66% of patients die within the first year after diagnosis [62]. In the past, considerable efforts were made to identify

markers for the prediction of malignant progression. Several markers like Galectin-3, increased expression of telomerase or Ki-67 labeling index above 10% have been suggested. Recently, repeated histopathological investigations in resected pituitary tumors revealed the loss of the MSH6 protein to be a factor of adenoma to carcinoma transformation in pituitary tumors under temozolomide treatment [55, 51]. Nevertheless none of those markers seems to be applicable to all carcinomas and so the exact mechanisms of progression to malignity remain unclear [46, 63, 6, 44].

2.1.2.3. Current Treatment Options for Pituitary Tumors

Pituitary tumors can be treated in many different ways, depending on their histological subtype and their clinical presentation. The aims of all treatment approaches are to i) reduce tumor mass, ii) normalize hormone levels, iii) recover normal pituitary function and iv) prevent the formation of metastases.

Surgery

Surgical treatment of pituitary neoplasms is the first line treatment for all pituitary neoplasms except prolactinomas. The advantages of a surgical intervention are an inexpensive and rapid relief from symptoms and the possibility to obtain tumor material for histological characterization [64]. Two techniques are currently performed: the transsphenoidal approach, which is preferred in 90 to 95% of cases and the more invasive transcranial approach [65]. The remission rates after transsphenoidal surgery vary between 54% and 70% depending on the criteria used for the definition of 'remission', the experience of the surgeon and the selection of adenomas [65]. Though the mortality rate in transsphenoidal surgery is quite low (0,9%), complications like (transient) anterior pituitary insufficiency and diabetes insipidus occur frequently (19,4% and 17,8%) [66]. Transcranial surgery is used in cases where size or extension of the tumor render a transsphenoidal approach impossible [67].

Radiotherapy

The goals of radiotherapy regarding the treatment of pituitary tumors lies primarily in the treatment of tumor recurrence as well as the removal of non-surgically resectable residues. 10 years after completion of radiotherapy, tumor growth was locally controlled in up to 98% of tumors, depending on the tumor type [68]. Effects of radiation therapy emerge only with a considerable time delay of 6 to 10 years on average [69]. Delivery of radiation can be carried out either as a high single dose treatment or it can be fractionated into many sessions with smaller doses. In terms of efficacy, both ways perform equally well [70]. The single dose approach, also called stereotactic radiosurgery, is more

convenient to the patient, as it requires only one visit, but achievement of hormonal control might be slow. The conventional fractionated radiotherapy appears to be superior in cases where the tumor lies close to the optic chiasm, because it is more gentle to the surrounding tissue [69]. Side effects of radiotherapy include development of hypopituitarism after several years, an increased risk for stroke and optic neuropathy. Around 50% of patients treated with radiotherapy for pituitary adenomas require hormone replacement therapy after 19 years [71].

Dopamine Agonists

Dopamine agonists mediate their antiproliferative and hormone suppressive effects via the D2 and D1 dopamine receptors of pituitary cells. The currently most used compounds are cabergoline, bromocriptin and quinagolid. Dopamine agonists are the treatment of choice in the therapy of prolactinomas. Cabergoline normalized prolactin levels in 86% of patients in a large study with 455 patients [72]. A reduction in tumor mass (i.e. more than 20% change to basal) was reported in more than 80% of patients [73]. Relief from compressive effects of the tumor is experienced after several days of treatment. In other types of adenomas, dopamine agonists show some effect, but their use is restricted to cases where other treatment options fail. In patients with growth hormone producing adenomas, cabergoline was proven to be effective in suppressing GH below 2µg/L in 46% and tumor shrinkage was observed in 13 of 21 patients [74]. In the case of corticotroph adenomas, a hormonal control rate of 40% after two years administration of cabergoline has recently been demonstrated [75]. In NFPA, dopamine agonists achieved a reduction of tumor size in approximately 27,6% of cases [76] whereas in thyrotroph adenomas, there is no clear evidence for a benefit [36]. The most common side-effects of dopamine agonist therapy are nausea and vomiting, postural hypotension and headache. Special attention must be given to the development of cardiac valve disease in very rare cases [77].

Somatostatin Analogs

Somatostatin analogs act mainly on the somatostatin receptor subtypes 2 and 5. The presently available drugs are octreotide, lanreotide and pasireotide. These drugs are mainly used in the treatment of acromegaly, in which case they were shown to suppress growth hormone levels below 2 µg/L in 60-70 % and to normalize IGF-1 levels in 50-80 % of patients [78]. Reduction of tumor size by somatostatin analogues varies between 20 and 70 % of acromegalic patients [78]. In patients with thyrotroph adenomas, a more than 30 % reduction in fT4 and fT3 blood levels could be achieved with octreotide in 7 of 8 patients [79]. In patients with NFPA, a decrease in tumor volume was seen in only

12 % [76] and in patients with prolactinomas, somatostatin analogs were not effective in lowering prolactin levels [80]. Only recently, a phase III trial with the somatostatin analog pasireotide for the treatment of Cushing's disease showed, that 50 of 103 patients had a reduction of more than 50 % of their baseline cortisol levels[81]. This strong effect on cortisol levels is attributed to the comparable high affinity of pasireotide to the somatostatin receptor subtypes 5, which is strongly expressed in adrenocorticotroph adenoma [82, 83]. The most common side effects of somatostatin analogs are gastrointestinal disturbances, gall stones and impaired glucose tolerance [84].

Other Methods Of Treatment

Besides the aforementioned established treatment options, several therapies have been applied to reduce the impact of symptoms: Pegvisomant is a growth hormone analog that blocks the peripheral action of growth hormone and it is used as a third line treatment in acromegaly[85]. For the treatment of Cushing's disease bilateral adrenalectomy and the use of inhibitors of steroidogenesis such as Ketokonazol, Metyrapone, Mitotane and the only recently discovered compound LCI699 represent third line treatment options that effectively control hypercortisolism [86, 87]. However, all these treatment options are directed towards the peripheral hormonal control and cannot control tumor size. In TSH secreting adenomas thyroidectomy and antithyroid drugs such as thiamazol and propylthiouracil were shown to reduce symptoms of hyperthyroidism.

2.2. Temozolomide

2.2.1. Metabolism

Temozolomide is an imidazotetrazine and stable in acidic solutions. Its pharmacological importance is based on its usage as a methylating agent that can methylate nucleic acids such as DNA. The molecular mechanism of action involves the formation of reactive methyldiazonium ion in aqueous solutions and the consecutive methylation of DNA by this reactive species (see figure 2.2). Temozolomide is activated spontaneously in aqueous solutions with physiological pH by hydrolysis of the tetrazine ring structure and the formation of monomethyl triazenoimidazole carbocamide (MTIC). Acidic catalyzed hydrolysis of MTIC forms a stable amino imidazole carboxamide (AIC) and a reactive methyldiazonium ion [88, 89]. This reactive species contains an electrophilic methyl group that reacts with nucleophilic groups, such as O6 and N7 of guanosine as well as N3 of adenosine (see figure 2.2). Due to the above described molecular mechanisms, temozolomide possesses properties that render it well-suited for medical applications. As

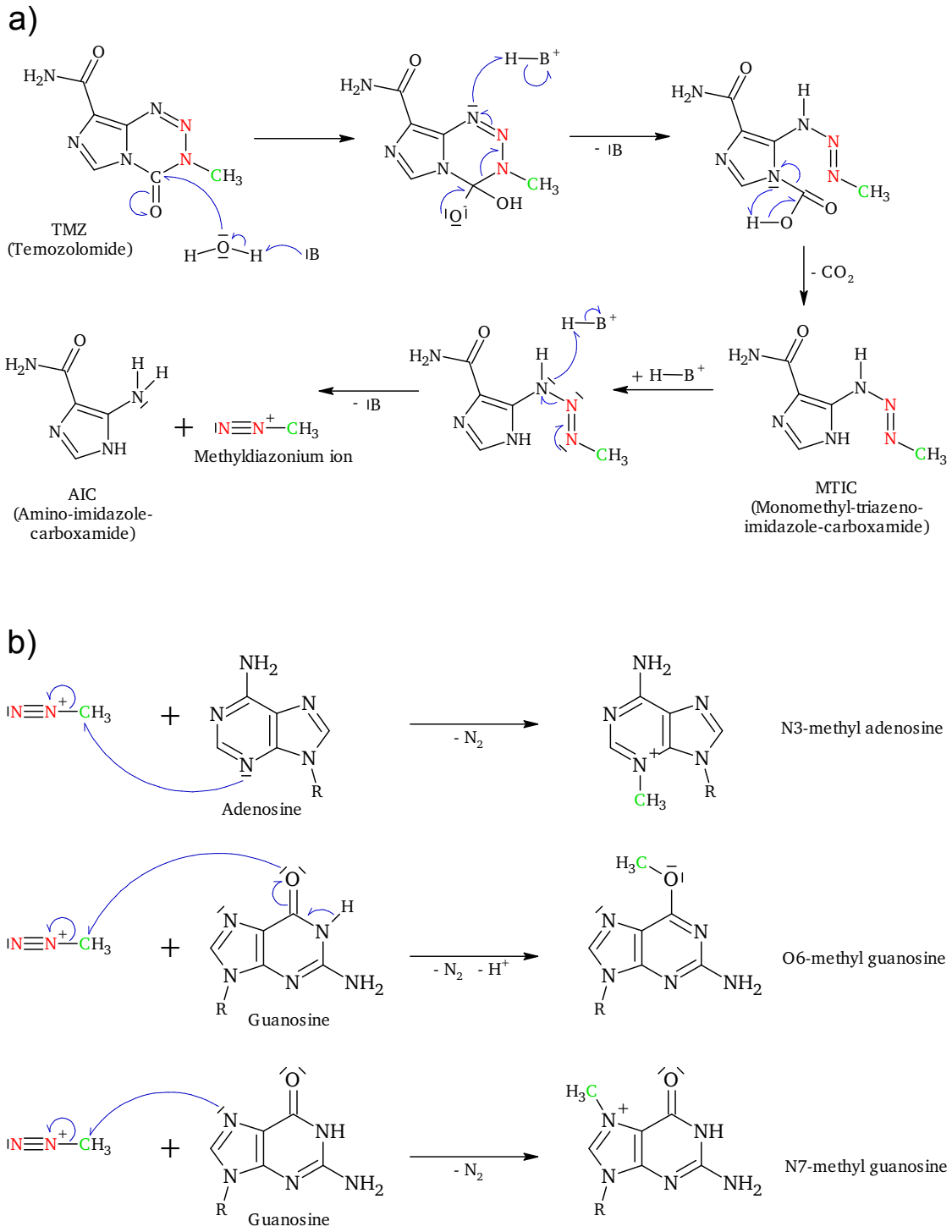

Figure 2.2. **DNA methylation by temozolomide**
a) Temozolomide is activated upon reacting with water leading to the release of the reactive species, methyldiazonium ions. b) The released methyldiazonium ions methylate various biomolecules, among which are different DNA bases. Here, the methylations of adenosine and guanosine are illustrated.

the first step of activation is inefficient in acidic solutions, temozolomide is not affected by the stomach passage and can thus be given orally. In contrast to other methylating agents, such as the structural analogue dacarbazine, the formation of MTIC occurs spontaneously and, in particular, does not require hepatic metabolism. Furthermore, studies of temozolomide have shown promising biokinetic properties such as an oral bioavailabilitiy of 100 % [90], a time to maximal plasma concentration of approximately 1 h (0,33-2,5 h) and a serum half-life of 109 min [91]. Considering that the simultaneous consumption of food results in a 9 % reduction of absorption [92], the drug should consequently be taken in a fasting state. The biodistribution of temozolomide is satisfactory as it crosses the blood-brain barrier readily and reaches concentrations of 2,9 to 6,7 µg/ml in human brain tumors [93]. The main excretion of temozolomide occurs via the urine, wherein 5,5 % of the dose are excreted as the original compound, 12 % as AIC and the rest in smaller degradation products [94]. As schedule dependency of temozolomide treatment due to depletion of MGMT has been demonstrated [95, 96], different treatment schedules have been suggested so far [97]. Most studies apply the schedule proposed in the phase 1 trial: Temozolomide is given in a 5 day course with a daily dose of 150 mg/m^2 in the first course. If there is no considerable myelotoxicity observed at day 22 of treatment, the following courses are given with 200 mg/m^{2}daily dose. Cycles of treatment are repeated every 4 weeks [90]. Other possible schedules include dose dense regimens with comparable low doses (50-150 mg/m^2) of temozolomide for longer time periods (7-28 subsequent days) [98].

2.2.2. Cellular Mechanism of Action

Temozolomide is an alkylating drug and its application leads to several products of DNA methylation (see figure 2.3). The methylation sites of temozolomide are N7 of guanin, N3 of adenine and O6 of guanine and methylation efficiency was found to be 70 %, 9.2 % and 5 %, respectively [89]. Like every other DNA damage, DNA methylation severly impacts cellular processes such as DNA replication. Consequently several DNA repair mechanisms can be found in human cells. The repair efficiency of the different mechanisms determines the severity of the respective DNA damage. In the context of temozolomide induced methylation, three different repair mechanisms need to be considered.

Base Excision Repair

The lesions at the N3 of adenine and the N7 of guanine, which represent the major part of the methylated bases, are removed completely by the base excision repair [99].

Consequently, methylation at these sites is cytotoxic only in cases where base excision repair is defective [100] or saturated by very high concentrations of temozolomide [101]. Potentiation of the cytotoxicity of these lesions can be achieved by inhibition of the base excision repair.

Mismatch Repair

The methylation at the O6 site of guanine is the main cause for the cytotoxicity of temozolomide, although it represents only 5 % of the methylated bases [102]. Methylated guanine mispairs with thymine instead of cytosine because this pairing provides less distortion to the DNA strand [103]. In the first replication phase, the O6-methylguanine-cytosine pair is readily replicated, resulting in an intact double strand carrying a G/C pair and the methylated strand carrying the O6-methylG/T pair. In the second S-phase, the mismatch repair removes the mispaired thymine as well as several up- and downstream nucleotides before a new complimentary strand is synthesized [104] (see figure 2.3). This effort is doomed to failure, because no perfectly matching base can be found for the O6-methylguanine [105]. Consequently the mismatch repair repeatedly attempts to fill the gap and replication of the DNA is unable to advance. In mismatch repair proficient cells, increased DNA synthesis is observed, reflecting the futile attempt of mismatch repair in processing the methylated base [106]. As a result, O6-methylation of guanine leads to G:C to A:T transitions, single- and double-strand breaks, sister chromatid exchanges and eventually to a stop of DNA replication and

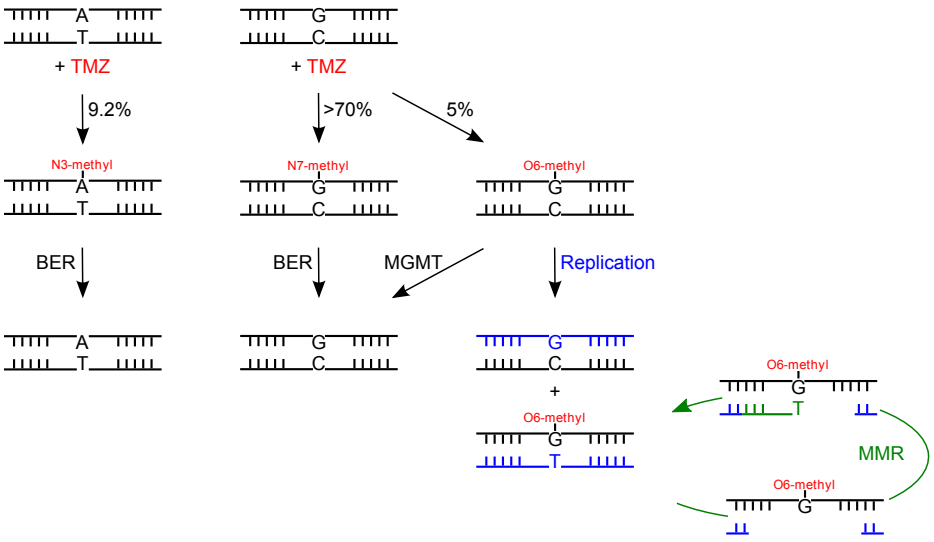

Figure 2.3. **Mechanisms of DNA repair**
BER refers to Base excision repair and MMR to Mismatch repair.

apoptosis [107]. Comparative studies in mismatch proficient and deficient cell lines show that cells with a non-functional mismatch repair are more tolerant to effects induced by O6-methylguanine lesions [106, 108].

Alkyltransferase-Mediated DNA Repair

A third repair mechanism relies on the enzyme methyl guanine methyl transferase (MGMT), an alkyltransferase that is involved in the removal of the methyl group at the O6 position of guanine. Although expression of MGMT is ubiquitous, expression levels vary between the investigated tissues and among individuals [109]. MGMT transfers the methyl group to an internal cysteine [110]. This step is irreversible and leads to the ubiquitination and depletion of the enzyme after prolonged temozolomide administration [96, 111]. The presence of high levels of MGMT can therefore promote tolerance to temozolomide treatment, because the toxic lesion is removed by the enzyme.

In a study by Wang et al. 86,3 % of 197 pituitary tumors were found to have low MGMT levels [112]. Prolactinomas exhibited the lowest levels of MGMT expression [113]. In the treatment of glioblastomas, the level of MGMT expression is predictive for the response to temozolomide [114]. The applicability of MGMT status to predict response to temozolomide in pituitary adenomas is still a matter of discussion. A recent review found 17 of 22 pituitary adenomas with low MGMT expression responsive to temozolomide treatment, whereas 3 of 3 pituitary adenomas with high MGMT expression showed a poor response [115] (see also tables 2.5 and 2.6). Despite of that, there are several limitations to the general application of MGMT levels as an indicator whom to treat and not to treat with temozolomide: Exact cut-off values for low or high expression as well as definition of response or failure of drug treatment are not well defined. Furthermore there are tumors with high MGMT expression that showed good response to temozolomide and there are cases where poor response was observed though MGMT level was low [97]. Table 2.5 highlights the negative correlation between reported response rates of pituitary adenomas that have already been treated with temozolomide and their MGMT expression level.

There are two commonly used techniques for the determination of MGMT expression levels: immunohistochemistry and promoter methylation analysis. Studies showed that the correlation between the two methods as well as the correlation of the determined MGMT levels with treatment outcome is not satisfactory [116, 117] (See appendix A). The currently preferred method to determine MGMT levels for pituitary tumors is immunohistochemistry. Compared to the analysis of MGMT promoter methylation, MGMT expression determined by immunohistochemistry was superior regarding the prediction of temozolomide sensitivity in 16 cell lines [118]. In the case of glioblastomas,

MGMT promoter methylation is the method of choice of determining temozolomide responsiveness [119].

