Glioblastoma
The effects of the dual kinase inhibitor PI-103 on glioblastoma cells

Dissertation submitted in partial fulfilment of the requirements for the degree of ‘Doktor der Medizin’ (Dr. med.) at the Medical Faculty of Ulm University

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Abbreviations

4E-BP1  Binding protein 1  
AGF  Anti-growth factor  
AKT  Protein kinase B  
AMAR  Adhesion-mediated apoptosis resistance  
AML  Acute myeloid leukaemia  
Apaif-1  Apoptotic protease activating factor 1  
APS  Ammoniumpersulfate  
ATM  Ataxia-telangiectasia mutated  
ATP  Adenosine triphosphate  
ATR  Ataxia-telangiectasia and Rad3-related protein  
BAD  Bcl-2 antagonist of cell death  
B27  B27 supplement  
Bak  Bcl-2 homologous antagonist/killer  
Bax  Bcl-2-associated X protein  
BBB  Blood brain barrier  
Bcl-2  B-cell lymphoma 2  
Bcl-xL  B-cell lymphoma-extra large  
BCNU  Bis-chlorethyl-nitrosourea  
bFGF  Basic fibroblast growth factor  
BH  Bcl-2 homology  
BID  BH3 interacting-domain death antagonist  
BSA  Bovine serum albumin  
BSA  Body surface area  
°C  Degrees Celsius  
CAF  Cancer-associated fibroblasts  
cAMP  Cyclic adenosine monophosphate  
Caspase  Cysteine-aspartic proteases  
CD  Cluster of differentiation  
CKI  Cyclin-dependent kinase inhibitor  
c-myc  c-myc regulator gene  
CNS  Central nervous system  
CREP  cAMP response element binding protein  
CSC  Cancer stem cell
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>dATP</td>
<td>Deoxyadenosine triphosphate</td>
</tr>
<tr>
<td>DC</td>
<td>Differentiated cell</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-inducing signalling complex</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DMEM/F-12</td>
<td>Dulbecco’s modified Eagle’s medium: nutrient mixture F-12</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleid acid</td>
</tr>
<tr>
<td>DNA-PK</td>
<td>DNA-dependent protein kinase</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemoluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>e.g.</td>
<td>Exempi gratia</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>eIF-4E</td>
<td>Eukaryotic initiation factor 4E</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
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<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
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<tr>
<td>Fas</td>
<td>First apoptosis signal</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Human fibroblast growth factor</td>
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<tr>
<td>FOXO3a</td>
<td>Forkhaed box O3a</td>
</tr>
<tr>
<td>G</td>
<td>Glioblastoma</td>
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<tr>
<td>g</td>
<td>Gram</td>
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<tr>
<td>G0 phase</td>
<td>Growth 0 phase</td>
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<tr>
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</tr>
<tr>
<td>Gata-1</td>
<td>Globin transcription factor-1</td>
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<td>GAPDH</td>
<td>Glycerinealdehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GF</td>
<td>Growth factor</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GRB</td>
<td>Growth factor receptor-bound protein</td>
</tr>
<tr>
<td>GSC</td>
<td>Glioblastoma stem cell</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3 beta</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>H₂O</td>
<td>Dihydrogen monoxide</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrogen chloride</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>hrs</td>
<td>Hours</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>Half-maximal inhibitory concentration</td>
</tr>
<tr>
<td>i.e.</td>
<td>Id est</td>
</tr>
<tr>
<td>IDH-1</td>
<td>Isocitrate dehydrogenase 1</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>l</td>
<td>Litre</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDM2</td>
<td>Mouse double minute 2 homolog</td>
</tr>
<tr>
<td>MEK</td>
<td>Mitogen-associated protein kinase kinase</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>MGMT</td>
<td>O6 methylguanine-DNA-methyltransferase</td>
</tr>
<tr>
<td>min</td>
<td>Minutes</td>
</tr>
<tr>
<td>mLST-3</td>
<td>Mammalian lethal with SEC13 protein 3</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>M phase</td>
<td>Mitotic phase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mSin1</td>
<td>synonymous with MAPKAP1 (mitogen-activated protein kinase-associated protein 1)</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin complex</td>
</tr>
<tr>
<td>MTT</td>
<td>Tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa-light chain enhancer of activated B cells</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>p53</td>
<td>Protein 53</td>
</tr>
<tr>
<td>pAKT</td>
<td>Phosphorylated-AKT</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PDK1</td>
<td>Phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PIP</td>
<td>Phosphatidyl-4-phosphate</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidyl-4,5-biphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol-3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PRAS 40</td>
<td>Proline-rich AKT substrate of 40 kDa</td>
</tr>
<tr>
<td>pS6</td>
<td>Phosphorylated-S6</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RAF</td>
<td>Rapidly accelerated fibrosarcoma</td>
</tr>
<tr>
<td>Raptor</td>
<td>Regulatory-associated protein of mTOR</td>
</tr>
<tr>
<td>RAS</td>
<td>Rat sarcoma</td>
</tr>
<tr>
<td>Rheb</td>
<td>RAS homolog enriched in brain</td>
</tr>
<tr>
<td>Rictor</td>
<td>Rapamycin-insensitive companion of mTOR</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>S6</td>
<td>S6 ribosomal protein</td>
</tr>
<tr>
<td>S6K1</td>
<td>S6 ribosomal protein kinase 1</td>
</tr>
<tr>
<td>SC</td>
<td>Stem cell</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>Ser 473</td>
<td>Serine 473</td>
</tr>
<tr>
<td>SOS</td>
<td>Son of sevenless homolog protein</td>
</tr>
<tr>
<td>SOX 2</td>
<td>Sex determining region Y-box 2</td>
</tr>
<tr>
<td>S phase</td>
<td>Synthesis phase</td>
</tr>
<tr>
<td>Src</td>
<td>Sarcoma</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylenediamine</td>
</tr>
<tr>
<td>Thr 308</td>
<td>Threonine 308</td>
</tr>
<tr>
<td>TMZ</td>
<td>Temozolomide</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumour protein p53</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF receptor-associated death domain</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumour necrosis factor-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>Tris</td>
<td>Trishydroxymethyl-aminomethane</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous sclerosis complex</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>uPAR</td>
<td>Urokinase-type plasminogen activator receptor</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
</tr>
<tr>
<td>Wnt</td>
<td>Wingless-type MMTV (mouse mammary tumour virus) integration site</td>
</tr>
</tbody>
</table>
1 Introduction