Recent studies analyzed potential mechanisms to enhance the cytotoxicity of temozolomide by depleting MGMT from the cells. With the discovery of O6-benzylguanine and O6-bromothenylguanine two potent inhibitors of MGMT were identified and combination of treatment with temozolomide is currently investigated [101] (compare chapter 2.2.3). A twice daily temozolomide regimen has also been proved as another mechanism of depleting MGMT without an increase in toxicity [120].

Role of MSH6 in the Context of Mismach Repair

MSH6 is a protein involved in mismatch repair. Together with MSH2 it forms a complex that recognizes DNA damage and promotes DNA repair. *In vitro* it has already been shown that inactivation of MSH6 mediates resistance to alkylating drugs since mismatch repair is no longer recognizing DNA mismatches. The lethal effects of alkylating drugs like temozolomide are therefore averted (Compare chapter 2.2.2). Hunter et al. were the first to report that *in vivo* MSH6 mutations arise in glioblastoma after temozolomide treatment as a consequence of clonal evolution [121]. These observations have been supported by two other studies: MSH6 inactivation in previously MSH6 proficient tumors was strongly associated with tumor recurrence [122, 123]. Inactivation of mismatch repair *in vitro* resulted in hypermutability, hence promoting cell growth and leading to drug resistance [124]. Treatment with alkylating drugs seems to produce selection pressure on the cells. *In vitro*, it could be observed that MSH6 deficient cells showed a markedly enhanced growth under temozolomide treatment, compared to MSH6 proficient cells [122]. In 2011, Murakami et al. described the first pituitary adenoma acquiring temozolomide resistance due to MSH6 inactivation. The previously MSH6 immunopositive atypical prolactinoma progressed to a MSH6 immunonegative pituitary

MGMT activity / Adenoma	High		Intermediate		Low		Unknown		*Total*
	+	-	+	-	+	-	+	-	
PRL	1	3	1	0	10	4	4	1	24
ACTH	3	4	0	0	6	1	3	1	18
GH	0	2	1	0	0	0	0	1	4
FSH/LH	0	0	2	0	0	0	0	1	3
NFPA	5	5	0	0	2	1	3	2	18
Total	9	14	4	0	18	6	10	6	67

Table 2.5. **Temozolomide efficacy in pituitary adenomas**
+= response to TMZ, - = no response to TMZ; MGMT staining: <10 %=Low, 11-50 %=Intermediate, >51 %=High. Status on April 15, 2015.

carcinoma [55]. Hereafter, a Japanese group retrospectively analyzed 13 tumor samples of patients with pituitary adenomas who were treated with temozolomide. They found a strong correlation between resistance to temozolomide treatment and absence of MSH6 immunostaining. All three of the adenomas with a progressive disease were immunonegative and all three adenomas with a complete response showed high immunopositivity for MSH6 (50 % or above). Only one patient with negative MSH6 staining had an initial response for nine months. The remaining six adenomas with partial response or stable disease showed intermediate to high immunostaining [125, 51]. The findings from the glioblastomas as well as the pituitary adenomas investigated so far suggest that MSH6 inactivation is independent of MGMT status and p53 mutations [126, 51]. These observations strongly suggest MSH6 to become an important factor to predict response to temozolomide treatment and moreover, MSH 6 is a promising target for future research in temozolomide resistant tumors.

2.2.3. Clinical Application of Temozolomide

After the first chemical synthesis of temozolomide in 1984 by Stevens et al. [127] and the phase I trial by Newlands et al. in 1992 [90], the efficacy of temozolomide treatment was tested in a huge variety of tumors and combined therapies were assessed with many different compounds. Due to its structural and functional similarity to dacarbazine, which has previously been used in the treatment of metastatic melanoma, temozolomide was investigated as a potential alternative to dacarbazine in melanoma therapy. A consequent study showed equal effectiveness to dacarbazine [128] and additional superiority in the treatment of brain metastases [129]. Today temozolomide is well integrated in the standard therapeutic strategy of metastatic melanoma [130]. Because of its good biodistribution and ability to pass the blood-brain barrier, the efficacy of temozolomide in malignant brain tumors was assessed in the phase I trial. In this study, responsiveness to temozolomide was observed in 2 of 4 high grade gliomas [90]. Since then, several studies have investigated the usage of temozolomide in the treatment of glioblastomas [131, 132, 133, 134]. The combination of temozolomide and radiotherapy became standard of care in many places after the study of Stupp et al. was released in 2005. The study analyzed the benefits of concomitant temozolomide treatment in radiotherapy and showed that the combined therapy increased the two-year survival rate from 10,4 % to 26,5 % [135].

Clinical trials with temozolomide have been performed in many tumor entities including: hematologic neoplasms, carcinomas, sarcomas, neuroendocrine tumors and different sorts of brain tumors [136]. But up to date, FDA and EMA approval of temozolomide

is restricted to anaplastic astrocytoma and newly diagnosed glioblastoma multiforme in combination with radiotherapy [137, 138].

Recent treatment strategies focus on the potentiation of temozolomide toxicity with other agents. The major role is attributed towards the disabling of two mechanisms of temozolomide resistance, i.e. inactivation of MGMT and base excision repair:

O6-benzylguanine acts as a false substrate to MGMT. Due to its structural similarities to the natural substrate O6-methylguanine, the MGMT enzyme binds to the benzyl group from O6-benzylguanine. This is an irreversible step and leads to the degradation of MGMT [139]. The use of temozolomide with concomitant O6-benzylguanine has been evaluated in several studies, but showed only little success [140, 141]. Another representative of MGMT inhibitors is the oral available agent O6-bromothenylguanine, which is ten times more potent than O6-benzylguanine [142]. However, no convincing benefits of the combination with temozolomide have been shown so far [143].

The second mechanism of temozolomide enhancement is the inhibition of poly (ADP-ribose) polymerase (PARP). The physiological function of PARP is to detect and bind to DNA damage. Subsequently it starts to recruit other enzymes involved in base excision repair [144]. Through the inhibition of PARP, the temozolomide induced methylation at N7-guanine and N3-adenine cannot be repaired anymore. The lesions that had previously sufficiently been repaired by the base excision repair can now contribute to temozolomide induced toxicity. The efficacy of PARP inhibitors in many tumor types is currently investigated in phase II trials. The combination of the PARP inhibitor ABT-888 and temozolomide has demonstrated good efficacy in temozolomide resistant tumor tissues [145].

A very recent development is the combination of temozolomide and the thymidylate synthase inhibitor capecitabine. The combination has first been approved by Strosberg et al. in neuroendocrine tumors and had showed good efficacy with good tolerability [146]. Gulati et al. first successfully applied this regimen to a pituitary tumor of a patient with a MEN1 associated prolactin secreting pituitary adenoma. The group found that a pretreatment with capecitabine enhanced toxicity, while cotreatment showed less synergistic effects. The reasons therefore remain still unclear [147].

Another promising substance for future combination treatments is pyrimethamine, an inhibitor of folic acid synthesis which is currently used for the treatment of malaria. After it was first described to improve temozolomide toxicity in melanoma cells [148], a consequent study in mice bearing GH3-tumor xenografts showed that pyrimethamine inhibited tumor growth, lowered hormone levels and provided improved survival to the mice [149]. Many other therapies have been evaluated for the concomitant use with

temozolomide which are summarized in figure 2.4. [142]

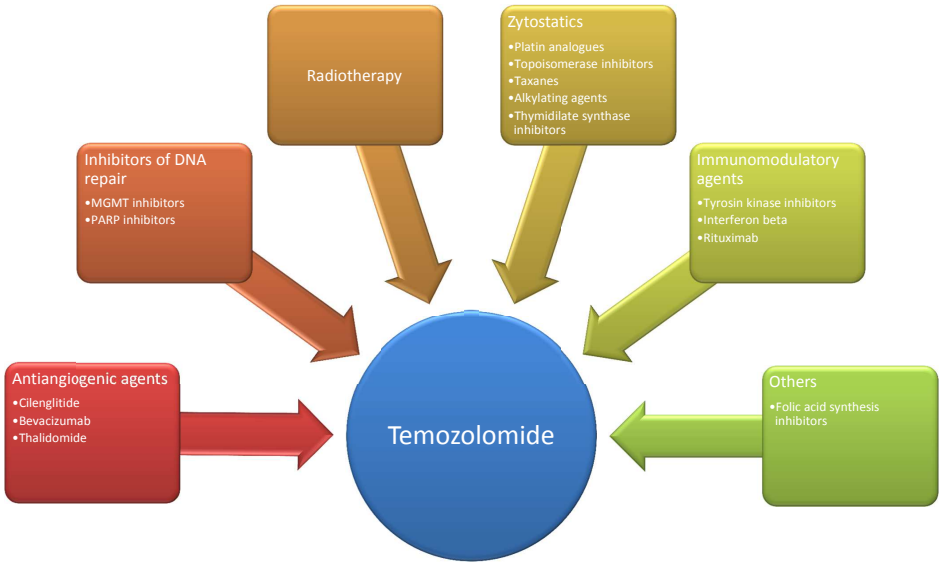

Figure 2.4. **Therapeutic combinations with temozolomide.**
Temozolomide can be combined with several other forms of treatment in order to achieve a maximal tumor response. Figure modified after **[142].**

2.2.4. Usage of Temozolomide in Treatment of Pituitary Tumors

The first use of temozolomide was reported by Zhu et al. at the annual meeting of the US Endocrine Society in 2004. They described the case of a 61-year old patient with a prolactin producing carcinoma, that responded to temozolomide with tumor shrinkage, reduction in hormone level and clinical improvement [150]. Two years later, other groups started to use temozolomide in treatment resistant, aggressive pituitary adenomas and pituitary carcinomas. In 2006 and 2007, five successful reports were published. These observations gave rise to larger case series in the following years. A detailed list of all published cases of temozolomide treated pituitary tumors is shown in appendix A. Until date, 103 cases of the usage of temozolomide in pituitary tumors have been documented. The median patient age was 45 years, with the youngest being 1 and the oldest 73 years old. Tolerability seemed to be good, comparable to that seen under

temozolomide in the treatment of other tumors. The tumors that were deemed applicable to a trial of temozolomide treatment in the literature were mostly aggressive in nature. Many showed locally invasive behavior, multiple recurrences and resistance to previous treatment options. 36 of the patients had pituitary carcinomas. The response rate of 58 % (60 of 103 tumors) is convincing, considering that these tumors were treatment resistant to other therapies (see table 2.5 and table 2.6). Reported response rates were greater in the beginning, probably due to "reporting bias". Subsequent larger case series found lower response rates. Treated patients usually showed improvement of clinical signs, reductions in hormone level and tumor shrinkage in brain scans (see figure 2.5 and appendix A). Tumors also showed changes in morphology after treatment with the drug. Kovacs et al. reported a case in which temozolomide induced necrosis, hemorrhage and even neuronal transformation of the tumor [151].

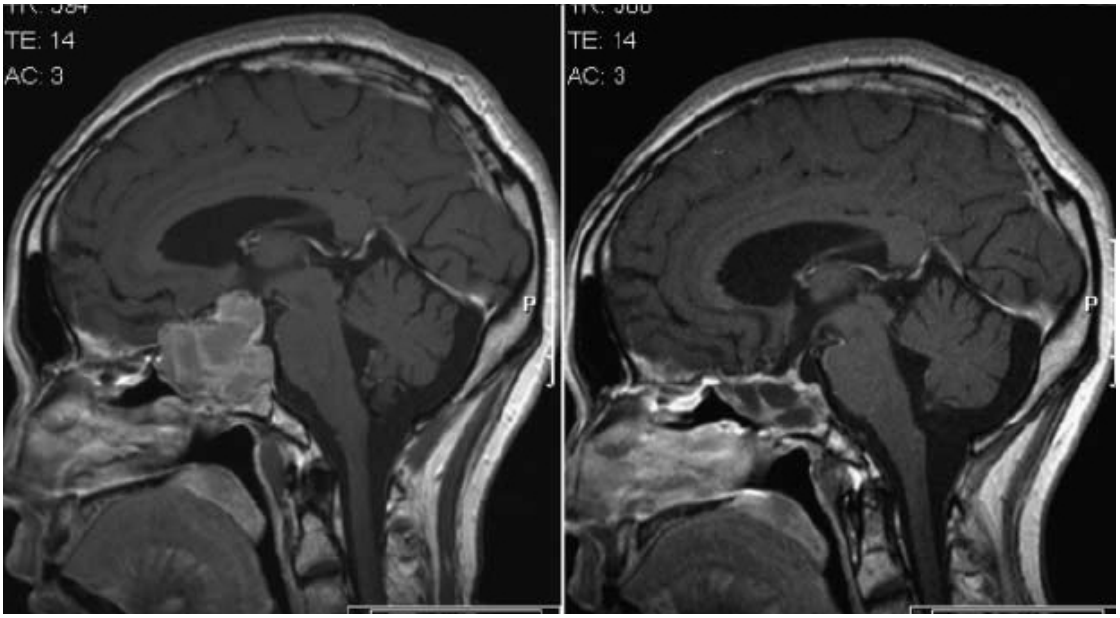

Figure 2.5. **Pituitary adenoma pre- and post-treatment**
The left scan shows the patient before and the right after temozolomide treatment. A marked reduction in tumor size can be seen.
Pictures obtained from Hagen et al. [152]

Response to temozolomide was not always permanent. Progression of disease after an initial response was noticed in several cases [153, 154, 55, 59, 47]. Response rates were different in the respective tumor types. Prolactin and ACTH producing tumors were most likely to respond (16 of 24; 66 % and 12 of 18; 66 %), followed by non-secretory tumors (10 of 18; 55 %). For other tumors, a positive response upon temozolomide treatment was reported for 1 of 4 growth hormone producing tumors as well as for 2 of

3 gonadotroph adenomas. Of the four described growth hormone producing tumors 3 showed no response and of the 3 gonadotroph adenomas, 2 responded. No difference in sensitivity to temozolomide treatment was found among adenomas versus carcinomas. Furthermore no significant difference in response to temozolomide between female and male patients has been reported.

18 of 24 adenomas (75 %) with low MGMT staining responded to temozolomide, compared to 9 of 23 adenomas (39 %) with high MGMT staining. These observations contribute to the hypothesis that response to temozolomide is linked to the presence of MGMT (See chapter 2.2.2). Regarding the pituitary carcinomas listed in table 2.6, a promising response rate of 53 % (19 of 36) was observed for these aggressive tumors. In the pituitary carcinomas, MGMT status also seems to play a role as only 2 of 6 carcinomas (32 %) with high MGMT expression responded, compared to 8 of 11 (73 %) with low MGMT status. However, in the adenoma group listed in table 2.5 as well as in the carcinoma group in table 2.6, the MGMT status of many tumors remains unknown, making a reliable statement difficult.

2.2.5. Adverse Reactions of Temozolomide Treatment

Throughout the therapy with temozolomide, monitoring of hepatic enzymes, renal function and blood counts should be performed. Elevation of liver enzymes has not been described in the big clinical trials with temozolomide, but some smaller case series witnessed a moderate increase [155, 156]. Renal function seems not to be affected by the drug [157]. Concerning the possible teratogenic potential of the drug, appropriate methods for contraception should be performed. Though evidence for potential fetal harm has been found in animal models [138], there have been several case reports of healthy babies, born to mothers on temozolomide treatment. [158]

MGMT activity / Carcinoma	High		Intermediate		Low		Unknown		*Total*
	+	-	+	-	+	-	+	-	
PRL	0	1	0	2	2	1	6	2	14
ACTH	1	2	1	2	4	1	1	3	15
GH	0	1	0	0	1	0	0	0	2
FSH/LH	0	0	0	0	0	0	1	0	1
Non-functioning	1	0	0	0	1	1	0	1	4
Total	2	4	1	4	8	3	8	6	36

Table 2.6. **Temozolomide efficacy in pituitary carcinomas**
+= response to TMZ, - = no response to TMZ; MGMT staining: <10 %=Low, 11-50 %=Intermediate, >51 %=High. Status on April 15, 2015.

Myelotoxicity was the dose limiting factor in the phase I study performed by Newlands et. al. in 1992 [90]. Lymphopenia evolved in approximately 50 % of patients treated for melanoma or neuroendocrine tumors and cases of opportunistic infections were also observed [159, 160]. The nadir of lymphopenia is predictable between days 21 and 28 of treatment and reduction of dose is advisable in these cases.

Due to the occasional occurrence of opportunistic infections with pneumocystis jirovecii, especially in patients receiving concomitant radiation and steroid therapy, a prophylactic treatment with Trimethoprim-Sulfamethoxazol is recommended by some clinicians [161]. Considering the incidence of less than 1 % of pneumocystis jirovecii pneumoniae and regarding the side effect profile of the chemoprophylaxis, its usage is subject to current discussions [162].

Recently a less common side effect concerning the posterior pituitary lobe has been reported by Faje et. al. [163]. The retrospective study identified 5 patients who developed central diabetes insipidus limited to the time of temozolomide treatment. Regarding the potential of temozolomide to induce secondary malignancies, several studies have linked temozolomide to the development of hematological malignancies such as leukemia [164]. Allergic reactions to temozolomide treatment have been reported [165] but are usually restricted to cutaneous symptoms.

In summary the most frequent encountered side effects linked to temozolomide use are fatigue, alopecia, nausea, vomiting, anorexia, headache and constipation [138]. Most side effects were mild to moderate in degree and usually treatable with appropriate medication. The dose limiting side effects are leukopenia (2 %) and thrombocytopenia (7 %), but less than 1 % of patients discontinued treatment due to adverse effects [131].