1.1 Cancer

Cancers - also known as malignant neoplasias - consist of a heterogeneous mass of more or less differentiated cells (Bignold 2007) that can divide in an unregulated fashion (Krontiris 1983) and are able to invade other tissues (Wittekind and Neid 2005). Although it seems to be older than the human race, and was first mentioned around 3000 BC in the Edwin Smith Papyrus, where it was described as a grave disease without treatment (Hajdu 2011), we are still dealing with cancer 5000 years later as a life-threatening, often incurable, disease.

Thirty-two point six million people worldwide are suffering from cancer diagnosed in the past five years and the incidence of new cases is rising rapidly. The incidence of cancer in 2012, the last year for which a full global estimate is available, was estimated at 14 million, and this number is expected to almost double by the year 2040 (WHO 2018). Interestingly, both incidence and mortality rates are higher in the developed countries than in developing countries. In 2012, 8.2 million deaths were due to cancer, which is the second most common cause of death after cardiovascular diseases (see Figure 1A) (Ferlay et al. 2014). In both sexes combined, cancer of the lungs, liver, stomach, colorectum and breast make up more than 50% of deaths due to cancer each year (see Figure 1B). Tumours of the brain and central nervous system (CNS) account for approximately 2% of all cancer-related deaths and are therefore the 12th most common cause of cancer death (Cancer Research UK).
Cancer was described by Willis as “an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissue, and persists in the same excessive manner after cessation of the stimulus which evoked the change” (Willis 1960). In 2000, Hanahan and Weinberg updated this definition by postulating that six hallmarks of cancer exist. These hallmarks are:

- **Independence of extrinsic growth signals**
  Normal cells depend on extracellular growth factors in order to proliferate. Cancer cells, however, can proliferate without the need for these signals due to autocrine and paracrine stimulation and receptor overexpression, leading to constitutive activation of growth signalling pathways (Hanahan and Weinberg 2000).

- **Resistance to growth-inhibitory signals**
  Anti-growth factors can force cells into a quiescent or post-mitotic state (Hanahan and Weinberg 2000), therefore preventing the continuation of the cell cycle and inducing a period of cellular inactivity or dormancy. The retinoblastoma gene, however, is an example of where this all goes wrong and the very opposite of
quiescence occurs. Mutations in this gene can enable cancer cells to proliferate in
an uncontrolled fashion and to go through endless cell cycles (Burke et al. 2012).

- Formation of new blood vessels
  The tumour needs to stimulate angiogenesis and to form new blood vessels in
  order to grow and proliferate. Angiogenesis is an obvious step in carcinogenesis
  (Hanahan and Weinberg 2000).

- Avoidance of apoptosis
  Apoptosis is a protective mechanism through which damaged cells can induce
  their own death. Cancer cells have acquired various features to enable them to
  avoid apoptosis, either by increasing anti-apoptotic signals or by decreasing pro-
  apoptotic ones (Hanahan and Weinberg 2000).

- Unlimited potential for proliferation
  Non-cancerous cells are limited in their proliferation rate by the shortening of
telomeres on the end of chromosomes. Cancer cells avoid this process, called senes-
cence, by producing telomerase enzymes that permanently elongate the telomeres
  (Hanahan and Weinberg 2000).