3. Aim of the Study

Atypical pituitary adenomas account for 8,9 % - 15 % of all surgically resected pituitary tumors but to date, no FDA approved therapy for this tumortype and for pituitary carcinomas exists[45, 166]. Atypical adenomas are usually large tumors that are resistant to dopamine agonists and somatostatin analogs. They demonstrate a high rate of invasiveness, require multiple surgeries and radiotherapy and show strong tendency to recur. The same is true for pituitary carcinomas and consequently this aggressive nature results in a death rate of 66 % within the first year after diagnosis [62]. For an optimal treatment of these tumors there are several main goals: i) control of tumor growth, ii) control of hormone excess, iii) prevention of metastases and finally iv) preservation of normal pituitary function. With a percentage of 58% clinical responders in the adenoma group and 52 % among the carcinomas (Compare table 2.5 and table 2.6), temozolomide has demonstrated good effectiveness even if all other treatment options have failed. Until now, temozolomide induced reduction of proliferation and a hormone reductive effect for FSH, LH and PRL in pituitary cell lines was demonstrated in two studies [167, 168]. The goal of this thesis was to expand the knowledge of temozolomide induced effects on pituitary cells. We wanted to investigate the influence on proliferation and hormone secretion of normal and tumoral pituitary cells of different origin. A special focus was set on the question whether the observed effects on hormone secretion were simply a result of mass reduction or due to a specific hormone suppressive effect of temozolomide. Another question that we wanted to answer was whether we could detect any effect on metastasizing potential. For answering this question we applied a clonogenic assay and we investigated the influence on angiogenesis of pituitary tumors.

4. Experimental Procedures

4.1. Material

4.1.1. Equipment

Table 4.1. **List of equipment used in this study**

Product	**Provider**
ARCTIS 1300 GS Refrigerator	AEG (Nürnberg, Germany)
Automatic pipet - Pipetus®	Hirschmann Laborgeräte (Eberstadt, Germany)
Autoradiography Cassette - Hypercassette	GE Healthcare (Buckinghamshire, England)
Burner - Fireboy plus	Integra Biosciences (Fernwald, Germany)
Cell counter - Scepter™ Handheld automated Cell Counter	Millipore Corporation (Billerica, MA, USA)
Centrifuge - Biofuge primo	Thermo Scientific (Waltham,USA)
Centrifuge - Centrifuge 5415 R	Eppendorf (Hamburg, Germany)
Cooling-Thermomixer - KTMR-133	Haep Labor Consult (Bovenden, Germany)
Electrophoresis chamber - Mini Trans Blot-Cell	Biorad (Hercules, USA)
Electrophoresissystem for gel preparation - Mini PROTEAN Tetra	Biorad (Hercules, USA)
GTN 2701 Refrigerator	Bosch (Munich, Germany)
HFU 586 TOP HERA freeze Refrigerator	Thermo Scientific (Waltham,USA)
HFU 686 TOP HERA freeze Refrigerator	Thermo Scientific (Waltham,USA)
Icemachine - ZBE-150	Ziegra (Isernhagen, Germany)
Incubator - Heraeus Instruments Type BB 6220 O2	Thermo Scientific (Waltham,USA)
KGK 4585 Refrigerator	Liebherr (Biberach an der Riss, Germany)

Product	**Provider**
KGT 4066 Premium Refrigerator	Liebherr (Biberach an der Riss, Germany)
KT 1483 Refrigerator	Liebherr (Biberach an der Riss, Germany)
KT 5R 18.1 Refrigerator	Siemens (Munich, Germany)
Laminar Flow - LaminAir®HB 2472	Thermo Scientific (Waltham,USA)
Microplatereader - Dynatech MR 5000	Dynex Technologies (Chantilly, USA)
Microscope - IMT-2	Olympus (Hamburg, Germany)
Microwave - HF12M240	Siemens (Munich, Germany)
Multichannel pipet	Eppendorf (Hamburg, Germany)
Multipipet - Multipette® plus	Eppendorf (Hamburg, Germany)
Neubauer counting chamber	Paul Marienfeld GmbH & Co KG (Lauda-Königshofen, Germany)
Pipet - Reference® variabel	Eppendorf (Hamburg, Germany)
Power supply - Power Pac 1000	Biorad (Hercules, USA)
Precision balance - AT261	Mettler Toledo GmbH (Greifensee, Switzerland)
Scanner - Aficio MP C 3300	RICOH Deutschland GmbH (Hannover, Germany)
Scintillation counter - LS 6000 IC	Beckman Coulter (Krefeld, Germany)
Scraper knife - Scraper Knife 45mm	NISAKU KUY TOOLS JAPAN (Niigata, Japan)
Shaker - Duomax 1030	Heidolph (Schwabach, Germany)
Spectrophotometer - Smart Spec™ Plus	Biorad (Hercules, USA)
Suction pump - Type N735.3 AN.18	KNF Neuberger (Freiburg, Germany)
Vortex - Genie 2™	Bender & Hobein AG (Zürich, Switzerland)
Waterbath - Type 1004	Gesellschaft für Laborbedarf (Burgwedel, Germany)

4.1.2. Consumables

Table 4.2. **List of consumables used in this study**

Product	**Provider**
Autoradiography films - Lumi-Film Chemiluminescent Detection Film	Roche (Indianapolis, USA)
Blotting filter paper 2.5mm thick	Invitrogen (Carlsbad, USA)
Cell Culture flasks	TPP (Trasadingen, Switzerland)
Cell scraper 13mm	TPP (Trasadingen, Switzerland)
Centrifuge tubes	TPP (Trasadingen, Switzerland)
Gloves - Semper guard latex powderfree	Semperit Technische Produkte GmbH (Wien, Austria)
Gloves - Semper guard nitril powderfree	Semperit Technische Produkte GmbH (Wien, Austria)
Insulin syringes - Micro Fine+ U-40	Beckton Dickinson (Franklin Lakes, USA)
Multiwell plates	Thermo Scientific (Waltham, USA)
Multiwell plates for ELISA - Corning® 96 Well Clear Polystyrene High Bind Stripwell™ Microplate	Corning B.V. Life Sciences (Amsterdam, Netherlands)
Nitrocellulosis membrane - Hybond™ ECL	GE Healthcare (Buckinghamshire, England)
Parafilm®	Pechiney Plastic Packaging (Menasha, USA)
Pasteur pipets	Roth (Karlsruhe, Germany)
Reaction vessels	Eppendorf (Hamburg, Germany)
Serological pipets	TPP (Trasadingen, Switzerland)
Tips - epT.I.P.S. Standard	Eppendorf (Hamburg, Germany)
Tips for Multipipets	Eppendorf (Hamburg, Germany)
Tips for Scepter™ Cell Counter	Millipore Corporation (Billerica, MA, USA)
Tubes - PP tubes	Greiner Bio-One (Kremsmünster, Austria)

4.1.3. Reagents

Table 4.3. **List of reagents used in this study**

Product	**Provider**
^{3}H-Thymidine 1mCi/ml	Perkin-Elmer (Boston, USA)
Acetic Acid	Merck (Darmstadt, Germany)
Acridine Orange	Sigma-Aldrich (Steinheim, Germany)
Acrylamide-Bis	Merck (Darmstadt, Germany)
Ammonium Persulfate 30%	Sigma-Aldrich (Steinheim, Germany)
Aqua ad iniectabilia	Braun (Melsungen, Germany)
Bovine Serum Albumin	Roth (Karlsruhe, Germany)
Bromophenol Blue	Sigma-Aldrich (Steinheim, Germany)
Chloramine-T hydrate	Sigma-Aldrich (Steinheim, Germany)
Cobalt (II) Chloride	Sigma-Aldrich (Steinheim, Germany)
Collagenase Type 1	Worthington (Lakewood, NJ, USA)
Copper(II) sulfate ($CuSO_4$)	Sigma-Aldrich (Steinheim, Germany)
Corticotropin Releasing Hormone	Bachem (Weil am Rhein, Germany)
Crystal Violet	Sigma-Aldrich (Steinheim, Germany)
Desoxyribonuklease	Sigma-Aldrich (Steinheim, Germany)
Developer Solution	Kodak (Stuttgart, Germany)
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich (Steinheim, Germany)
Disodium Hydrogen Phosphate Dihydrate ($Na_2 \cdot HPO_4$ $2H_2O$)	Merck (Darmstadt, Germany)
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen (Carlsbad, USA)
DuoSet® ELISA Development System for human VEGF	R & D Systems (Minneapolis, MN, USA)
ECL ™ Western Blotting Analysis System	GE Healthcare (Buckinghamshire, England)
Ethanol 70%	Medizet (Munich, Germany)
Ethidium Bromide 1%	Roth (Karlsruhe, Germany)
Ethylenediaminetetraacetic Acid (EDTA)	Sigma-Aldrich (Steinheim, Germany)
Fetal Calf Serum (FCS)	Invitrogen (Carlsbad, USA)
Ficoll 400	Sigma-Aldrich (Steinheim, Germany)
Fixer Solution	Kodak (Stuttgart, Germany)

Product	**Provider**
Glucosis	Roth (Karlsruhe, Germany)
Glutaraldehyde	Sigma-Aldrich (Steinheim, Germany)
Glycine	Roth (Karlsruhe, Germany)
Growth Hormone Releasing Hormone	Bachem (Weil am Rhein, Germany)
Hepes	Sigma-Aldrich (Steinheim, Germany)
Hyaluronidase Type II (from sheep testes)	Roche (Indianapolis, USA)
Hydrochloric acid	Sigma-Aldrich (Steinheim, Germany)
Insuline (from bovine pancreas)	Sigma-Aldrich (Steinheim, Germany)
Isopropylalcohol	Roth (Karlsruhe, Germany)
L-Glutamine	Biochrom AG (Berlin, Germany)
Methanol Roripuran®	Roth (Karlsruhe, Germany)
Milk powder	Granovita GmbH (Heimerlingen, Germany)
Monopotassium Phosphate (KH_2PO_4)	Sigma-Aldrich (Steinheim, Germany)
Non-essential amino acids solution (x 100)	Invitrogen (Carlsbad, USA)
Nonidet P-40	Invitrogen (Carlsbad, USA)
Particin	Biochrom AG (Berlin, Germany)
Penicillin/Streptomycin	Biochrom AG (Berlin, Germany)
Ponceau S Solution	Sigma-Aldrich (Steinheim, Germany)
Potassium chloride (KCl)	Merck (Darmstadt, Germany)
Precision Plus Protein™ Dual Color Standards	Bio-Rad (Hercules, USA)
Protease Inhibitor Cocktail	Sigma-Aldrich (Steinheim, Germany)
Reagent Diluent	R & D Systems (Minneapolis, MN, USA)
Roti® Load 1 Loading buffer	Roth (Karlsruhe, Germany)
Sodium Chloride (NaCl)	Roth (Karlsruhe, Germany)
Sodium Deoxycholate	Sigma-Aldrich (Steinheim, Germany)
Sodium Dodecyl Sulfate (SDS)	Sigma-Aldrich (Steinheim, Germany)
Sodium Hydroxide (NaOH)	Sigma-Aldrich (Steinheim, Germany)
Sodium Selenite	Sigma-Aldrich (Steinheim, Germany)
Stop Solution(2N H_2SO_4) for ELISA	R & D Systems (Minneapolis, MN, USA)
Substrate Solution for ELISA	R & D Systems (Minneapolis, MN, USA)
Temozolomide	Sigma-Aldrich (Steinheim, Germany)
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich (Steinheim, Germany)
Thyreotropine Releasing Hormone	Bachem (Weil am Rhein, Germany)

Product	**Provider**
Transferrin	Sigma-Aldrich (Steinheim, Germany)
Trichloroacetic Acid	Sigma-Aldrich (Steinheim, Germany)
Triiodo-L-Thronine (T3) Sodium Salt	Sigma-Aldrich (Steinheim, Germany)
Tris Pufferan®	Roth (Karlsruhe, Germany)
Triton x -100	Roth (Karlsruhe, Germany)
Trypsin Inhibitor	Roth (Karlsruhe, Germany)
Trypsin/EDTA Solution (0,25%)	Invitrogen (Carlsbad, USA)
Tween® 20	Biochrom AG (Berlin, Germany)
Xylene Cyanol	Sigma-Aldrich (Steinheim, Germany)

4.1.4. Buffers And Solutions

Table 4.4. **List of buffers and solutions used in this study**

Cell Staining Solution	50µl	Acridine Orange (200mg/l)
	50µl	Ethidium Bromide (10mg/l)
	900µl	PBS
Colony Staining Solution	6%	Glutaraldehyde
	0.5%	Crystal violet (2.3%)
Culture Medium	500ml	DMEM
	10%	FCS
	5ml	L-Glutamin (200mM)
	5ml	Penicillin/Streptomycin (10000 U/ml / 10000 µg/ml)
	5ml	Particin (1%)
HDB+ buffer	25mM	Hepes
	5mM	KCl
	0.7mM	Disodium Hydrogen Phosphate Dihydrate
	10mM	Glucose
	25µg/ml	Particin
	105 E/l	Penicillin/Streptomycin
		Adjusted to pH 7,3

Mix of collagenases	100ml	HDB+ Buffer
	370mg	Collagenase (1000U/ml)
	10mg	Hyaluronidase
	10mg	Trypsin Inhibitor
	400mg	Bovine Serum Albumin
	500ml	Desoxyribonuclease
PBS	8g/l	Sodium Chloride
	0.2g/l	Potassium Chloride
	1.44g/l	Disodium Hydrogen Phosphate Dihydrate
	0.2g/l	Potassium Phosphate Monobasic
RIPA buffer	50mM	Tris Hydrochloric acid pH8
	150 µM	Sodium Chloride
	1%	Nonidet P-40 (0,08mM)
	0.5%	Sodium deoxycholate (>97%)
	0.1%	SDS (>99,0%)
Running buffer	25mM	Tris
	192mM	Glycin
	0.1%	SDS
		Adjusted to pH 8,3
Stripping Solution	25mM	Glycine
	1%	SDS (>99,0%)
		Adjusted to pH 2
TBS (10x)	24.2g	Tris
	80g	Sodium Chloride
		Adjusted to pH 7,6
TBST	1000ml	TBS (1x)
	0.1%	Tween® 20 (1.095 g/ml)
Transfer buffer	25mM	Tris
	192mM	Glycine
	10%	Methanol

Tumor medium	500ml	Culture medium
	5ml	Non-essential amino acids solution (100x)
	500µl	Insulin Novo 40 I.E.
	2.5mg	Transferrin
	10µg	Triiodo-L-Thronine (T3) Sodium Salt
	10µg	Sodium Selenite

4.1.5. Antibodies

Table 4.5. **List of antibodies used in this study**

Product	**Provider**
Anti-mouse IgG (horse HRP linked Antibody)	Cell Signaling Technology (Danvers,USA)
Anti-human ß-Actin clone C 4 (mouse monoclonal Antibody)	Millipore (Billerica, USA)
Anti-human HIF - 1 α Antibody (mouse monoclonal Antibody)	R & D Systems (Minneapolis, MN, USA)

4.2. Methods

4.2.1. Cell culture

4.2.1.1. Cell Culture of Cell Lines AtT-20 and GH3

Both cell lines(AtT-20 and Gh3) are immortalized cell lines and were obtained from the American Type Culture Collection (Manassas, VA, USA). The GH3 cell line derives from a prolactin and growth hormone secreting rat tumor and the AtT-20 cell line from an ACTH secreting mouse tumor. The cell lines were kept in aliquots at -179° C for long-time storage. For thawing, the tubes were heated up to 37 °C in the water bath and the cell suspension was diluted with culture medium. This was followed by a centrifugation with 1200 rpm for 3 min at room temperature. The supernatant was discarded and the pellet resuspended in fresh culture medium.

Cells were kept in 75 cm^2 flasks in an incubator with 37 °C, 95 % air humidity and 5 % CO_2 concentration. All works with the cells were performed under sterile conditions on an appropriate work bench. The medium was changed and cells splitted every 3 to 4 days according to cell density under the microscope and the color of the medium. This

was accomplished by removing the old medium and washing the cells with 15ml of PBS. Then 4 ml of trypsin/EDTA solution was added, followed by an incubation time of 3 min in the incubator in order to detach the cells from the flask. If necessary, remaining attached cells were mobilized by handclaps against the flask. 10 ml of culture medium was added to stop the reaction. The cell suspension was transferred into a 50 ml tube and centrifuged with 1200 rpm at room temperature for 4 min. The supernatant was discarded and cells were resuspended in fresh culture medium. Approximately 1 million cells per flask were distributed in new flasks and further incubated.

In order to count the cells, an aliquot of the resuspended cells was mixed with staining solution (50 µl of 200 mg/l acridine orange and 10 mg/l ethidium bromide and 50 µl of cell suspension) and introduced into a Neubauer counting chamber. While acridine orange is incorporated in viable cells and colors them green, ethidium bromide can only enter dead cells and causes an orange stain. By counting the green cells of 4 x 16 squares and multiplication with 2 x 10^4 the average numbers of cells per milliliter was determined. All cell assays were performed using either 6-, 48 or 96-well plates. In the case of the 48- and 96-well plates, only the inner wells were used for the assay, while the outer wells were filled with PBS solution only. Wells on 96-well plates and 48-well plates were filled with 100 µl/well and 500 µl/well, respectively. 6-well plates were filled with 2 ml/well for seeding and 1 ml/well for consecutive treatment. Before seeding new plates, the cell density of the corresponding cell suspensions was determined as described above. All plates were finally covered with the respective plastic lids.

4.2.1.2. Primary Cell Culture of Human Pituitary Adenomas

The tissue of human pituitary adenomas was obtained by transpheniodal surgeries in the Neurosurgical clinics of the Universitätsklinikum Erlangen and the Klinikum Großhadern and Klinikum Rechts der Isar in Munich. The utilization of the tissue was approved by the local ethics committee. The patients were informed about the usage of the extracted material prior to the surgery and signed a declaration of agreement. Immediately after extraction, the tissue was transferred into a tube containing cell culture medium and send to the lab, where it arrived the following day and further preparation was continued. The tumor was washed several times with HdB+ buffer to eliminate remaining blood contamination. The tissue was cut with scalpels in a petri dish and transferred into a tube containing a mixture of collagenases. This tube was incubated in a waterbath at 37 °C and retrieved every 15 min to break up the tissue by pipetting it multiple times with a Pasteur pipet. The reactions of the collagenases were stopped by adding 10 ml culture medium when no more cell debris was observed by the eye, but earliest 1 hour

after addition of the collagenases. The suspension was centrifuged at 1200 rpm for 4 min at room temperature and the supernatant was discarded. The centrifuged material was resuspended with 10 ml culture medium and centrifugation was repeated. Cells were resuspended once more and viable tumor cells were counted and seeded using the same procedure as described above for cell lines. For the seeding and for all further experiments with the adenoma cells tumor medium as described in the material section was used.

4.2.1.3. Primary Cell Culture of Rat Pituitaries

Besides murine cell lines and primary human adenoma cells, cell assays were also performed with six week old male Sprague Dawley rats. Animals were killed by carbon dioxide euthanasia and subsequently decapitated in a miniature guillotine. The scalp was shifted towards the nose so that the skull could be cut on both sides using scissors. The skull was flapped towards the nose and the cerebrum was removed. The pituitary gland was now lying free in the sella. Under complete removal of the surrounding connective tissue it could be withdrawn and stored in HdB+ buffer until further preparation. When all required pituitary glands were collected, cell preparation was performed corresponding to the protocol for the human adenomas.