- Ability to invade and metastasize
  The ability to invade and metastasize is the characteristic feature that distin-
guishes malignant neoplasias from benign ones. Integrins, cadherins and other
cell-cell adhesion molecules are important in maintaining membrane barriers.
  When these become modified, cancers gain the ability to invade surrounding tis-
sue (Hanahan and Weinberg 2000).

Eleven years later, Hanahan and Weinberg added novel emerging hallmarks (Hanahan
and Weinberg 2011):

- Evasion of immune destruction
  It is an accepted theory that the majority of developing cancer cells are detected
  and eliminated by the immune system. Thus, cancer cells that are able to form
  a tumour mass must somehow have acquired the ability to evade destruction by
  the immune system (Hanahan and Weinberg 2011).
Deregulation of the cellular metabolism

Otto Warburg has previously stated that cancer cells prefer glycolysis to oxidative phosphorylation for energy generation. Although glycolysis provides less ATP, it might have other advantages like the provision of substrates for biosynthetic pathways (Hsu and Sabatini 2008).

To achieve all these hallmarks, a normal cell must undergo several changes. It is for good reason that cancer is sometimes called the ‘disease of mutations’ (Loeb 2016). Cancer can be seen as the result of a multistep process in which the genome of one single cell of the human body has acquired all the mutations necessary to transform itself into a cancerous cell. In general, these mutations can be divided into driver mutations and passenger mutations. Driver mutations occur during tumorigenesis and provide a proliferative advantage, thereby leading to the selection of the mutated cells. Passenger mutations, however, are somatic mutations that occur in every genome throughout life, but do not confer a growth advantage and therefore do not lead to tumour formation (Stratton et al. 2009). Basing his theory on age-incidence statistics, Renan postulated that only 3-12 mutations, all of these possibly driver mutations, are necessary for cancer to develop (Renan 1993). This number naturally varies depending on the tumour type. Furthermore, only the rate-limiting mutations were analyzed during the long-lasting process of tumour formation. It is quite possible that other mutations of equal importance also exist (Hahn and Weinberg 2002). Once mutations have occurred in previously stable genes and the genetic stability of the cell is compromised, further mutations can take place more easily. This results in the affected cell developing a ‘mutator phenotype’ and that allows thousands of mutations to occur in a tumour cell (Loeb 1991; Loeb et al. 2003). Consequently, tumour formation can be seen as a process of mutation and selection (see Figure 2). These mutations generally comprise point mutations, deletions, insertions and amplifications, chromosome translocations and losses or gains of chromosomes (Lengauer et al. 1998). It is often pointed out that these mutations leading to a tumour occur in the tumour stem cell, which leads us to examine the cancer stem cell hypothesis.
Carcinogenesis is a long-lasting process of repetitive mutation and selection of the fittest. Through endogenous and environmental sources, DNA damage is induced, resulting in driver and passenger mutations as well as mutations in mutator genes. Mutations that provide survival advantage are selected, leading to the achievement of hallmarks of cancer. Finally, a clinically detectable cancer develops.

DNA = Deoxyribonucleic acid, GF = Growth factor; AGF = Anti-growth factor.

Based on Loeb (2011) and Hanahan and Weinberg (2011).

1.1.1 Cancer stem cell hypothesis

The cancer stem cell hypothesis assumes that there exists a specific subgroup of cells with stem cell-like characteristics within every tumour; and these cells are consequently called cancer stem cells (Shipitsin and Polyak 2008). Like normal stem cells, cancer stem cells also possess the ability to self-renew and the capability to give rise to any cancer cell within the tumour (Clarke et al. 2006). This hypothesis presumes a hierarchical organisation of tumours (Bonnet and Dick 1997), supposing that all cells of a tumour emerge out of cancer stem cells and that only the cancer stem cells are tumorigenic (Cheng et al. 2010), whereas the more differentiated ones that compose the tumour...
bulk are not (Shackleton et al. 2009). Moreover, the model suggests that cancer stem cells are more resistant to therapy than other cells (Bao et al. 2006; Li et al. 2008b), and that destroying the cancer stem cells within a tumour is enough to eliminate the whole tumour. Many different studies have supported the cancer stem cell hypothesis for several cancers like leukaemia (Bonnet and Dick 1997), breast cancer (Al-Hajj et al. 2003), colon cancer (O’Brien et al. 2007) or brain cancers (Singh et al. 2004). However, the cancer stem cell hypothesis is not universally accepted nowadays. For some cancers, it has been shown that they are not organized hierarchically but follow the clonal evolution model (Williams et al. 2007), which contrasts with the cancer stem cell hypothesis. In this model, it is claimed that mutations over time lead to a selective growth advantage for some cells and that these cells out-compete other ones (Nowell 1976). However, it is important to notice that the cancer stem cell and the clonal evolution theory are not mutually exclusive, but can co-exist (Magee et al. 2012), as cancer stem cells have a distinct advantage and would be selected. One should above all be aware that neither theory makes a statement about the cell of origin and that the cancer stem cell especially is not to be equated with the cell of origin.