4.2.2. Treatment with Temozolomide

After the respective time of attachment ranging from 3 to 4 days, cells of primary culture were washed with PBS to remove nonattached cells and cell detritus. In experiments with cell lines this step was not necessary and the time of attachment was only 24 h. Afterwards the old medium was pipetted off and replaced with temozolomide - containing medium solutions. Prior to any further usage the solutions were freshly prepared with thawed temozolomide aliquots and culture medium which was preheated to 37 °C. All experiments were performed using several temozolomide concentrations as indicated.

4.2.3. ^{3}H-thymidine Incorporation

The incorporation of Tritium-enriched thymidine (^{3}H-thymidine) was determined as an indicator of proliferation. Human adenoma cells were seeded in 48-well plates with a density of 10^5 cells per well, AtT-20 and GH3 cells with 10^4 cells per well. Human primary adenoma cells were simultaneously treated with medium containing temozolomide and 2 % ^{3}H-thymidine solution for 24 h at 37 °C, 95 % air humidity and 5 % carbon dioxide concentration. Cell lines were incubated for 24 h with temozolomide, followed by an incubation period of for 4 h with ^{3}H-thymidine. Cells were then precipitated by

addition of 500 µl/well ice cold 10 % trichloroacetic acid for 1 h at 4 °C. After removal of trichloroacetic acid, cells were washed with PBS and hydrolyzed with a solution containing 0.5 M NaOH and 1 % Triton X-100 for 24 h at 4 °C. Thereafter plates were gently vortexed for 5 min and the cell suspension of every well was transferred into tubes with scintillation liquid. The tubes were thoroughly mixed by vortexing and radioactivity was measured using a scintillation counter.

4.2.4. Colony Formation Assay

The capacity of forming colonies as well as the reduction of cell number was measured with a clonogenic assay, following the protocol of Franken et al. [169]. Cells were seeded in 6-well plates with a density of 1 x 10^3 cells per well for the GH3 cell line and 0.5 x 10^3 cells per well for the AtT-20 cell line. Due to the long incubation period of 1 or 2 weeks, culture medium with 2 % FCS was used. As we investigated two different settings, cells were left for attachment for either 1 day or 1 week and then treated with different concentrations of temozolomide for 2 or 1 weeks, respectively. Change of medium was performed according to the pH-indicating color of the culture medium approximately every 3 days. The cells were first washed with PBS and then stained for 30 min with a solution containing 6 % glutaraldehyde and 0.5 % crystal violet. Staining solution was removed, plates were washed two times with water and then left to dry at room temperature in normal air. To count the colonies, plates were scanned with 200 dpi as a jpg file and colors were adjusted to a grey scale using Adobe Photoshop. At a magnification of 120 % the colonies of one well of each plate were first counted manually. Parameters for threshold and minimum were adjusted so that the automated count of the Clono program [170] matched the manually counted colony number of this well. These parameters were used to count the rest of the scanned plate by the program.

4.2.5. Cell Counting

In order to obtain exact cell numbers for each well and to still have the possibility to measure the hormone concentration in the respective well after the cell count, the handheld Scepter® cell counter was used. The Scepter® cell counter is able to count cells using an impedance-based method, where cells are aspirated and the change of electrical resistance when a cell is passing the sensor is registered and counted. Cells were seeded in 48-well plates with a density of 1 x 10^5 cells per well and left for attachment for 24 h. Stimulation with different concentrations of temozolomide was performed for 24 h. The wells were washed with PBS and solubilized with 250 µl trypsin/EDTA solution per well

for 10 min at 37 °C in the incubator. Trypsinization was stopped by the addition of 750 µl culture medium per well. The content of each well was mixed by pipetting up and down right before measuring it with the Scepter®.

4.2.6. Hormone Measurement using Radioimmunoassay (RIA)

The secretion of ACTH, GH, PRL and TSH were measured for different cell types. For each hormone that was investigated, a labelled equivalent was needed. In the case of the ACTH measurement labeling of the antigen had to be performed prior to the RIA. The other hormones, which had to be artificially tyrosinated before where purchased as kits from Dr. Parlow, National Hormone & Peptide Programme, Harbor-UCLA Medical Center, CA, USA.

4.2.6.1. ACTH ^{125}I Labeling

10 µl of ACTH standard was diluted with 90 µl of 0.05 M phosphate buffer and 5 µl of this solution was given into a siliconized glass tube. Subsequently 25 mg Chloramine T as well as 25 mg sodium bisulfate were dissolved each in 8 ml 0.05 M phosphate buffer. 10 µl of radioactive iodine (^{125}I) was mixed with 20 µl of 0.5 M phosphate buffer and added into the glass tube together with the solved Chloramine T. The glass tube was vortexed for 5 s and the reaction was stopped by adding the previouly dissolved sodium bisulfate and vortexing for 5 s. Chloramine T oxidizes iodine and therby acts as an activator for the ^{125}I by facilitating the iodination of the aromatic system of tyrosine residues in ACTH. In contrast, sodium bisulfate reduces iodine to form iodide and therefore stopps iodination of tyrosine side chains. In order to prevent attachment to the glass, 1 ml 10 % FCS in culture medium was added. Next, separation of the excessive iodine and the iodinated ACTH was achieved by vortexing the glass tube with 5 mg CUSO for 1 min and centrifuging for 30 s. Like that, the ACTH attached to the CUSO and was spun down. The supernatant was discarded and the pellet was washed with 1 ml 0.05 M phosphate buffer. ACTH was eluted from CUSO with 300 µl elution solution, vortexed for 2 min and centrifuged for 2 min. The supernatant was immediately transferred in 8 ml of buffer A, aliquoted in 1 ml eppendorf tubes and frozen at 20 °C.

4.2.6.2. Binding of Antibodies

Prior to the measurement, approximately five random samples were taken from one 48-well plate. The approximate hormone concentration in these samples was determined in order to make sure if a dilution step needs to be conducted prior to the actual measure-

ment. The following steps are based on a competitive principle. For the measurement, a known amount of radiolabeled antigens was mixed with the appropriately diluted tumor antigen. The respective primary antibodies were added and the tubes were incubated at room temperature for 24 hours. This allowed for a competitive binding of the labeled and unlabeled antigen to the limited number of binding sites of the antibody. The more antigens the sample contained, the less radiolabeled antigen was bound to the primary antibody. Then, a secondary antibody, directed towards the Fc part of the primary antibody was added. This step led to the precipitation of the antigen-antibody-antibody complexes. After centrifugation and disposal of the unbound antibodies and antigens in the supernatant, samples were resolved. Radioactivity was measured using a γ-counter. In parallel, dilutions with known concentrations of the respective hormone antigen were prepared and measured using the same procedure as described above. A standard binding curve was established for each hormone and the respective hormone concentration was calculated according to the curve.

4.2.7. Measurement of VEGF-production using ELISA

Cellular expression levels of VEGF were measured with an ELISA Kit (R&D Systems, MN, USA) according to the manufacturer's instructions following the principle of a sandwich ELISA. Cells were seeded in 48-well plates with 1 x 10^5 cells per well and grown for 24 h before being treated with different concentrations of temozolomide. A set of experiments was performed with an additional stimulation of 125 µM cobalt (II) chloride, which has previously been shown to increase basal VEGF production [171]. After 24 h of stimulation by temozolomide, plates were stored at -70 °C until further usage. Fresh 96-well ELISA plates were incubated overnight at room temperature with 100 µl anti-human VEGF antibody (1 µg/ml) per well and plates were covered with Parafilm®. After incubation, wells were washed three times with 400 µl Wash Buffer using a multichannel pipette. After each washing step, plates were tapped against a paper towel to ensure proper removal of the Wash Buffer. Subsequently, 300 µl Reagent Diluent per well was added, followed by an incubation time of 1 h at room temperature and three more washing steps as performed before. Seven VEGF standards with concentrations ranging from 31,25 pg/ml to 2000 pg/ml were prepared. 100 µl of the thawed samples or standards were added to each well, plates were covered with Parafilm® and left for 2 h at room temperature. Plates were washed three times as described previously and 100 µl of the Detection Antibody (100 ng/ml) was added and plates covered and left to incubate at room temperature for 2 h. After another three washing steps, plates were incubated with 100 µl of Streptavidin-HRP (5 µl/ml) per well for 20 min at room temperature and

under light protection. Three more washing steps were performed and then 100 µl of Substrate Solution was added to each well and plates were left to incubate under light protection. The HRP-catalyzed reaction was stopped by adding 50 µl Stop Solution to each well. Plates were gently mixed and absorption at 450 nm was determined by an ELISA plate reader while absorption values at 550 nm were subtracted to achieve higher accuracy. The plate reader fitted a standard curve for each plate and calculated VEGF concentrations of each sample according to this curve.

4.2.8. Measurement of Hif-1α-secretion using Western Blot

The production of the transcription factor Hif-1α, which plays an important role in angiogenesis, was measured by Western Blot.

4.2.8.1. Protein Extraction

Cells were seeded in 48-well plates with a density of 2 x 10^5 cells per well and treated with temozolomide after an attachment time of 24 h. After 1 h cobalt(II) chloride with a final concentration of 250 µM was added and incubated for 3 h to enhance basal Hif-1α production. Plates were put on ice and medium was replaced by 100 µl PBS per well. Content of 8 wells with the same condition was scratched off with a Cell scraper and transferred into a 1.5 ml tube. Centrifugation at 4000 rpm at 4 °C was performed for 3 min and supernatant was discarded. The pellet was resuspended in 20 µl of 99 % RIPA buffer and 1 % Protein Inhibitor Cocktail solution. Proteins were extracted by passing the cell suspension several times through a narrow needle and thereby breaking up the cell membranes. The tubes were stored at -20 °C until further usage.

4.2.8.2. SDS-PAGE

A polyacrylamide gel electrophoresis was performed in order to separate the proteins by molecular weight. First, a resolving gel was produced and filled between two glass plates, which were hold together by a gel caster. Second, the stacking gel was filled on top of the polymerized resolving gel and a comb was placed in it to preform the slots for the samples. Ingredients of the stacking gel and the resolving gel are shown in table 4.6. After 20 min the glass plates with the gel were wrapped in plastic foil and stored at 4 °C until the next day. The gel was then put into an electrophoresis chamber which was filled with Running buffer. Samples were thawed, homogenized by passing through a narrow needle and centrifuged with 13,200 rpm at 4 °C for 4 min and 20 µl of the supernatant was mixed with 8 µl of Loading buffer. After a heating period at 95 °C for 7.5 min the

Stacking gel		Resolving gel	
2.1 ml	Water	4 ml	Water
0.38 ml	1.5 M Tris (pH 6.8)	2.5 ml	1.5 M Tris (pH 6.8)
0.03 ml	SDS 10 %	0.1 ml	SDS 10 %
0.5 ml	Acrylamide mixture	3.3 ml	Acrylamide mixture
0.03 ml	Ammonium persulfate 10 %	0.1 ml	Ammonium persulfate 10 %
0.003 ml	TEMED	0.004 ml	TEMED

Table 4.6. **Ingredients for SDS-PAGE**

samples were loaded in the slots of the gel. One slot was filled with 7 µl of a 1 kb marker to observe the progress of the protein bands and later on to determine the molecular weight of the labeled proteins. The gel was now set under an electric field. Due to the building of negatively charged micelles caused by SDS, the proteins migrated towards the anode. Under a constant voltage of 70 V the proteins passed through the large pores of the stacking gel within 15 min. The voltage was then switched to 120 V and ran for 2 h. The dense matrix of the resolving gel allowed smaller proteins to run faster, while bigger proteins were slower. In that way a separation according to molecular weight was achieved.

4.2.8.3. Membrane Transfer

For the transfer of the proteins from the SDS gel onto a nitrocellulose membrane the gel was cut into the right size and placed in a cassette between filter paper and the membrane. To avoid drying of the gel, the filter paper, the nitrocellulose membrane and the gel itself were immersed in transfer buffer. Bubbles between the different layers were removed by a roller. The cassette was closed, placed into an electrophoresis chamber and orientated in a way that the gel was facing towards the negative pole while the nitrocellulose membrane faced the positive pole. An electrical voltage of 55 V was applied for 2 h and the gel was permanently cooled by a block of ice. Meanwhile the negatively charged proteins could move towards the anode and bind to the membrane.

4.2.8.4. Detection and Analysis

The following steps were performed on a shaker. After blotting the sample proteins to the membrane, the efficacy of the transfer was verified with a Ponceau dye which colored the protein bands red. Before and after this step, the membrane was washed 3 times

with TBST. Then the membrane was saturated with 2.5 % milk in TBST solution for 1 h at room temperature. This was necessary to prevent unspecific antibody binding. The membrane was incubated overnight at 4 °C with Hif-1α antibody which was diluted in 5 % milk solution. Through the previous blocking process all binding sites were occupied with either sample or milk protein and the detection antibody could therefore exclusively bind to the sample protein. At the next day the membrane was rinsed three times with TBST for 5 min and incubated for 1.5 h with the secondary antibody. The secondary antibody was directed against the Fc part of mouse antibodies and was linked to a horseradish peroxidase enzyme at its own Fc part. The membrane was washed three times with TBST for 5 min to remove redundant antibody. Afterwards it was put into a plastic foil and incubated for 1min in the dark with ECL detection reagent. The horseradish peroxidase interaction with the ECL substrate results in enhanced chemiluminescence. The membrane was transferred into a hypercassette and covered with an autoradiography film in a darkroom. The incubation time was varied to achieve an optimal contrast range. The autoradiography film was taken out and immersed in developer solution, rinsed with water and with fixer solution. Wherever the sample protein bound with the antibodies and the horseradish peroxidase was attached to the membrane, chemiluminescence was detected by the autoradiography film. In order to relate the received bands to the total protein amount, another detection process was performed with a ß-actin antibody as primary antibody. ß-actin is a structure protein, which is present in all eukaryotic cells. To perform this detection, the membrane first had to be deliberated of the bound antibodies. Therefore it was washed 1 min with water followed by 30 min incubation with stripping solution, another washing step with water and another 30 min of stripping. After another blocking step, the procedure was the same as done with the Hif-1α antibody. The autoradiography films were sticked on a white paper and scanned as a tif file. The bands were analyzed with Image J.

4.2.9. Statistical Data Analysis

Quadruplicate wells were used for the individual experiments. Each experiment was repeated thrice. Data were analyzed with one way analysis of variance, followed by post hoc analysis using the Bonferroni method. A p value of $<0{,}05$ was considered significant and marked with an asterisk.

5. Results

5.1. Influence of Temozolomide on Proliferation

An antiproliferative effect on pituitary tumor cells was postulated based on previous experiments with temozolomide, but only sparse *in vitro* data exists so far. In this study different methods were used to assess the influence of temozolomide on proliferation and metabolic activity of pituitary cells in cell culture. For the experiments we used rodent pituitary cells lines (GH3 and AtT-20) and human pituitary adenomas in primary culture. Additionally, some experiments were performed using primary culture of rat pituitaries in order to obtain information on how temozolomide acts on healthy pituitary cells.

5.1.1. Direct Cell Counting

For the GH3 cell line, a dose dependent reduction of the cell number was observed as measured by the Scepter® tool (see figure 5.1). The effect was more prominent after 4 hours than after 24 and 48 hours. After 4 hours the number of cells stimulated with

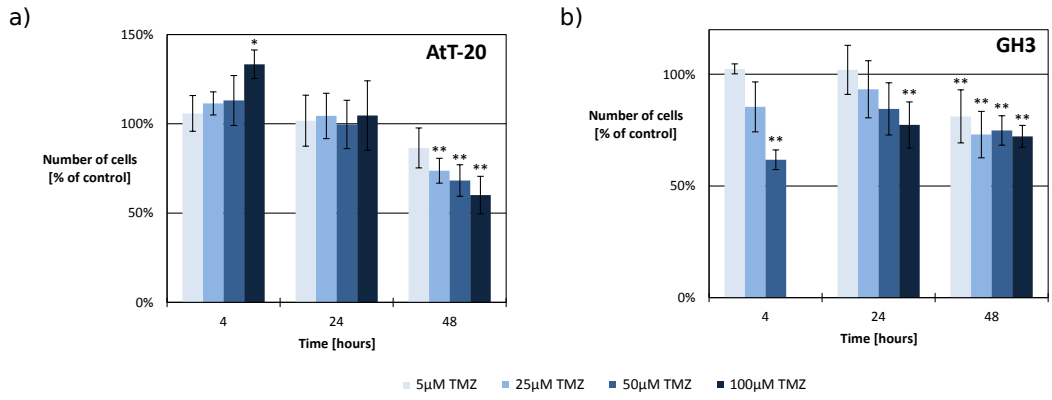

Figure 5.1. **Cell count of cell lines**
Cells were stimulated with different concentrations of temozolomide. The number of cells per well was measured with the Scepter® tool after 4, 24 and 48 hours. Columns indicate the number of cells in percentage of control. Results for AtT-20 cells are shown in **a)**. Results for GH3 cells are shown in **b)**.

100 µM temozolomide was reduced to 42% [±8%], after 24 hours the reduction accounted for 77% [±10%] and after 48 hours the cell number was reduced to 72% [±5%]. In the AtT-20 cell line, after an initial dose dependent increase in cell number to 133% [±8%]l after 4 hours of stimulation with 100 µM temozolomide, a strong decrease to 60% [±10%] was evident after 48 hours.