However, according to the cancer stem cell hypothesis, which claims that cancer stem cells are intrinsically different from ‘normal’ tumour cells, cancer stem cells should be identifiable by means of specific molecular markers. One frequently applied marker to detect CSC is CD133 (prominin). Other CSC markers include CD44 and CD22 for breast cancers or CD34, CD38 and IL3Rα for leukaemia stem cells (Visvader and Lindeman 2012). However, these markers are not a hundred per cent reliable, as tumorigenic cells exist that are CD133− (Wu and Wu 2009; Beier and Beier 2011). From this it can be deduced that several cells within a tumour could be tumorigenic. This was proved by Schneider et al., who were able to show that even differentiated cells transplanted into a mouse were able to create a new tumour by dedifferentiating (Schneider et al. 2016). By the concept of dedifferentiation, both theories (the hierarchical one and the clonal evolution one) might be unified, as the ability to dedifferentiate might be an inherited capacity (hierarchical theory) or acquired by mutations (clonal evolution model) (Plaks et al. 2015) and depend on the tumour cells’ microenvironment. Stem cells in general are known to need their stem cell niche to survive (Morrison and Spradling 2008; Voog and Jones 2010). Similarly, cancer stem cells are found in special microenvironments (Plaks et al. 2015). Consequently, stemness might not be a characteristic of the cancer stem cell itself but a product of interaction between the cancer cell and the microenvironment (Kise et al. 2016; Aponte and Caicedo 2017). Moreover, tumour recurrence
after therapy does not necessarily imply therapy resistance of tumour stem cells, as it might be a result of a protective microenvironment (Shackleton et al. 2009).

To sum up, a tumour consists of a heterogeneous mass of cells with various differentiation statuses and some of the cells within a tumour may have stem cell character. However, there is a permanent plasticity of the tumour cells, and cells may change from one differentiation state into another.

As mentioned above, cancer stem cells harbour many mutations. These mutations frequently occur in proto-oncogenes and tumour suppressor genes.

1.1.2 Proto-oncogenes and tumour suppressor genes

In tumours, mutations in proto-oncogenes and tumour suppressor genes can be found. Mutations, amplifications and translocations in proto-oncogenes can modify gene function, upregulate gene expression and consequently turn proto-oncogenes into oncogenes. Proto-oncogenes normally produce proteins which regulate cell proliferation and control programmed cell death as well as differentiation. When proto-oncogenes are activated, normal cellular homeostasis is shifted in the direction of cell growth (Croce 2008). One of the best known proto-oncogenes is Rat sarcoma (RAS) which is found mutated in 20-30% of all cancers (Bos 1989). Other examples of proteins that result from the transcription and translation of proto-oncogenes include constitutively activated growth factors such as platelet-derived growth factor (PDGF), modified growth factor receptors such as epidermal growth factor receptor (EGFR), tyrosine-protein kinases such as Sarcoma (Src), and finally serine/threonine-specific protein kinases, like Protein kinase B (AKT). In addition, transcription factors and chromatin remodelers can also function as oncogenes (Croce 2008). As there is a long latency period between activation of a proto-oncogene and the onset of cancer, it is assumed that the transformation of one single proto-oncogene into an oncogene is not sufficient. The prevailing theory is that at least five mutations that can take place in proto-oncogenes as well as in other genes like tumour suppressor genes are necessary (Hahn and Weinberg 2002). These tumour suppressor genes are also often called anti-oncogenes. Weinberg hypothesised that these anti-oncogenes play an even more important role in carcinogenesis than the oncogenes (Weinberg 2014). In oncogenes, activation of one allele alone is sufficient to lead to the development of cancer. In contrast, tumour suppressors require biallelic inactivation, i.e. both alleles of the tumour suppressor gene need to be knocked
out, before tumorigenesis can occur (Sherr 2004). This phenomenon, also known as the two-hit-hypothesis, was first proposed by Knudson in 1971 in retinoblastoma (Knudson 1971).

Tumour suppressor genes generally act to inhibit uncontrolled cell growth by regulating various cellular activities. Examples of this include the suppression of growth factors, the regulation of the cell cycle, the repair of DNA damage or, if DNA damage cannot be repaired, the activation of cellular apoptosis (Sherr 2004). The gene products of tumour suppressor genes have also been shown to inhibit metastasis (Yoshida et al. 2000).

Tumour suppressor genes can be divided into gatekeepers, caretakers (Kinzler and Vogelstein 1997) and landscapers (Michor et al. 2004). Inactivation of caretakers and landscapers will contribute indirectly to cancer formation. Landscapers inhibit the formation of an abnormal stromal environment, which is otherwise necessary for carcinogenesis to take place (Michor et al. 2004). In breast cancer, for example, myoepithelial cells are known as landscapers because they prevent the luminal epithelial cells from transforming into cancer (Bissell and Radisky 2001). Caretakers, however, act by maintaining the stability of the genome. If they become inactivated, many mutations in proto-oncogenes and gatekeepers can arise and accumulate (Oliveira et al. 2005). Gatekeepers control the delicate balance between cell proliferation and cell death (Kinzler and Vogelstein 1996), and each cell usually has only 1-3 gatekeepers (Kinzler and Vogelstein 1997). If they are knocked out, cells can proliferate uncontrollably, which leads directly to tumorigenesis. In carcinogenesis, therefore, mutations in caretaker genes occur first, causing further mutations in gatekeeper genes; and this is then what leads directly to tumour formation. In hereditary cancers, only one gatekeeper allele is mutated and inherited, so that during the patient’s lifetime only the second corresponding allele remains to be knocked out (Kinzler and Vogelstein 1997).

One well-known and often mutated gatekeeper is the Protein 53 (p53) gene. In addition to the maintenance of genetic stability, p53 normally inhibits angiogenesis. Another important role of p53 is to prevent stressed cells from proliferating, for example by means of cell cycle inhibition. If this cannot be prevented, then p53 will induce programmed cell death (Vogelstein et al. 2000).

Proteins of altered proto-oncogenes and tumour suppressor genes are often part of central intracellular pathways. One of these pathways is the PI3K pathway, which is the subject of the next section.
1.1.3 The PI3K/AKT signalling pathway

The PI3K pathway is an important intracellular pathway in cell cycle regulation and therefore in controlling growth, proliferation, cell death, migration and survival. Cell signalling in this pathway is very complex and far from completely understood. The pathway consists of many activators, inhibitors, effectors and second messengers with many feedback loops and branches (Marone et al. 2008). Essentially, activation of PI3K triggers a signalling cascade. For example, activated PI3K leads to the activation of AKT; this again phosphorylates and activates or inhibits many downstream effectors.

In the following section only the most important players will be described. The PI3K superfamily can be divided into 3 subgroups (class I, II and III PI3Ks). As so far only class IA is known to be related to cancer, the focus will lie on class IA PI3K signalling. Class I PI3Ks are lipid kinases which phosphorylate phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP$_2$). They are heterodimers consisting of a regulatory and a catalytic subunit. There exist different kinds of regulatory and catalytic subunits. Consequently, different heterodimers can be composed. Depending on the kind of catalytic as well as regulatory subunits the heterodimer is composed of, PI3Ks are further divided into class IA and class IB. Examples of class IA PI3K catalytic subunits are p110$\alpha$, p110$\beta$ and p110$\delta$, and IA PI3K regulatory subunits are p85$\alpha$, p55$\alpha$, p50$\alpha$, p85$\beta$ and p55$\gamma$, whereas class IB PI3Ks have the p110$\gamma$ catalytic subunit and the p101 regulatory subunit (Carpenter et al. 1990; Franke 2008). The PI3Ks mentioned below all belong to the class IA family.

PI3Ks are mostly activated downstream of the different receptor tyrosine kinases (RTKs) (see Figure 3). After their autophosphorylation, PI3Ks can bind to these receptor tyrosine kinases, directly through their regulator subunit or indirectly via adaptor molecules such as the insulin receptor substrate (IRS-1). In both cases, the regulatory subunit stops inhibiting the catalytic subunit and the PI3K becomes activated. PI3Ks can also be activated via small membrane-bound GTP-RAS, a central intracellular signalling protein (Cantley 2002). Activated PI3K migrates to PIP$_2$ and consequently converts it into Phosphatidylinositol-3,4,5-trisphosphate (PIP$_3$)

Levels of PIP$_3$ are tightly regulated by Phosphatase and Tensin homolog (PTEN). The phosphatase dephosphorylates PIP$_3$ and therefore prevents cells from excessive signalling via the PI3K/AKT pathway (Hopkins et al. 2014).

Increased levels of PIP$_3$ lead to the migration of the threonine/serine kinase AKT
towards PIP₃ bound to the plasma membrane. This brings AKT into proximity of phosphoinositide-dependent kinase 1 (PDK 1), which is also bound to PIP₃. PDK 1 then phosphorylates AKT at the AKT phosphorylation loop on threonine 308, partially activating it. For full activation of AKT, however, phosphorylation of the AKT carboxyterminal hydrophobic domain on serine 473 is necessary. This is carried out by PDK 2 kinases like the mammalian target of rapamycin complex 2 (mTORC2), consisting of mTOR, Rictor, mLST-8 and mSin1, or by DNA-PK (Song et al. 2005; Marone et al. 2008).