5.1.2. ^{3}H-thymidine Incorporation

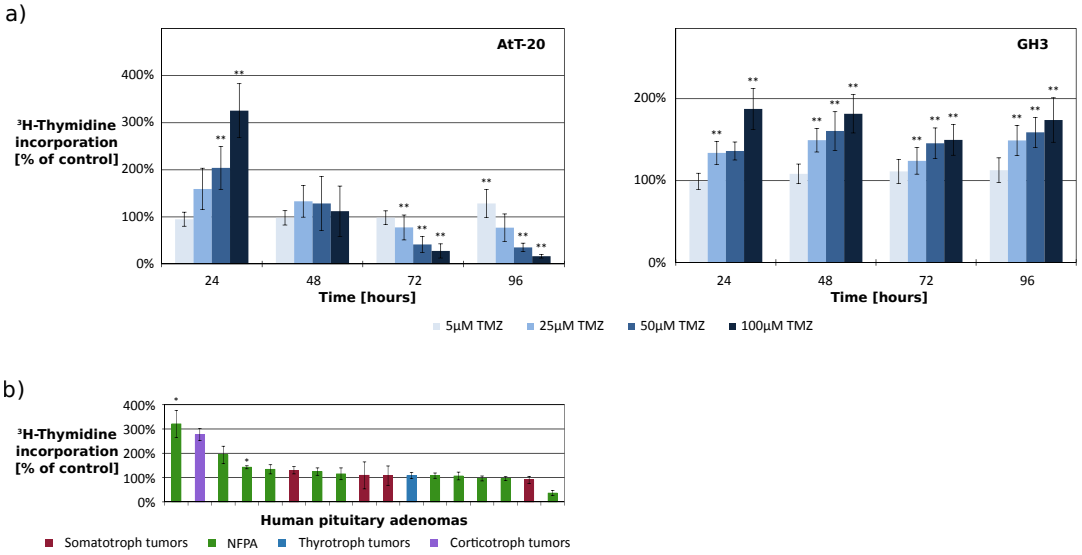

Figure 5.2. **^{3}H-thymidine incorporation in cell lines and tumors**
Cells were stimulated with different concentrations of temozolomide. ^{3}H-thymidine was added for 24 hours to the adenoma cells and for 4 hours to the cell lines. After the respective time points, radioactivity was measured in a scintillation counter. Results are shown for AtT-20 (left) and GH3 cells (right) in **a)**. Incorporation rates of different pituitary tumors are shown in **b)** and each column represents ^{3}H-thymidine incorporation at 100µm temozolomide from one tumor.

Figure 5.2 shows the temozolomide induced changes in ^{3}H-thymidine incorporation in the rodent pituitary cell lines. In the GH3 cell line, ^{3}H-thymidine incorporation was dose-dependently increased to 174% [±27%] of control after 96 hours [p<0,001]. The increased incorporation was evident throughout all 4 days of treatment. For all tested temozolomide concentrations, no time-dependent trend was observed since levels of ^{3}H-thymidine incorporation remain constant of time. In contrast to GH3 cells, a time dependent change was observed for AtT-20 cells. While the incorporation was strongly increased to over 300% of control after 24 hours [p<0,001], this trend was inverted in the following days. After 24 hours of temozolomide treatment, the incorporation rates in all measured temozolomide concentrations are reduced to the level of incorporation

observed for the control. Strikingly after three and four days of temozolomide treatment a clear dose-dependent trend towards a strong reduction of ^{3}H-thymidine incorporation was observed [p<0,001]. After four days of temozolomide treatment a maximal reduction to 17% with 100 µM temozolomide was evident in the AtT-20 cell line.

Alongside the measurements in the cell lines, ^{3}H-thymidine incorporation of different human pituitary adenomas after 24 hours treatment with 100 µM temozolomide was also determined. Investigation of longer incubation time periods showed a time-independent response regarding ^{3}H-thymidine incorporation and therefore no significant changes compared to 24 hours of treatment were observed (data not shown). Due to the limited amount of material, a standard incubation period of 24 hours was chosen. Changes of ^{3}H-thymidine incorporation after 24 hours in the human adenomas are also shown in figure 5.2c. Only cells of one adenoma showed a clearly visible, but not significant reduction of ^{3}H-thymidine incorporation [p=0,079]. In contrast, an increased incorporation of ^{3}H-thymidine (more than 10% compared to control) was observed for eight tumors. Two tumors showed a highly increased incorporation that doubled the incorporation rate of the control. However, the majority of adenomas (15 of 17) showed no significant change in ^{3}H-thymidine incorporation, similar to the observations made with the GH3 cells. There was no significant difference between the secretion types of the investigated adenomas [p=0,613].

5.1.3. Colony Formation Assay

The number of colonies formed after temozolomide treatment was strongly reduced in both cell lines (see figure 5.3). In the AtT-20 cell line, the number of colonies was reduced to 57% [±17%] after one and to 28% [±14%] after two weeks of treatment with 100 µM temozolomide. A significant reduction of colony number was already present with 25 µM temozolomide. It should be noted that no changes in colony size were induced by temozolomide (see figure 5.4). Colonies of GH3 cells were reduced to 57% [±6%] after one and to 43% [±22%] after two weeks treatment with 100 µM temozolomide. No significant reduction of colony formation of GH3 cells was observed for low temozolomide concentrations, indicating that the cells were less sensitive towards temozolomide treatment compared to the AtT-20 cells. In agreement with the findings for GH3 cells, no changes were observed regarding colony size after temozolomide treatment.

5.2. Influence of Temozolomide on Hormone Production

The influence of temozolomide on the production of hormones of cells from the AtT-20 and GH3 cell lines and cells from primary culture of human adenomas and pituitaries of healthy rats was investigated using radioimmunoassays.

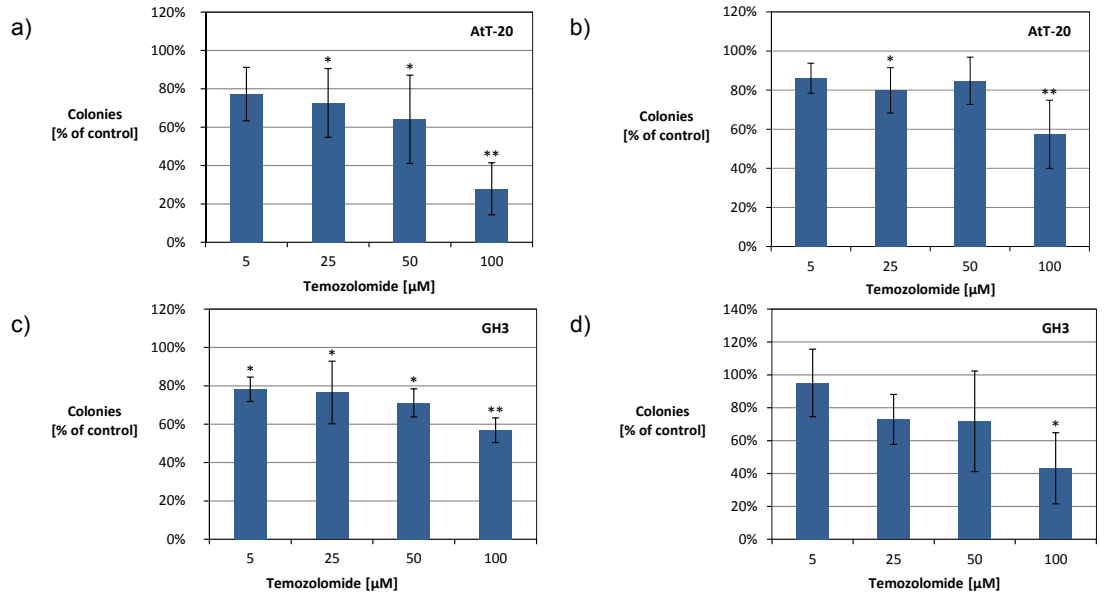

Figure 5.3. **Colony formation in pituitary cell lines**
Cells were seeded out in 6-well plates with low density and stimulated with different concentrations of temozolomide. After the respective time periods, colonies were stained and dried. The plates were scanned and analyzed with the Clono program [170]. **a)** shows the number of AtT-20 cell colonies in % of control after 1 week of attachment followed by 1 week of temozolomide stimulation. **b)** shows the number of AtT-20 cell colonies in % of control after 1 day of attachment followed by 2 weeks of temozolomide stimulation. **c)** shows the number of GH3 cell colonies in % of control after 1 week of attachment followed by 1 week of temozolomide stimulation. **d)** shows the number of GH3 cell colonies in % of control after 1 day of attachment followed by 2 weeks of temozolomide stimulation.

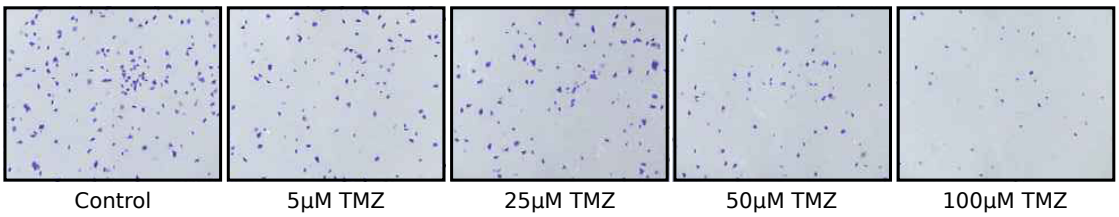

Figure 5.4. **Colony formation in AtT-20 cells**

5.2.1. Effects on Cell Lines

The cell number of each well was measured with the Scepter® while hormone production was determined by radioimmunoassay and normalized to the number of cells. In total, there was a decrease in the normalized ACTH production (see figure 5.5). The results show that after 4 hours of temozolomide treatment, the normalized ACTH production in AtT-20 cells decreased to 70% of control, whereas after 48 hours the hormone concentration was increased to 135% by treatment with 100 µM temozolomide compared to untreated cells. PRL and GH production were investigated in the GH3 cell line. For both hormones, production per cell was dose-dependently increased after 4 and 24 hours.

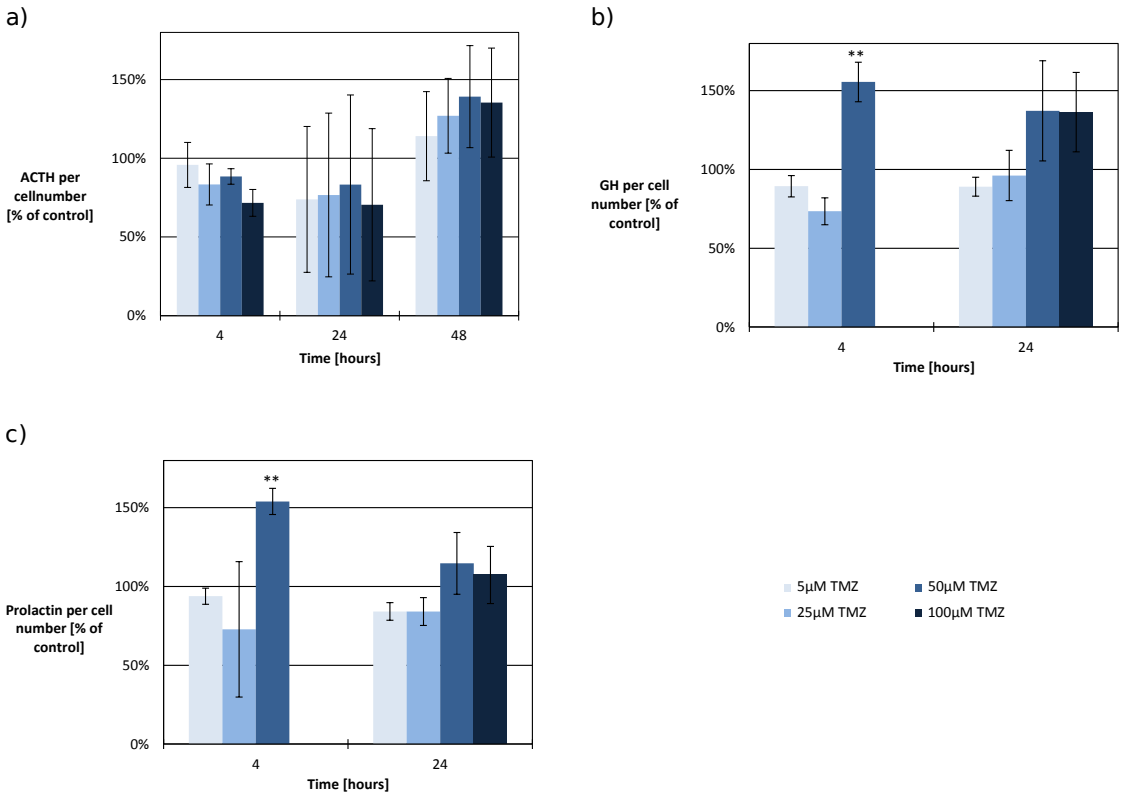

Figure 5.5. **Hormone production in pituitary cell lines**
a) shows ACTH production per cell number in AtT-20 cells after 4, 24 and 48 hours stimulation with different concentrations of temozolomide.
b) shows GH production per cell number in GH3 cells after 4 and 24 hours stimulation with different concentrations of temozolomide.
c) shows PRL production per cell number in GH3 cells after 4 and 24 hours stimulation with different concentrations of temozolomide.

5.2.2. Effects on Primary Culture of Rat Pituitary Cells

In primary culture of normal rat pituitaries, ACTH production after 4 as well as 24 hours of treatment was not affected by temozolomide treatment (see figure 5.6). The same was true when cells were additionally treated with CRH to achieve higher basal levels of ACTH. Similar results were observed regarding the influence of temozolomide

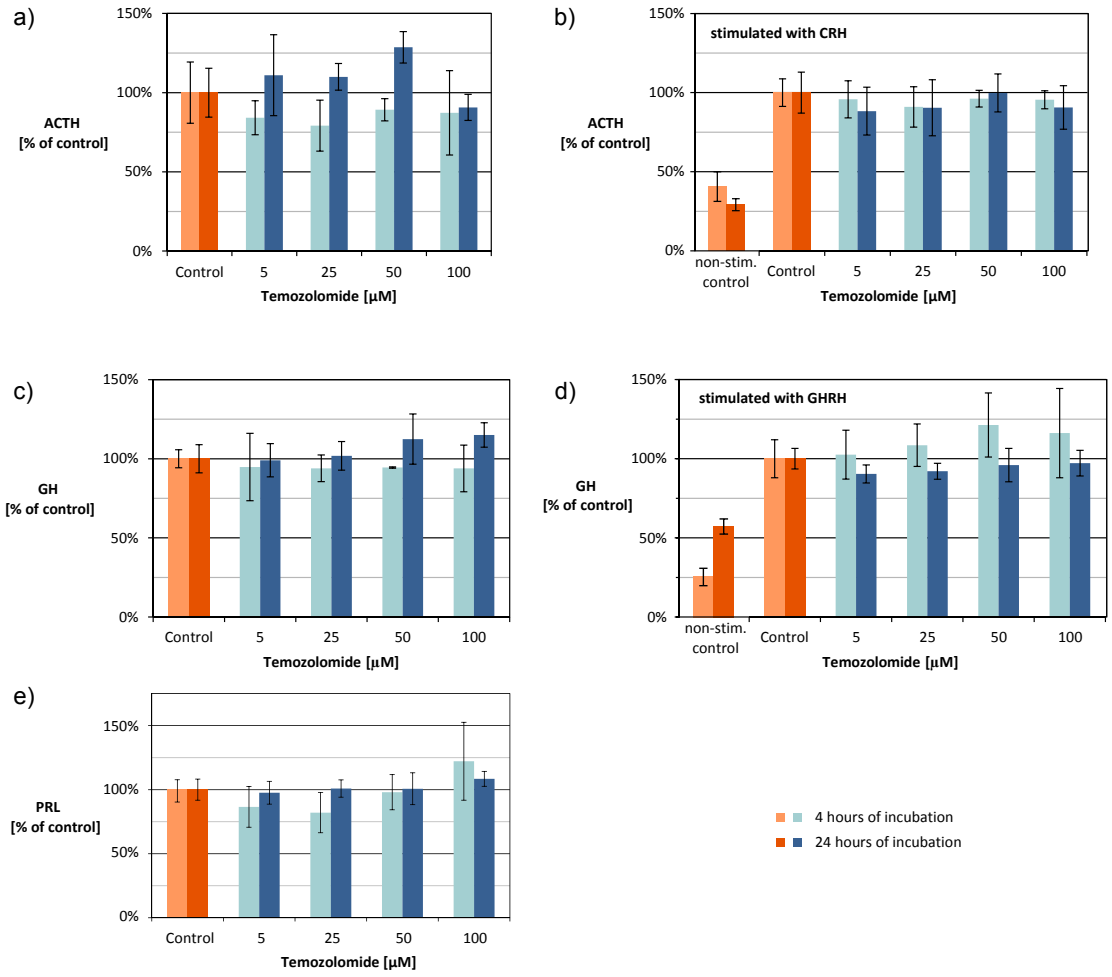

Figure 5.6. **Hormone production in rat pituitary cells**
ACTH production in rat pituitary cells after stimulation with different concentrations of temozolomide is shown in **a)**. ACTH production after stimulation with different concentrations of temozolomide and additional corticotropin releasing hormone for enhanced ACTH secretion is shown in **b)**. GH production in rat pituitary cells after stimulation with different concentrations of temozolomide is shown in **c)**. GH production after stimulation with different concentrations of temozolomide and additional growth hormone releasing hormone for enhanced GH secretion is shown in **d)**. PRL production in rat pituitary cells after stimulation with different concentrations of temozolomide is shown in **e)**.

on the production of growth hormone. There was no change in production after 4 or 24 hours temozolomide treatment. No change in hormone production was seen when cells were additionally stimulated with GHRH to generate higher basal levels of growth hormone. Comparatively, prolactin production was also not affected by 4 or 24 hours treatment.

5.2.3. Effects on Primary Culture of Human Adenomas

Production of growth hormone in five acromegalic tumors was not changed by 24 hours treatment with maximal temozolomide concentrations of 100 µM (see figure 5.7). Additional GH-releasing hormone stimulation did not affect the results (data not shown). ACTH production in a corticotroph adenoma was insignificantly reduced from 177 ng/ml [±30 ng/ml] in control to 143 ng/ml [±18 ng/ml] with 100 µM temozolomide. Similarily in one investigated thyrotroph tumor, TSH levels were reduced from 0,56 mlU/l [±0,2 mlU/l] to 0,47 ml/U [±0,06 mlU/l].

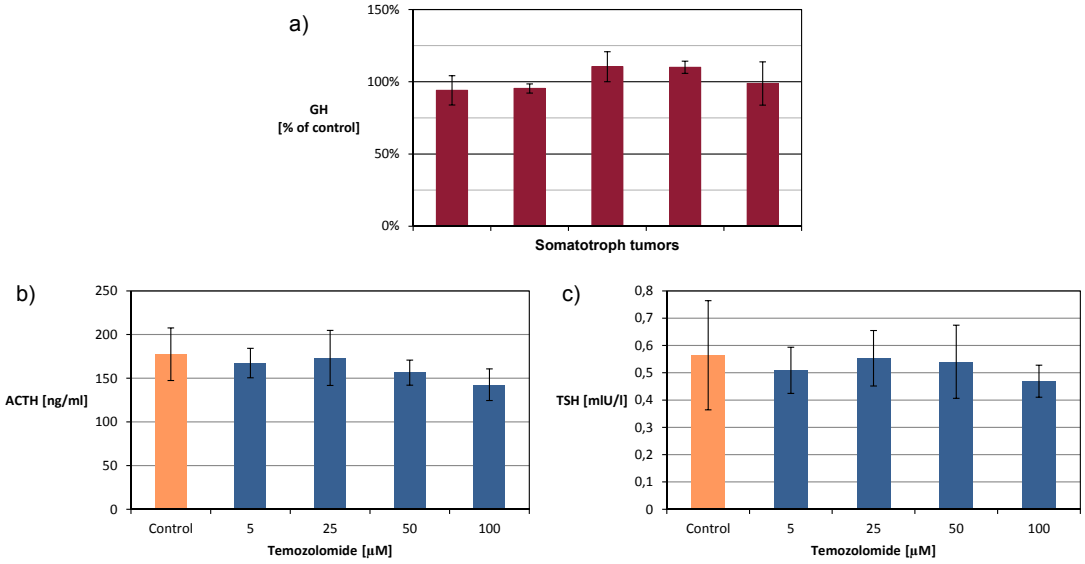

Figure 5.7. **Hormone production in pituitary tumors**
Percentual changes in growth hormone production, as measured by radioimmunoassay in human acromegalic adenomas after stimulation with 100µm temozolomide are shown in **a)**. Each column represents one somatotroph tumor. **b)** Changes in ACTH production of one adrenocorticotroph tumor after stimulation with different concentrations of temozolomide. **c)** Changes in TSH production of one thyrotroph tumor after stimulation with different concentrations of temozolomide

5.3. Influence of Temozolomide on Neovascularization

The influence of temozolomide on the production of two factors involved in the process of angiogenesis, Hif-1α and VEGF, was examined using ELISA and Western Blot. Measurements were performed in primary culture of human pituitary adenomas. Additional treatment with $CoCl_2$ was performed in order to mimic hypoxic conditions, since under hypoxic conditions, a higher basal VEGF and Hif-1α production is measured.

5.3.1. Influence on VEGF-production

VEGF-production was assessed using specific ELISA in 11 pituitary adenomas in the presence and absence of a simultaneous $CoCl_2$ stimulation, which increases basal VEGF production levels (see figures 5.8a) and 5.8b, respectively). With $CoCl_2$ stimulation, the decrease after 100 µM temozolomide treatment was 13% [±8,6%]. The inhibitory effect was also apparent in cells of five adenomas that were not treated with $CoCl_2$ (reduced VEGF production of 13% [±6,1%]). Yet, the observed inhibition in both experiments was not significant. The tumors used in these experiments included non-functioning or somatotroph adenomas. However, no difference between the types of tumors with regard to VEGF production was observed (compare figure 5.8).

5.3.2. Influence on Hif-1α Production

Production of Hif-1α by 7 human pituitary adenomas (6 non-functioning and 1 somatotroph) was determined using western blots. In order to achieve higher basal levels of Hif-1α, cells were exposed to 125 µM $CoCl_2$. Results were normalized to basal ß-actin levels as measured by the western blot. The effects of temozolomide treatment between

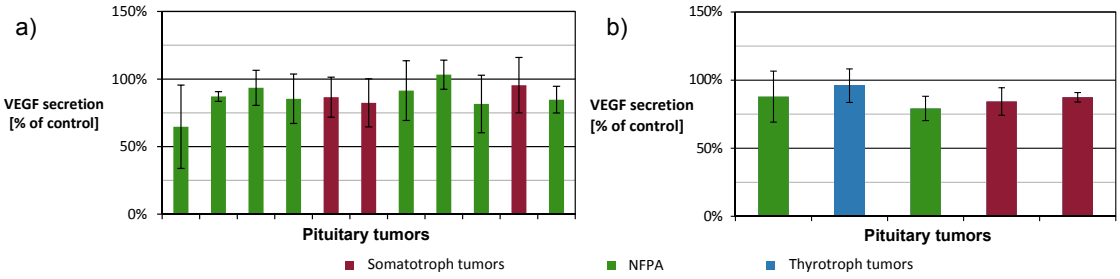

Figure 5.8. **VEGF production in pituitary tumors**
VEGF was measured by ELISA after 24 hours of stimulation with temozolomide. Columns in **a)** show VEGF production in pituitary tumors after stimulation with 100µm temozolomide and additional $CoCl_2$ treatment. Columns in **b)** show VEGF production in pituitary tumors after stimulation with 100µm temozolomide.

adenomas ranged from strong enhancement in two tumors to strong depression of Hif-1α production in one tumor (see figure 5.9). Each experiment was performed using temozolomide doses ranging from 5 to 100 µM, but no dose dependency was observed.

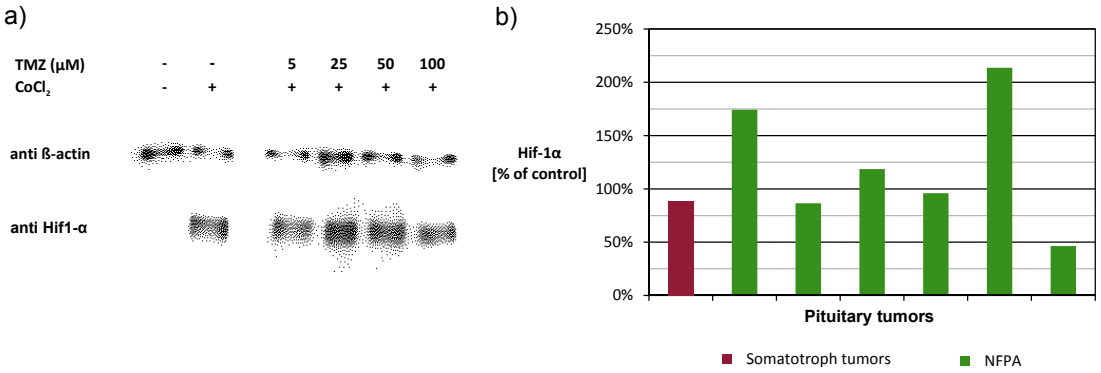

Figure 5.9. **Hif-1α production in pituitary tumors**
Hif-1α expression levels were measured using western blotting. **a)** Exemplary western blotting result of one tumor. Bands for ß-actin and Hif-1α are shown. $CoCl_2$ treatment and temozolomide treatment are indicated. **b)** Each column represents Hif-1α production after stimulation with 100µm temozolomide in one tumor.

6. Discussion

The goal of this study is the *in vitro* characterization of temozolomide regarding its effect on cell proliferation and hormon production. In this chapter, the limitations of the methods will be discussed and finally the results of the *in vitro* experiments will be discussed. The chapter finishes with an outlook and a summary.

6.1. Discussion of Methods

Some limitations of the methods used in this study trace back to uncertainties and missing knowledge regarding the adenoma samples obtained from patients after the surgery. The classification of the used human pituitary adenomas into subtypes was performed by the operating surgeon. Criteria used for the classification were therefore excessive hormone secretion and clinical features that proposed the classification to a certain adenoma subtype. Data of the immunohistological identification of the tumor type were not obtained, because there was not enough material left for a histopathological classification. We assume, that the pre-surgical laboratory investigations of the patients from which the pituitary tissue in this study was obtained, included an evaluation of all pituitary hormones as recommended by the WHO guidelines [172].

Moreover no differentiation between tumors that had received prior treatment and those that hadn't been treated at all was done in this study. Consequently, no distinction was made between the types of pretreatment.

The tumors that were obtained from the surgeons mainly include those tumors that could not be controlled medically and therefore needed surgical removal. As a result, the set of tumors used in this study is biased towards more aggressive pituitary tumors. This fact is however reflecting clinical reality as temozolomide is only applicable for those tumors failing conventional medical treatment or surgery. The results consequently do not represent an overview about all types of pituitary adenomas, but only about those failing medical treatment or requiring surgery for any other reason. As a consequence, the investigated adenomas included mainly non-funtioning pituitary adenomas and somatotroph tumors. Lactotroph or gonadotroph tumors could not be obtained for preparation.

Another potential bias lies within the acquisition of the tumors. Although the viability of the tumor cells was verified for every obained sample, it remains unknown how resection, transport and processing steps required for cell culture have influenced the tumors response to the chemotherapeutic agent. In order to achieve a better picture of the *in vivo* situation, experiments using different pituitary cell types were combined. The murine pituitary cell lines serve as a model for the fast growing pituitary tumors and have the advantages to be easy to handle and to generate reproducible results. The constant cultivation however might lead to different results than would be obtained regarding the original tumor cells. In order to compensate for this effect, we also used cells in primary culture.

Other limitations of the study arise from the *in vitro* design: of course, the results obtained from cell culture studies cannot be translated into the *in vivo* situation without critical assessment. Additional aspects must be considered in the complex body. *In vivo* it has been shown, that temozolomide reduces tumor mass and has an inhibitory effect on angiogenesis, which might add to the observed good clinical outcome [173]. Besides that, temozolomide has been shown to cause local hemorrhage, fibrosis and necrosis [151, 174, 175]. While measuring effects on cell proliferation *in vitro* is easily achieved, measuring effects on angiogenesis is more difficult. Angiogenesis and the supply of oxygen is not a limiting factor for the growth of single cells in cell culture, but it is essential for tumor cells growing in tissue. The measurement of a single factor involved in angiogenesis was used in this study as a read-out for effects on angiogenesis, but it should be noted that it is only a single component of the complex system of angiogenesis. More factors need to be taken into account to achieve a more accurate picture.

Previous *in vitro* studies that showed a strong effect of temozolomide in GH3 and AtT-20 cells such as Dai et al.[149] used a concentration of 250 µM TMZ to stimulate cells. In this study, a maximum dose of 100 µM TMZ was chosen to more accurately reflect plasma and intratumour concentrations that have been determined in previous clinical trials. In the conventional schedule with a daily dose of 200mg/m^2 TMZ, plasma concentrations range from 0,1 to 16 µg/mL, corresponding to doses of 0,5 to 82 µM TMZ. Accumulation over the treatment period of 5 days has not been observed [176, 177, 178, 93]. In this study, temozolomide concentrations range from 5 to 100 µM and therefore perfectly mimic the temozolomide concentrations found *in vivo*.

As a measure of cell proliferation, the ^{3}H-thymidine incorporation method is often used in cell culture. ^{3}H-thymidine incorporation and clonogenic assays have been shown to highly correlate with clinical outcome [179]. In this study, the mechanism of action of temozolomide generates increased mononucleotide uptake before inducing apoptosis

in the cell. Therefore, an initial increase in ^{3}H-thymidine incorporation was expected. After all, for a direct measurement of proliferation, the ^{3}H-thymidine incorporation could not be used as solely method, because a decrease in incorporation could either be the effect of a reduction in cell number, or a decrease in metabolic activity of a greater cell number. Hence, the study was extended to complementary methods that measure cell proliferation: Direct cell counting with the Scepter® and a clonogenic assay. As suggested by Hueng et al. the measured total hormonal production was normalized to the number of cells allowing to evaluate a specific effect on hormone production [180]. To this end, the data obtained with the Scepter® tool were used, because it is a fast and accurate method of determining cell numbers [181].

Clonogenic assays are used to provide information on how the drug influences the capacity of the cells to build colonies and their capacity to proliferate. It was shown that the capacity of colony formation influences the *in vivo* capacity of the tumor to develop micrometastases. The colony formation assay that we used had a simple, monolayer design. Obviously this is just a model and allows no definite conclusions for the transfer to the *in vivo* situation. It provides only two-dimensional cell to cell contacts and does not acclaim for the multiple other factors involved for the formation of a metastasis *in vivo* [182]. Noticeably in a study where single layer culture methods and three-dimensional spheroid models were compared, alkylating drugs showed no difference in toxicity [183]. A possible methodical flaw of this assay is the erroneous confusion of cell clumps and cell colonies during the counting process.

Neovascularization is an important mechanism for tumors to acquire access to nutrients and oxygen, which is needed for tumor growth. VEGF has been shown to be one of the most important factors in angiogenesis. For measuring a possible effect of temozolomide on neovascularization, we measured VEGF expression in human pituitary adenomas with or without additional treatment with CoCl2. CoCl2 has been shown to closely mimic hypoxic conditions and is therefore used in our experiments as a model for hypoxia [184]. To create a better picture of the effects on neovascularization, we also evaluated the production of Hif-1α. Hif-1 is a transcription factor that is responsible for the regulation of multiple important genes for a cellular adaption to hypoxic stress. It is constitutively expressed and degraded under normoxic conditions. In hypoxia, degradation is prevented by a redox-dependent pathway [185].

6.2. Results in the Context Of Literature

6.2.1. Temozolomide Inhibits Proliferation and Clonogenic Potential of Pituitary Cells

Evaluating the effects on proliferation of the tumoral pituitary cells was one of the main goals of this thesis. In this study, proliferation was measured by direct cell counting, a clonogenic assay and ^{3}H-thymidin incorporation. These experiments were conducted with murine pituitary cell lines and murine and human pituitary cells in primary culture.

The direct cell counts of the pituitary cell lines showed a significant reduction after temozolomide treatment. The AtT-20 cells were reduced to 60% after 48 hours and the GH3 cell number was reduced to 72%. The reduction in proliferation that we observed in the pituitary cell lines are in agreement with the study of Sheehan et al. [167] and Ma et al.[168] who already demonstrated suppression of growth in the MMQ, GH, AtT-20 and αT3-1 pituitary cell lines.

In the clonogenic assay we showed for the first time that temozolomide could effectively suppress the clonogenic potential of pituitary cells. Both, the AtT-20 and the GH3 cell line demonstrated a markedly decreased number of cell colonies. Moreover temozolomide has already been shown to reduce the metastatic potential in cells of several derivations [186].

The results of the ^{3}H-thymidine assay show that incorporation of labeled thymidine in AtT-20 cells has two phases: first, a dose-dependent increase after 24 hours and secondly a strong reduction after 72 and 96 hours. A possible explanation for this observation lies within the mechanism of temozolomide function. As already mentioned previously, the O6 methylated guanine mispairs with thymine, therefore leading to activation of mismatch repair which in turn leads to increased thymidine incorporation. After the cells died from the cytotoxic effects of temozolomide, incorporation decreases as observed for long incorporation times. Interestingly, the GH3 cells showed a constant increase in ^{3}H-thymidine incorporation, though direct cell counting revealed a reduction in cell number. This can be explained by a strongly increased ^{3}H-thymidine incorporation. However, levels of the corresponding proteins MGMT or MSH6 wer not determined in this study. Sheehan et al. observed a similar dynamic using the MTT assay, an assay that assesses metabolic activity to determine cell viability. GH3 cells first showed good response to temozolomide, but then seemed to recover. We assume that the GH3 cells are less sensitive to temozolomide than the AtT-20 cells. By whatsoever mechanism, they show upregulated DNA synthesis in the remaining cells. Understanding the underlying mechanism should be the goal of future studies.

The human pituitary adenomas that we investigated showed mostly no change in ^{3}H-thymidine incorporation. Only one tumor showed a strong, but not significant reduction of incorporation. Instead, eight tumors demonstrated increased incorporation. These results do not correspond to the observations reported in the literature. A possible reason might be the same effect that we already observed with the GH3 cells: ^{3}H-thymidine incorporation might not have been an accurate method to determine changes in cell viability or proliferation induced specifically by temozolomide because of its interference with the mechanism of action of temozolomide. Other metabolism based assays would have been more appropriate for this purpose.

In general, the primary culture cells that we investigated seemed to be less responsive to temozolomide compared to the pituitary cell lines. This is a common phenomenon, because cell lines have a higher proliferative index and are assumed to mimic the situation in an aggressive tumor. The primary cells thus have low growth rates and less metabolism. In the literature that is summarized in appendix A, the effect of temozolomide in pituitary tumors creates a mixed picture, with 58% of the treated patients responding. In some patients large and quick reductions in hormone levels and tumor size were observed while for other patients a slow but steady decline of hormone levels was observed. But still, there are many patients who either did not show any response or suffered from recurrences. This heterogeneous situation resembles the observations in cell culture that we investigated. These differences strongly suggest the presence of mechanisms of resistance that remain to be unvealed.

6.2.2. Temozolomide Reduces Hormone Levels Through Reduction of Cell Number

In the treatment of patients with pituitary neoplasms, it is not just important to know whether the treatment has an effect on the tumor itself, but also how it affects the surrounding healthy pituitary tissue. While radiotherapy and surgery leave patients with a high rate of hypopituitarism, this does not occur under medical treatment with dopamine agonists or somatostatin analogues. Our results in primary culture indicate that there is no effect on secretion of ACTH, PRL and GH of normal, healthy rat pituitary cells. We investigated this with and without additional stimulation with CRH and GHRH in the case of ACTH and GH measurements. These results are in agreement with the observations gathered from the clinical experience with temozolomide. Regular measurements of pituitary hormone levels in patients treated with temozolomide gave no indications of a temozolomide induced hypopituitarism. Nevertheless, a previous study based on five cases suggested temozolomide to potentially cause central diabetes

insipidus [163]. However, it remains unknown whether diabetes insipidus was caused by a temozolomide induced change of ADH production, secretion or storage. Considering the large number of patients treated with temozolomide and the low number of reported cases of diabetes insipidus, the development of this side effect seems to be a rare event. Further studies and longer experience with the use of the drug will reveal a possible connection between central diabetes insipidus and temozolomide use.

Oversecretion of pituitary hormones is one of the most important harms caused by pituitary tumors. Dopamine agonists and somatostatin analogues are successfully used to reduce hormonal excess in the majority of patients. However, some patients never show any response or develop resistance throughout the therapy. In the case reports listed in appendix A, a temozolomide induced reduction in hormone secretion was observed in 79% of the prolactin secreting tumors, in 81% of the corticotroph tumors and in 50% of the growth hormone secreting tumors. The single gonadotroph tumor that was reported in the literature showed no reduction in hormone secretion. Apart from that we found no case, where temozolomide had been used in a thytotrop tumor. The correlation between a positive radiological response and a biochemical decrease in hormones is very high. All of the tumors, where a reduction in hormone secretion is mentioned also show a radiological response. So far, there is only one case report, where a noticeable drop in hormone levels was not accompanied by a reduction in tumor size [187].