AKT has many downstream effects. More than 100 AKT substrates have so far been reported (Manning and Cantley 2007). Mammalian target of rapamycin complex 1 (mTORC1), consisting of mTOR, Raptor and mLST8, is known as one of the main substrates of AKT. It can be activated by AKT through two different mechanisms. On the one hand, AKT phosphorylates and consequently inhibits Proline-rich AKT substrate of 40 kDa (PRAS 40), which usually acts to inhibit mTORC1. On the other hand, AKT phosphorylates Tuberous sclerosis complex 2 (TSC2) at multiple sites, leading to the accumulation of RAS homolog enriched in brain Guanosine triphosphate (Rheb GTP). This activates mTORC1, which plays an important role in cell growth by up-regulating messenger ribonucleic acid (mRNA) translation of proteins of the synthetic apparatus (Manning and Cantley 2007). Substrates of mTORC 1 are, for example, the 70 kDa S6 kinases 1 and 2 (S6K1 and S6K2) which phosphorylate S6 ribosomal protein. Levels of phosphorylated S6 ribosomal protein correlate with increased translation of mRNA transcripts which are characterised by an oligopyrimidine tract in their 5’ untranslated regions (Thomas 2002). These mRNAs encode for elongation factors and ribosomal proteins involved in translation as well as for proteins that are important to cell cycle progression (Peterson and Schreiber 1998; Dufner and Thomas 1999). Additionally, mTORC1 phosphorylates and therefore inhibits the eukaryotic initiation factor 4E (eIF-4E) and binding protein-1 (4E-BP1). Both S6K1 and 4E-BP1 are important proteins for the regulation of different eukaryotic initiation factors of translation (Steelman et al. 2011). Both S6K1 and mTORC1 trigger a negative feedback loop by inhibiting IRS-1 (Manning and Cantley 2007). Other substrates of AKT are involved in cell cycle progression, apoptosis, DNA repair and glucose metabolism. Mouse double minute 2 homolog (MDM2), for example, is activated through phosphorylation, and can thus inhibit p53 more strongly. This can lead to increased survival, less DNA repair and uncontrolled cell cycle progression (Vivanco and Sawyers 2002). Survival is also increased by phosphorylation of caspase 9 and Bcl-2 antagonist of cell death (BAD), a
pro-apoptotic protein that is inactivated through phosphorylation (Manning and Cantley 2007). Cell cycle progression can be mediated by various substrates of AKT. The levels of cyclin-dependent kinase inhibitors (CKIs), like p27 or p21, are negatively influenced by AKT. As a result, CKIs are less inhibited and the cell cycle can continue (Gesbert et al. 2000; Zhou et al. 2001). Cyclin D1 is normally marked for degradation by Glycogen synthase kinase 3 beta (GSK3β), another substrate of AKT. As GSK3β is inactivated through phosphorylation, cyclin D is no longer degraded and can accumulate, encouraging cell cycle progression (Vivanco and Sawyers 2002). In addition, GSK3β is part of the Wingless-type mouse mammary tumour virus (Wnt) pathway, an important pathway for cell proliferation and migration. As GSK3β is inhibited through AKT, β-catenin is not degraded and accumulates. β-catenin can then induce transcription in proteins such as c-myc or cyclin D1 (Rao and Kühl 2010). Moreover, GSK3β interferes with the cell metabolism as it normally prevents glycogen synthesis (Embi et al. 1980). Finally, the published literature also reports that GSK3β is responsible for differentiation of glioblastoma cells (Li et al. 2010). Additionally, forkhead box O3a (FOXO3a), a transcription factor that induces transcription of many different genes, has been reported to promote differentiation. As FOXO3a is also inhibited by AKT, de-differentiated cells can result through activation of the PI3K/AKT signalling pathway (Sunayama et al. 2011).
Figure 3: The PI3K pathway

In glioblastoma, both the PI3K pathway and the RAS/RAF/MEK/ERK pathway are frequently upregulated. Important functions of the PI3K pathway like cell proliferation, cell cycle progression, inhibition of apoptosis, translation, differentiation and motility are shown. PI3K is activated by receptor tyrosine kinases resulting in the activation of AKT and its many downstream effectors. mTORC2 and S6K start a negative feedback loop, inhibiting IRS-1 and consequently further PI3K activation. The PI3K pathway is normally tightly regulated by the phosphatase PTEN converting PIP₃ into PIP₂. Also illustrated is the important interaction between the PI3K and the RAS protein.

PI3K = Phosphatidylinositol-3-kinase; RAF = Rapidly accelerated fibrosarcoma; RAS = Rat sarcoma; MEK = Mitogen-associated protein kinase kinase; ERK = Extracellular regulated kinase; AKT = Protein kinase B; mTORC2 = Mammalian target of rapamycin complex 2; S6K = S6 ribosomal protein kinase; IRS-1 = Insulin receptor substrate 1; PTEN = Phosphatase and Tensin homolog; PIP₃ = Phosphatidylinositol-3,4,5-trisphosphate; PIP₂ = Phosphatidyl-4,5-biphosphate.