In our experiments we found no change in growth hormone production in the case of five somatotroph adenomas as well as slight, but not significant reductions of ACTH in one corticotroph and of TSH in one thyrotroph adenoma after treatment with a maximum dose of 100 µM TMZ. Unfortunately, no prolactin producing adenoma could be acquired. In comparison with the clinical experience that has already been made with temozolomide, this *in vitro* response rate seems to be low. A possible reason might be the low growth and metabolic rate of human pituitary adenomas in primary cell culture. To account for this effect, we provided additional stimulation of basal hormone secretion via GHRH and CRH, which enhanced basal secretion, but had no effect on temozolomide response. Finally, when considering the missing effect on proliferation of human pituitary adenomas seen in the ^{3}H-thymidine incorporation experiment and the lack of a specific hormone reducing effect as seen in the cell lines, these results contribute to the hypothesis, that there is no specific hormone suppressive effect of temozolomide.

Pituitary cell lines are used as a model for fast growing pituitary tumors. A hormone suppressive effect on pituitary cell lines has already been demonstrated by Sheehan et al. and Ma et al. The Ma group demonstrated the reduction of FSH and LH production in the gonadotroph αT3-1 cell line while Sheehan et al. already demonstrated a temo-

zolomide induced reduction in PRL secretion in the pituitary GH3 and MMQ cell lines. [167, 168]. In both studies non-physiological temozolomide concentrations were used and only total hormon secretion was measured. In order to reveal the specific effects on hormone secretion, we measured hormone production per cell. In the corticotroph AtT-20 cells, no significant change in ACTH production per cell was observed. The GH3 cell line showed no change on PRL production per cell but a slight increase in GH production per cell.

The findings suggest that a reduction of hormone secretion might rather be due to a reduction in cell number, than to a specific hormone suppressive effect on a cellular level. The considerable reductions in hormone levels that were found by Ma et al could therefore be explained by the deleterious effect on cell viability. Consequently in cases where tumor growth under temozolomide treatment is observed, a change of drug seems warranted as the mechanism of action of temozolomide induced effects on hormone secretion seem to be obtained by tumor shrinkage and not via specific inhibition of hormone secretion.

6.2.3. Temozolomide Inhibits Neovascularization

Neovascularization is a necessary step in tumorigenesis for gaining access to nutrients and oxygen and for the removal of unneeded metabolic products. Most human tumors exhibit an increased rate of angiogenesis compared to normal tissue. Pituitary tumors appear to be different. In a study consisting of 157 pituitary adenomas and 7 carcinomas, microvessel density as a parameter of angiogenesis was investigated. A small, but not significant correlation between microvessel density and invasiveness of the tumor was found. Microvessel density was higher in the investigated pituitary carcinomas in this study, but not in another study. In general pituitary adenomas seem to have a lower microvessel density than healthy pituitary tissue, which is supposed to result in the comparatively slow pace of pituitary tumor growth [188, 189].

A similar observation is made regarding VEGF expression of pituitary adenomas. VEGF is the most important signal protein involved in the formation of new blood vessels and recent findings even suppose a role in regulating growth of pituitary adenomas [190]. In benign pituitary adenomas, VEGF expression seems to be lower than in healthy pituitary tissue, but pituitary carcinomas have been shown to have upregulated VEGF expression compared to pituitary adenomas [191]. This is in agreement with the positive correlation between metastasizing and neovascularization that has been demonstrated for many other tumor types [192]. Discordant results were reported regarding the connection between pituitary tumor invasiveness, VEGF expression and microvessel density, partly due to methodological differences [193]. It is not yet known whether the low microvessel

density of pituitary adenomas is due to their low growth rate, an ingrowth of vessels of the pituitary gland or an inhibition of angiogenic factors. In spite of the inconsistent results in the literature, a VEGF reducing effect could be beneficial in case that future studies would reveal a definite link to tumor aggressiveness. A recent study using a mouse model of dopamine resistant prolactinomas showed that inhibition of VEGF resulted in a decrease of tumor size and reduction of prolactin content. Therapeutic targeting of VEGF therefore seems warranted in dopamine resistant prolactinomas [194].

By measuring VEGF and Hif-1α production of the temozolomide treated cells, we wanted to extend the current knowledge on how temozolomide influences angiogenesis. We compared basal to temozolomide induced changes in VEGF expression of human pituitary adenomas under normoxic and hypoxia-like conditions in primary culture. When cells were treated with a final dose of 100 µM temozolomide, a small, not significant reduction of 13% VEGF levels was apparent in five investigated adenomas. The same was true under hypoxia-like conditions that were induced by $CoCl_2$ treatment of the cells. A 13% reduction of VEGF levels of 11 human pituitary adenomas was generated by 100 µM temozolomide. These results indicate that there could be a small anti-angiogenic effect of temozolomide on pituitary adenomas. Restrictions must be made due to the small sample size and the fact that we examined mostly NFPA. Effects might be different among the adenoma types, but we did not have enough material to examine potential differences. Basal levels of VEGF protein expression and microvessel density have though been shown to differ among the adenoma types with growth hormone producing tumors having the lowest levels [189].

Hif-1 is a modulator of angiogenesis and promotes transcription of a large quantity of genes in response to hypoxic conditions, including VEGF. Hif-1 consists of two dimers, the α subunit which is oxygen and growth hormone regulated and the β subunit, which is constitutively expressed [195, 196]. We could not detect an effect of temozolomide on Hif-1α production in 7 human pituitary adenomas. The results varied greatly among the investigated tumors: Hif-1α production was strongly enhanced in two tumors and strongly suppressed in one tumor while no effect was observed in the remaining four tumors. However, since the sample size was very small, this complex respond pattern cannot be examined in detail. The great variation of Hif-1α levels could be explained by differences in temozolomide sensitivity or it might just have been a methodological problem. We did not assess the MGMT status of the investigated adenomas in order to correlate the findings. A greater sample size could maybe reveal if there is an effect of temozolomide on Hif-1α production or degradation. Earlier studies have shown the Hif-1 dependent VEGF production in common tumor angiogenesis, but Kim et al. found no

relation between Hif-1 and VEGF expression in a series of human pituitary adenomas [197, 198]. So far there have been no other studies investigating anti-angiogenic effects of temozolomide in pituitary cells, but reduction of microvessel density as well as Hif-1 and VEGF production by temozolomide have already been demonstrated in glioma cells [173, 199]. Likewise, angiogenesis of chicken chorioallantoic membranes was inhibited by temozolomide, namely by low, non-toxic concentrations of temozolomide [200]. Regarding the toxicity of temozolomide, angiogenesis does not seem to be a major factor, because the vascularization in pituitary adenomas is low in general. Nevertheless we could show a small VEGF-suppressive effect which might at least add up to the efficacy of temozolomide in the treatment of pituitary adenomas.

6.3. Outlooks

Temozolomide treatment is the last line treatment option for patients with aggressive pituitary adenomas and pituitary carcinomas. Although the observed response rate of 58% is quite high, there is still a large number of patients that do not benefit from temozolomide treatment. For these patients no clinically approved treatment options exist so far. Efforts have been made to identify new drugs that are effective in the treatment of resistant tumors. Studies on the mTOR inhibitors everolimus and rapamycin have demonstrated *in vitro* effectiveness in pituitary cell lines and primary culture of pituitary adenomas, especially in the combination with the somatostatin analogue octreotide [201, 202, 203]. However, a clinical application of an everolimus and octreotide combination in a patient with an ACTH producing pituitary carcinoma showed no success [204]. Nevertheless further trials with patients that do not respond to temozolomide treatment seem warranted, as laboratory results seem promising and other alternatives are rare.

Another option to receive higher response rates could be the combination of temozolomide and other drugs. Capecitabine, MGMT-inhibitors, PARP-inhibitors, pyrimethamine or antiangiogenic agents all have been used together with temozolomide *in vitro* or *in vivo*, but there is still insufficient data for a broad application of these combined treatments [142, 149, 147]. However, it must be ensured that the combination of two agents does not increase systemic toxicity or is potentially mutagenic.

New treatment regimens might also enhance temozolomide effectiveness. For glioblastomas, temozolomide has been shown to increase the sensitivity for radiation [205, 206]. This resulted in the remarkable improvement of survival rates from 10.4% for patients treated with radiation only to 26,5% treated with radiotherapy and concomitant temozolomide [135]. Studies in pituitary tumors investigating simultaneous radio-

chemotherapy and *in vitro* studies regarding a possible radio-sensitizing effect of temozolomide in pituitary cells remain to be done. Another possible strategy to improve treatment effectiveness is the initiation of temozolomide treatment before radiotherapy, a so called neoadjuvant approach. In patients suffering from glioblastoma, neoadjuvant treatment with temozolomide has already proven effective, but also in this case, studies in pituitary tumors haven't been performed yet [207, 208].

Besides from adding new drugs or radiation to the current treatment, variations of therapeutic schedules provide promising alternatives compared to the conventional temozolomide treatment of a $200\,mg/m^2$ daily dose during five-days every four weeks [209, 210]. Most regimens use lower doses of temozolomide for a longer time period, as laboratory findings suggested better efficacy without an increase in toxicity. However, some schedules have proven ineffective if compared with the conventional schedule [211]. One more point that still needs to be addressed is the optimal length of treatment. The experience with temozolomide has so far shown that on the one side, there are patients that develop recurrent disease after cessation of temozolomide [187], on the other side there are some patients that acquire temozolomide resistance, possibly due to selective pressure on the cells [123, 122]. A rationale for the optimal individual length of treatment still needs to be found.

Right now, temozolomide is only used in cases, where other treatment options already failed. Due to the low rate of side effects, it seems warranted to discuss the initiation of temozolomide at an earlier point of time. Therefore early identification of malignant tumors must be accomplished. First attempts to achieve better prognostic markers for malignant transformation or recurrence rate have already been suggested. A high Ki-67 index correlates with recurrency and therefore warrants close monitoring of MRI and hormone levels as well as early initiation of additional therapy [212, 213]. In addition to that, efforts should be made towards the better identification of patients that show temozolomide resistance. This will primarily be important as soon as alternatives to temozolomide treatment are found. Unlike in the treatment of glioblastomas, the MGMT status allows no accurate prediction of response to temozolomide in pituitary tumors. The MSH-6 status seems a promising marker to identify drug resistant tumors and it could be a potential target for future research (Compare chapter 2.2.2).

Finally a still futuristic approach might revolutionize antineoplastic therapy: local delivery of chemotherapeutic drugs through biodegradable polymer wafers is currently being approved for glioblastomas. A carmustine wafer has already been tested in phase II studies and showed promising results and a low side effect profile. Devices with temozolomide have already been designed and proved effective in a rodent gliosarcoma model

[214, 215, 216]. However safe and ready to use temozolomide containing devices still need to be designed and thoroughly tested before clinical application can be recommended.

In conclusion, based on the response rate of 58% in the published case reports, the low rate of side effects and the laboratory findings that support the antiproliferative and anti-angiogenic action of temozolomide, a further use in the following tumors is warranted: i) dopamine agonist and somatostatin analog resistant prolactinomas and somatotropinomas that require multiple surgeries and radiotherapy, ii) aggressive corticotroph, thyrotroph, gonadotroph and non-functioning pituitary adenomas and iii) pituitary carcinomas.

6.4. Summary

Pituitary adenomas are a common type of tumors with an estimated prevalence of 16.7% [9]. They present clinically either through excess of hormonal secretion or mass effects. The therapy of pituitary tumors has four main goals: i) reducing tumor mass, ii) reducing hormone levels iii), prevent or treat metastases and iv) preserve normal pituitary function. Usually, the tumors are benign and can be controlled through medication, radiotherapy or surgery. However, some tumors show locally aggressive behavior or recurrence to medical or surgical treatment regimens [45]. Until now, no legally approved treatment options for patients with these types of pituitary adenomas or pituitary carcinomas exist so far. Therefore, treatment options need to be urgently identified and characterized.

Temozolomide was shown to have anti-tumoral effects in several human neoplasms. Currently it is predominantly used for treating glioblastoma, anaplastic astrocytoma and malignant melanoma. In this study 103 cases of application of temozolomide in human pituitary adenoma and carcinoma are listed and summarized in appendix A. Response rates of temozolomide treatment varied among the investigated patients but with an average of 58 % responding, it is the best treatment option for this kind of patients. Patients with aggressive pituitary adenomas or carcinomas that responded to the treatment usually demonstrated tumor shrinkage and a reduction of hormone production in cases of hormone producing tumors. However the exact effects of temozolomide on pituitary cells remain poorly understood. To be precise, only two studies in pituitary cells, suggesting an antiproliferative effects or effects on hormone secretion have been published[167, 168].

The aim of this study is the investigation of the antiproliferative capacity of temozolomide in different types of pituitary cells and the effects on hormone secretion, neovas-

cularization and colony formation. In order to get a more precise picture of the effects, different pituitary cells were used in this study: Healthy rat pituitary cells in primary culture, human adenoma cells in primary culture as well as rat pituitary cells lines (GH3 and AtT-20 cell line). The set of different cell types was combined with a set of cell-based assays to determine cell proliferation, including ^{3}H-thymidine incorporation, direct cell counting and colony formation assays. To probe hormon production biochemical assays such as Western Blots, radioimmunoassays and ELISA were used.

In this study, proliferation of healthy rat pituitary and most human pituitary adenoma cells, both obtained from primary culture, were not affected according to ^{3}H-thymidine incorporation assays. However in the case of rat pituitary cell lines (GH3 and AtT-20 cell line), a reduction of cell proliferation was observed by ^{3}H-thymidine uptake as well as colony formation assays. The results obtained from cell lines further revealed that the thymidine incorporation in cells shows a complex time-dependence, which is suggested to be caused by an interfering increase of DNA repair activity induced by temozolomide stimulation. As a consequence, the antiproliferative effect of temozolomide was confirmed with direct cell counting assays for both cell lines.

Hormone measurements in primary culture of human pituitary adenomas showed no relevant hormone suppressive effects of temozolomide. Regarding healthy pituitary cells, no relevant effects on GH, ACTH and PRL production of normal rat pituitaries were found in primary culture. In contrast, the fast growing pituitary cell lines showed a decrease of the pituitary hormone ACTH. However, hormone production normalized to the cell number showed no reduction, suggesting that temozolomide has no specific inhibitory effect on hormone production.

Moreover, Hif-1α and VEGF production after temozolomide treatment was investigated to examine potential effects on neovascularization. VEGF production was slightly decreased, suggesting a modest inhibitory effect on neovascularization of human pituitary adenomas. In the case of Hif-1α production no clear trend was observed as temozolomide can increase or decrease the production in primary culture of human pituitary adenomas.

In this *in vitro* study, temozolomide was found to be a cytotoxic agent and capable of reducing the colony forming potential in the case of pituitary cell lines. These effects might be particular useful in the prevention of cerebral micrometastases deriving from pituitary carcinomas and therefore, the findings of this study allow to partially understand the previously reported successful application of temozolomide in the treatment of pituitary adenomas or carcinomas. The results of this study further suggest, that temozolomide solely acts as an antiproliferative agent. This renders temozolomide useful to reduce tumor size as well as the risk of metastasis, but in terms of hormon secretion of

pituitary tumors, a reduction is only achieved by reduction of tumor mass, not through a specific hormone reducing effect.

6.5. Zusammenfassung

Hypophysenadenome sind eine häufig vorkommende, gutartige Tumorart mit einer geschätzten Prävalenz von 16,7% [9]. In der Klinik wird oft eine erhöhte Hormonproduktion sowie eine Größenzunahme der Hypophysentumore beobachtet. Als Therapieziele gelten daher i) die Reduktion der Tumorgröße, ii) die Reduktion der Hormonproduktion, iii) die Vorbeugung oder Behandlung von Metastasen und iv) die Erhaltung der regulären Hypophysenfunktion. Größtenteils sind Hypophysentumore gutartig und können mittels medikamentöser Therapie, Bestrahlung oder operativer Resektion behandelt werden. Einige dieser Tumore zeigen jedoch infiltratives Wachstum und sind gegenüber konventionellen Therapiemethoden resistent[45]. Für Patienten, die an dieser Form von aggressiven Hypophysenadenomen oder Hypophysenkarzinomen erkrankt sind, gibt es kaum Behandlungsoptionen. Die Entwicklung neuer Therapiemethoden ist deshalb besonders wichtig.

Temozolomid hat in der Therapie verschiedener menschlicher Tumorerkrankungen bereits gute Erfolge erzielt. Es wird derzeit vor allem in der Behandlung von Glioblastomen, anaplastischen Astrozytomen und malignen Melanomen verwendet. In dieser Arbeit wurden insgesamt 103 publizierte Anwendungsfälle von Patienten gesammelt und zusammengefasst, die aufgrund eines Hypophysenadenoms oder -karzinoms mit Temozolomid behandelt wurden (siehe Anhang A). Die Ansprechrate der behandelten Patienten liegt bei durchschnittlich 58% und ist somit die momentan beste Therapieoption für Patienten mit dieser Art von Tumoren. Bei erfolgreichen Behandlungen aggressiver Hypophysenadenome oder Hypophysenkarzinome mit Temozolomid konnte eine Abnahme des Tumorvolumens sowie bei Hormon produzierenden Tumoren eine Reduktion der Hormonproduktion beobachtet werden. Die genauen Effekte von Temozolomid auf Hypophysenzellen sind allerdings noch nicht gut erforscht. Tatsächlich existieren lediglich zwei Studien mit Hypophysenzellen in denen ein antiproliferativer Effekt oder ein Effekt auf die Hormonsekretion gezeigt wurde[167, 168].

Das Ziel dieser Studie war es die antiproliferativen Fähigkeiten von Temozolomid in unterschiedlichen Hypophysenzellen zu untersuchen und die Auswirkungen auf Hormonsekretion, Neovaskularisation und Koloniebildung näher zu betrachten. Um möglichst aussagekräftige Ergebnisse über den zellulären Wirkmechanismus zu erhalten wurden gesunde Rattenhypophysenzellen in Primärkultur, menschliche Adenomzellen in Primär-

kultur und murine Zelllinien (GH3 und AtT-20 Zelllinien) untersucht. Diese Auswahl an Zelltypen wurde mit verschiedenen zellbiologischen Experimenten kombiniert, unter anderem die Quantifizierung des Einbaus an ^{3}H-Thymidin, direkte Messungen der Zellzahl sowie der Bestimmung der koloniebildenden Einheiten. Zusätzlich wurden Western Blots, Radioimmunassays und antikörperbasierte Nachweisverfahren (ELISA) verwendet um Hormonkonzentrationen zu bestimmen.