Based on www.lifetechnologies.com (2018), Steelman et al. (2011) and Manning and Cantley (2007).
As mentioned above, the PI3K pathway is tightly linked to the RAS protein and subsequently also to the RAS/RAF/MEK/ERK pathway. This pathway mediates transmission of extracellular signals to the nucleus, and, as a result, induces translation of many growth-promoting proteins. An activated growth factor receptor binds to the Growth factor receptor-bound protein 2 (GRB2) via an Src homology 2 domain-containing adapter protein. Consequently, the son of sevenless homolog protein (SOS) is recruited, which combines RAS with GTP. Activated RAS can then go on to activate rapidly accelerated fibrosarcoma (RAF) kinase, inducing phosphorylation of mitogen-activated protein kinase kinase 1 (MEK). In turn, MEK phosphorylates and activates mitogen-activated protein kinase kinase 1 (MAPK) (Steelman et al. 2011). Activated MAPK can control translation via the p90 ribosomal S6 kinases (Anjum and Blenis 2008). Moreover, MAPK also influences transcription via the cAMP response element binding protein (CREP), C-myc and Globin transcription factor 1 (Gata-1) (Steelman et al. 2011).

Interestingly, in 2014 Will et al. published a paper suggesting that the RAS/RAF/MEK/ERK signalling pathway can also be activated downstream of PI3K. They claimed that PI3K regulates wild-type RAS activation, inducing signalling of the RAS/RAF/MEK/ERK pathway (Will et al. 2014). One may conclude that both the PI3K and the RAS/RAF/MEK/ERK pathways play an important role in many different cancers, of which glioblastoma is an example. As can be seen in Figure 3, induction of apoptosis plays a central role in the PI3K pathway. Mutations in the PI3K pathway consequently lead to the avoidance of apoptosis, a central principle of cancer. Therefore, the next section will deal with apoptosis.

1.1.4 Apoptosis

There are two main pathways of apoptosis: the intrinsic and the extrinsic (see Figure 4). However, these pathways are not completely independent of one another, but can influence each other (Igney and Krammer 2002). Additionally, a perforin/granenzyme pathway exists. All three pathways end in a common execution pathway leading to DNA fragmentation (Elmore 2007).

The intrinsic pathway is also called the mitochondrial pathway because it is triggered by mitochondrial stress. Mitochondrial stress is detected by the BH3-only proteins, which are members of the Bcl-2 family. These BH3-only proteins now activate the
Figure 4: Apoptosis
Apoptosis can be induced either in an extrinsic or an intrinsic way. By the intrinsic way, cell injury results from mitochondrial stress and the dimerization of proapoptotic proteins, following the release of cytochrome c into the cytosol. Together with Procaspase 9 and Apaf-1, the Apoptosome is formed, activating Caspase 9. The extrinsic way is initiated by death receptors like the Fas receptor, leading to the activation of Caspase 8. Caspases 8 and 9 then induce the executioner caspases, resulting in cell death. Intrinsic and extrinsic ways are connected via BID. Survival factors activating the PI3K pathway can prevent apoptosis. The activation of apoptosis in the granenzyme way is not illustrated. Apaf-1 = Apoptotic protease-activating factor 1; BID = BH3 interacting-domain death agonist; Fas = First apoptosis signal; FADD = Fas-associated death domain; PI3K = Phosphatidylinositol-3-kinase; AKT = Protein kinase B; BAD = Bcl-2 antagonist-of-cell-death; BH = Bcl-2-homology domain; Bax = Bcl-2-associated X protein; Bcl-2 = B-cell-lymphoma-2; Bcl-xL = B-cell-lymphoma-extra large; BID = BH3 interacting-domain death antagonist; DNA = Deoxyribonucleic acid.

Based on Kumar et al. (2013) and Igney and Krammer (2002).
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pro-apoptotic proteins of the Bcl-2 family, simultaneously inhibiting the anti-apoptotic ones (e.g. Bcl-2 and Bcl-xL). The pro-apoptotic proteins Bcl-2 homologous antagonist/killer (Bak) and Bcl-2-associated X protein (Bax) dimerize, permeate the mitochondrial membrane and form channels within it. Due to the increased membrane permeability, cytochrome c and other proteins can escape into the cytosol (Kumar et al. 2013). The release of cytochrome c triggers the release of Apoptotic protease activating factor-1 (Apaf-1), deoxyadenosine triphosphate (dATP) and Procaspase 9, which together all combine to form a large quaternary protein called apoptosome. It is this apoptosome that activates the previously inactive Procaspase 9 (Cain et al. 2000).

The extrinsic pathway is initiated when a member of the Tumour necrosis factor receptor superfamily binds to a ligand. Two well-characterized ligands are Tumour necrosis factor (TNF) and First apoptosis signal (Fas). After the ligand has bound to the corresponding receptor, cytoplasmic adaptor proteins, i.e Fas-associated death domain (FADD) for Fas ligand/Fas receptor and TNF receptor-associated death domain (TRADD) for TNF ligand/TNF receptor, will diffuse towards the receptor. In the case of FADD, Procaspases 8 and 10 are also added on to form the Death-inducing signalling complex (DISC) (Kischkel et al. 1995). Procaspase 8 dimerises, resulting in cleavage and activation of Caspase 8 (Fulda and Debatin 2006; Elmore 2007). Beneath the induction of the executioner pathway, Caspase 8 cleaves BH3 interacting-domain death agonist (BID), which translocates to the mitochondria and cross-links the extrinsic with the intrinsic way (Igney and Krammer 2002). At this point two different cell types can be distinguished: type I and type II cells. Type I cells are independent of the mitochondria and Caspase 8 can directly cleave the executioner caspases, whereas in type II cells activation of mitochondria is necessary for apoptosis (Scaffidi et al. 1998; Özören and El-Deiry 2002).