In dieser Studie konnte anhand des Einbaus an ^{3}H-Thymidin keine Auswirkung von Temozolomid auf die Proliferation von gesunden Rattenhypophysenzellen oder menschlichen Adenomzellen, jeweils in Primärkultur gehalten, festgestellt werden. Im Falle der murinen Zelllinien (GH3 und AtT-20 Zelllinien) konnte jedoch mit Hilfe des Einbaus an ^{3}H-Thymidin eine Reduzierung der Zellproliferation sowie eine Verringerung der koloniebildenden Einheiten durch Temozolomid festgestellen werden. Außerdem zeigen die Daten der Zelllinien, das der Thymidineinbau in Zellen einer komplexen Zeitabhängigkeit unterliegt, welche vermutlich von einer interferierenden, von Temozolomid ausgelösten, erhöhten Aktivität der DNA-Reparatur beeinflusst wird. Daher wurde die antiproliferative Wirkung von Temozolomid auf die Zelllinien mit direkten Messungen der Lebendzellzahl bestätigt.

Messungen der Hormonsekretion der menschlichen Adenomzellen in Primärkultur ergaben keine hemmende Wirkung von Temozolomid. Auch bei gesunden Rattenhypophysenzellen in Primärkultur blieb die Produktion von GH, ACTH oder PRL nach einer Stimulierung mit Temozolomid konstant. Im Gegensatz dazu sank im Falle der schnellwachsenden Zelllinien die produzierte Menge des Hormons ACTH. Allerdings, blieb auch bei den beiden Zelllinien die auf die Zellzahl normierte Hormonproduktion konstant, weshalb eine spezifische Hemmung der Hormonproduktion durch Temozolomid ausgeschlossen werden kann.

Um Rückschlüße auf einen potentiellen Einfluß von Temozolomid auf die Neovaskularisierung zu untersuchen, wurde die zelluläre Produktion von Hif-1α and VEGF bestimmt. Da die VEGF Produktion minimal reduziert wurde, hat Temozolomid vermutlich nur einen sehr geringen oder keinen Einfluß auf die Neovaskularisierung. Für die Produktion von Hif-1α konnte in dieser Studie keine Aussage getroffen werden, da sowohl erhöhte als auch reduzierte Botenstoffkonzentrationen festgestellt wurden.

In dieser *in vitro* Studie wurde im Falle der murinen Zelllinien eine zytotoxische Wirkung und eine Hemmung der koloniebildenden Einheiten durch Temozolomid nachgewiesen. Diese Wirkungen können vor allem bei der Vorbeugung der zerebralen Metastasenbildung durch Hypophysenkarzinome helfen, weshalb mit den Ergebnissen dieser Studie die publizierten erfolgreichen Behandlungen von Hypophysenadenomen oder -kar-

zinomen mit Temozolomid teilweise erklärt werden können. Die Ergebnisse dieser Studie legen nahe, dass Temozolomid ausschließlich antiproliferativ wirkt. Damit ist Temozolomid geeignet um die Tumorgröße und das Risiko von Metastasen zu reduzieren, eine Reduzierung der Hormonsekretion wird jedoch nur indirekt über eine reduzierte Tumormasse, nicht aber eine spezifische Hemmung der Hormonproduktion erzielt.

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A. Application of Temozolomide in the Treatment of Pituitary Adenomas

Table A.1. **Usage of temozolomide in the treatment of pituitary tumors**
MGMT immunohistochemistry: <10%=Low, 11-50%=Intermediate, >51%=High
Age = Age at diagnosis. Status on April 15, 2015.

Tumor	**Age**	**Gender**	**Response**			**MGMT IHC**	**MGMT Promoter**	**Author**
			Clinical	**Radiological**	**Biochemical**			
Prolactin carcinoma	61	male	Yes	Yes	Yes	No data	No data	Zhu et al. (2004) [150]
Prolactinoma	46	male	Yes	Yes	PRL fell from 1838 µg/l to 30 µg/l	Low	No data	Syro et al. (2006) [174, 151]
Prolactin carcinoma	26	male	Only initially	Only initially	Yes	No data	No data	Fadul et al. (2006) [153]
Gonadotroph carcinoma	38	male	Yes	Yes	Not applicable	No data	No data	Fadul et al. (2006) [153]
Prolactin carcinoma	65	male	Yes	Yes	PRL fell from 73 000IU/l to 6,1 IU/l	No data	No data	Lim et al (2006) [217]
Prolactinoma	52	female	Yes	Yes	PRL fell from 7,000 ng/ml to 150-260ng/ml	No data	No data	Neff et al. (2007) [218]
Adreno-corticotroph carcinoma	23	male	No	No	No data	High	No data	Kovacs et al. (2008) [219, 50]
Non-functioning pituitary carcinoma	9	female	No	No	No	No data	No data	Guzel et al. (2008) [220]

Tumor	Age	Gender	Response			MGMT IHC	MGMT Promoter	Author
			Clinical	Radiological	Biochemical			
Somatotropinoma	48	male	No	No	No	High	No data	McCormack et al. (2009) [113]
Prolactin carcinoma	42	male	Yes	Yes	PRL fell from 504 602 mlU/l to 90 863 mlU/l	Low	No data	McCormack et al. (2009) [113]
Adrenocorticotroph carcinoma	46	female	Yes	Yes	ACTH fell from 15 000 pg/ml to 20,8 pg/ml	Low	No data	Takeshita et. al. (2009) [221]
Adrenocorticotroph carcinoma	64	female	Yes	Yes	ACTH fell from 2472 pmol/l to 389 pmol/l	Low	No data	Moyes et al. (2009) [222]
NFPA	28	female	No data	Yes	No data	No data	No data	Mohammed et al. (2009) [154]
Adrenocorticotroph carcinoma	60	male	Yes	Only initially	ACTH fell from 140 pmol/l to 21 pmol/l	High	No data	Mohammed et al. (2009) [154]
Adrenocorticotroph adenoma	43	female	Yes	Yes	Yes	Low	No data	Mohammed et al. (2009) [154]
NFPA	20	male	Yes	Yes	Not applicable	Low	No data	Hagen et al. (2009) [152]
Prolactinoma	60	male	Yes	Yes	PRL fell from 650 to 18 µg/l	Low	No data	Hagen et al. (2009) [152]

Tumor	Age	Gender	Response			MGMT IHC	MGMT Promoter	Author
			Clinical	Radiological	Biochemical			
Prolactin carcinoma	48	female	Yes	Yes	PRL fell from 738µg/l to 14 µg/l	Low	No data	Hagen et al. (2009) [152]
Prolactinoma	64	male	Yes	Yes	PRL fell from 504 000 mlU/l to 24 000mlU/l	No data	No data	Byrne et al. (2009) [223]
NFPA	52	male	No	Yes	No data	High	No data	Syro et al. (2009) [224]
Adreno-corticotroph adenoma	42	male	Yes	Yes	No data	Low	No data	Curto et al. (2010) [49]
Adreno-corticotroph carcinoma	No data	No data	Yes	Yes	Yes	No data	No data	Bode et al. (2010) [48]
NFPA	No data	No data	Stable	Stable	Not applicable	High	unmethyl-ated	Bush et al. (2010) [61]
Adreno-corticotroph adenoma	No data	No data	Yes	Yes	ACTH fell from 221pg/ml to 18pg/ml	Low	unmethyl-ated	Bush et al. (2010) [61]
Gonado-tropinoma	No data	No data	Stable	Stable	Not applicable	Inter-mediate	unmethyl-ated	Bush et al. (2010) [61]
NFPA	No data	No data	Stable	Stable	Not applicable	High	unmethyl-ated	Bush et al. (2010) [61]
Prolactinoma	No data	No data	Yes	Yes	PRL fell from 5702 ng/ml to 121ng/ml	Low	unmethyl-ated	Bush et al. (2010) [61]

Tumor	Age	Gender	Response			MGMT IHC	MGMT Promoter	Author
			Clinical	Radiological	Biochemical			
Carcinoma	No data	No data	No data	Stable	No data	High	No data	Bush et al. (2010) [61]
Carcinoma	No data	No data	No data	No	No data	Low	unmethylated	Bush et al. (2010) [61]
Adrenocorticotroph adenoma	57	male	No	No	No data	No data	No data	Losa et al. (2010) [54]
Adrenocorticotroph carcinoma	46	male	Yes	Yes	ACTH fell from 237pg/ml to 10pg/ml	Low	No data	Losa et al. (2010) [54]
Adrenocorticotroph adenoma	40	female	Stable	Only initially	ACTH fell from 6144 pg/ml to 3580 pg/ml, then rose to 6750 pg/ml	High	unmethylated	Losa et al. (2010) [54]
Adrenocorticotroph adenoma	28	female	No	No	No	High	unmethylated	Losa et al. (2010) [54]
Prolactinoma	55	male	Stable	Stable	PRL fell from 2800 ng/ml to 1232 ng/ml, then rose again	Low	unmethylated	Losa et al. (2010) [54]
Prolactinoma	53	female	Yes	Yes	PRL fell from 1845 ng/ml to 8 ng/ml	No data	unmethylated	Losa et al. (2010) [54]
Prolactin carcinoma	32	male	No data	Yes	PRL normalization	No data	No data	Raverot et al. (2010) [53]

Tumor	Age	Gender	Response			MGMT IHC	MGMT Promoter	Author
			Clinical	Radiological	Biochemical			
Prolactin carcinoma	52	male	No	No	No	Intermediate	unmethylated	Raverot et al. (2010) [53]
Prolactinoma	54	male	No	No	No	Low	8,5%	Raverot et al. (2010) [53]
Prolactin carcinoma	30	female	No	No	No	High	No data	Raverot et al. (2010) [53]
Adrenocorticotroph carcinoma	31	male	No data	No	No	Intermediate	2,6%	Raverot et al. (2010) [53]
Adrenocorticotroph adenoma	49	male	No data	No	No	Low	No data	Raverot et al. (2010) [53]
Adrenocorticotroph carcinoma	38	male	Yes	Yes	ACTH fell from 135 to 64 ng/l	Intermediate	No data	Raverot et al. (2010) [53]
Adrenocorticotroph adenoma	42	female	Yes	Yes	ACTH fell from 114 to 47 ng/l	Low	9,8%	Raverot et al. (2010) [53]
Prolactin carcinoma	54	female	Only initially	Only initially	PRL fell from 2355ng/ml to 42,2ng/ml, then rose to 714,4 ng/ml	Low	No data	Murakami et al. (2011) [55]
Adrenocorticotroph carcinoma	50	male	Only initially	Only initially	ACTH fell from 1,874 pg/ml to 309pg/ml	No data	No data	Thearle et al. (2011) [59]

Tumor	Age	Gender	Response			MGMT IHC	MGMT Promoter	Author
			Clinical	Radiological	Biochemical			
Pituitary blastoma	1	female	Ony initially	Only initially	No data	High	No data	Scheithauer et al. (2011) [225]
Adreno-corticotroph carcinoma	44	male	No	No	Not applicable	High	No data	Ortiz et al. (2011) [97]
Adreno-corticotroph carcinoma	65	male	Yes	Yes	ACTH fell from 5685ng/l to 2318 ng/l	Low	No data	Annamalai et al. (2011) [52]
Adreno-corticotroph adenoma	58	male	Yes	Yes	ACTH fell from 67.3 pmol/l to 15.2 pmol/l	No data	unmethyl-ated	Dillard et al. (2011) [226]
Adreno-corticotroph carcinoma	48	female	Only initially	No data	No data	No data	No data	Arnold et al. (2012) [47]
Somato-tropinoma	22	male	No	No	No	No data	No data	Morin et al. (2012) [227]
Prolactinoma	13	male	Yes	Yes	PRL fell from 49 000 to 3515 mlU/l	Low	No data	Whitelaw et al. (2012) [115]
Prolactinoma	34	male	Yes	Yes	PRL fell from 90 000 to 3222 mlU/l	Low	unmethyl-ated	Whitelaw et al. (2012) [115]

Tumor	Age	Gender	Response			MGMT IHC	MGMT Promoter	Author
			Clinical	Radiological	Biochemical			
Prolactinoma	32	male	Yes	Yes	PRL fell from 24 000 to 4540 mlU/l	Low	No data	Whitelaw et al. (2012) [115]
Adreno-corticotroph adenoma	49	female	Yes	Yes	No data	Low	No data	Rotondo et al. (2012) [228]
Carcinoma	58	male	Yes	Yes	Not applicable	Low	No data	Morokuma et al. (2012) [60]
Prolactin carcinoma	No data	male	Yes	No data	Yes	No data	No data	Phillipon et al. (2012) [229]
Gonado-tropinoma	50	male	Stable	Stable	No data	Intermediate	No data	Ersen et al. (2012) [175]
Prolactin carcinoma	25	male	No	No	No	No data	No data	Phillips et al. (2012) [230]
Prolactin carcinoma	54	male	Yes	Yes	PRL return to normal	No data	No data	Gulati et al. (2013) [147]
Somato-tropinoma	47	male	No	No data	No data	High	unmethyl-ated	Batisse et al. (2013) [231]
Prolactinoma	54	male	No	No	No	No data	No data	Zemmoura et al. (2013) [232]
Adreno-corticotroph carcinoma	40	male	No	No	No	No data	No data	Cornell er al. (2013) [233]

Tumor	Age	Gender	Response			MGMT IHC	MGMT Promoter	Author
			Clinical	Radiological	Biochemical			
NFPA	59	male	Yes	No data	Not applicable	High	No data	Hirohata et al. (2013) [51]
Adreno-corticotroph adenoma	42	female	Yes	No data	No data	Low	No data	Hirohata et al. (2013) [51]
Prolactinoma	60	female	Only initially	No data	No data	Low	No data	Hirohata et al. (2013) [51]
NFPA	23	male	Stable	No data	Not applicable	High	No data	Hirohata et al. (2013) [51]
Adreno-corticotroph adenoma	53	female	Yes	No data	No data	High	No data	Hirohata et al. (2013) [51]
Prolactinoma	60	female	Yes	No data	No data	High	No data	Hirohata et al. (2013) [51]
Adreno-corticotroph adenoma	57	male	Stable	No data	No data	High	No data	Hirohata et al. (2013) [51]
NFPA	73	female	Yes	No data	Not applicable	Low	No data	Hirohata et al. (2013) [51]
Prolactinoma	60	male	No	No data	No data	Low	No data	Hirohata et al. (2013) [51]
NFPA	61	female	No	No data	Not applicable	High	No data	Hirohata et al. (2013) [51]

Tumor	Age	Gender	Response			MGMT IHC	MGMT Promoter	Author
			Clinical	Radiological	Biochemical			
Prolactinoma	66	female	Yes	No data	No data	Low	No data	Hirohata et al. (2013) [51]
Prolactinoma	49	female	No	No data	No data	Low	No data	Hirohata et al. (2013) [51]
Adreno-corticotroph adenoma	45	female	No	No data	No data	High	No data	Hirohata et al. (2013) [51]
Adreno-corticotroph adenoma	55	female	Yes	Yes	ACTH fell from 1854pmol/l to 21,1pmol/l	No data	No data	Asimakopoulou et al. (2013) [234]
NFPA	30	female	Yes	Yes	Not applicable	No data	No data	Zhong et al. (2014) [235]
Prolactinoma	59	female	Yes	Yes	PRL fell from 3902 to 28,8ng/ml	No data	No data	Strowd et al. (2015) [236]
NFPA	39	male	No	No	Not applicable	Low	No data	Ghazi et el. (2015 [237]
NFPA	67	female	No	No	Not applicable	No data	No data	Ceccato et al (2015) [83]
Gonado-tropinoma	39	female	No	No	No	No data	Low	Ceccato et al (2015) [83]
NFPA	40	male	No	Yes	Not applicable	No data	Low	Ceccato et al (2015) [83]

Tumor	Age	Gender	Response			MGMT IHC	MGMT Promoter	Author
			Clinical	Radiological	Biochemical			
Adreno-corticotroph adenoma	32	male	Yes	Yes	Yes	No data	Low	Ceccato et al (2015) [83]
NFPA	47	male	Stable	Stable	Not applicable	No data	Low	Ceccato et al (2015) [83]
Somato-tropinoma	31	female	Yes	Yes	66% decrease in GH-levels	Intermediate	No data	Bengtsson et al. (2015) [187]
Prolactinoma	33	male	Yes	Yes	No data	Low	No data	Bengtsson et al. (2015) [187]
Prolactinoma	22	male	Only initially	Only initially	No data	High	No data	Bengtsson et al. (2015) [187]
Prolactinoma	34	male	Stable	Stable	73% decrease in PRL levels	Intermediate	No data	Bengtsson et al. (2015) [187]
Prolactinoma	45	male	No	No	No	High	No data	Bengtsson et al. (2015) [187]
Prolactinoma	55	male	Yes	Yes	73% decrease in PRL	Low	No data	Bengtsson et al. (2015) [187]

Tumor	Age	Gender	Response			MGMT IHC	MGMT Promoter	Author
			Clinical	Radiological	Biochemical			
Prolactinoma	23	male	No	No	No data	High	No data	Bengtsson et al. (2015) [187]
NFPA	45	male	Yes	Yes	Not applicable	High	No data	Bengtsson et al. (2015) [187]
NFPA	52	female	No	No	Not applicable	High	No data	Bengtsson et al. (2015) [187]
NFPA	59	male	No	No	Not applicable	High	No data	Bengtsson et al. (2015) [187]
NFPA	57	male	No	No	Not applicable	High	No data	Bengtsson et al. (2015) [187]
Adreno-corticotroph carcinoma	51	male	Only initially	Only initially	88% decrease in ACTH levels for 6 months	Intermediate	No data	Bengtsson et al. (2015) [187]
Adreno-corticotroph carcinoma	62	male	No	No	No data	High	No data	Bengtsson et al. (2015) [187]
Adreno-corticotroph carcinoma	70	male	Only initially	Only initially	98% decrease of urinary free cortisol	Low	No data	Bengtsson et al. (2015) [187]

Tumor	Age	Gender	Response			MGMT IHC	MGMT Promoter	Author
			Clinical	Radiological	Biochemical			
Somatotrop carcinoma	46	male	No	No	No data	High	No data	Bengtsson et al. (2015) [187]
Somatotrop carcinoma	40	female	Yes	Yes	Normalisation of IGF-1 levels	Low	No data	Bengtsson et al. (2015) [187]
Prolactin carcinoma	32	female	No	No	No data	Intermediate	No data	Bengtsson et al. (2015) [187]
Prolactin carcinoma	59	female	Yes	Yes	80% decrease in PRL levels	No data	No data	Bengtsson et al. (2015) [187]

B. Affirmation

Eidesstattliche Versicherung von Daniela Hartlmüller

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel

The Effects of Temozolomide on Normal and Tumoral Pituitary Cells

selbstständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.
Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, 23.05.19	Daniela Hartlmüller
(Ort, Datum)	(Daniela Hartlmüller)