The initiation Caspases 8, 9 and 10 cleave the executioner Caspases 3, 6 and 7, which go on to activate the execution pathway. Endonucleases and proteases become activated and degrade DNA as well as nuclear and cytoskeletal proteins. Chromatin begins to condense, the cells shrink and finally, apoptotic bodies develop (Elmore 2007). Apoptotic cells then display an ‘eat-me signal’ like phosphatydilserine, which is normally found on the inner leaflet of the plasma membrane. Phosphatydilserine is recognised by receptors displayed on macrophages, and phagocytosis of the apoptotic cells by macrophages takes place (Savill et al. 2003).

Additionally, the immune system can induce apoptosis via T-cells and NK-cells, which secrete perforin, allowing the simultaneously released granenzymes to enter the cell.
The granzymes can then directly activate the execution pathway by cleaving caspases (Igney and Krammer 2002).

1.2 Glioblastoma

Glioblastoma, originally called glioblastoma multiforme, is the most commonly occurring primary brain tumour in adults, with an incidence of 3 in every 100,000 people in the United States (Ostrom et al. 2014). In addition, it is also one of the most lethal cancers with a median overall survival of 15-18 months with current standard care (Mayra Paolillo 2018; Bi and Beroukhim 2014). Some characteristics associated with a better prognosis have been reported: young age, high Karnofsky Performance Status, high Mini-mental status, O6-methylguanine methyltransferase promoter methylation and mutant isocitrate dehydrogenase 1 (IDH 1) expression (Adamson et al. 2009). People suffering from glioblastoma often exhibit unspecific symptoms such as headache or seizures. Depending on the location within the CNS, signs and symptoms such as visual disturbances, aphasia or personality changes can also emerge (Liu et al. 2009). A preliminary diagnosis can already be made by Magnetic resonance imaging (MRI), with imaging depicting a ring-enhancing lesion with cystic and solid parts, diffuse infiltration of the adjacent brain tissue and peritumoural oedema (Sartor et al. 2006). However, diagnosis cannot be fully confirmed until (at least) a tissue biopsy is taken. The appearance of glioblastomas on MRI images already reflects the histological morphology which will be described in the next section.

1.2.1 Histology and pathology of glioblastoma

Glioblastoma is classified as a World Health Organization (WHO) grade IV astrocytoma and is defined as a tumour consisting of a hypercellular mass of less differentiated astrocytes with cellular polymorphisms, nuclear atypia and high mitotic activity (Marquet et al. 2007). Other features such as large necrotic areas, pathological vasculature and astrocyte-forming glomeruli-like patterns are also typical of glioblastoma (Hambardzumyan and Bergers 2015). In approximately 90% of all cases, glioblastoma develops de novo as a so-called primary glioblastoma. In contrast, secondary glioblastoma arises from less malignant precursor forms, e.g. anaplastic astrocytoma. While primary
glioblastoma can mainly be found in men with an average age of 62 years, secondary glioblastoma tends to occur in women with an average age of 45 years and has a significantly better prognosis (Ohgaki et al. 2004; Ohgaki and Kleihues 2007). Although both primary and secondary types of glioblastoma have a very similar morphology in terms of histology, their genetic profiles are different. Mutations of isocitrate dehydrogenase 1 (IDH1) can reliably be used to differentiate between primary and secondary glioblastomas. In 80% of secondary glioblastoma, IDH1 mutations are found, whereas only 5% of primary glioblastoma exhibit mutated IDH1 (Ohgaki and Kleihues 2013). Interestingly, glioblastomas with IDH mutations tend to be localised in the frontal lobe of the brain, show less necrosis and have a better prognosis (Lai et al. 2011). Another important distinguishing feature is tumour protein p53 (TP53) mutations which are primarily found in secondary glioblastoma (65%). However, mutations of TP53 can also be found in 28% of primary glioblastoma, perhaps as a result of the increasing genomic instability during carcinogenesis (Ohgaki and Kleihues 2007). Loss of heterozygosity (LOH) on chromosome 10q is a common feature of both types of glioblastoma, and therefore the most frequent genetic alteration. LOH on 10p or on the whole chromosome is only associated with primary glioblastomas. Also associated with LOH is the loss of PTEN, a tumour suppressor gene regulating the PI3K pathway, as PTEN is located on chromosome 10q (Ohgaki and Kleihues 2007). According to the Cancer Genome Atlas Research Network, 88% of all glioblastomas harbour mutations in various genes which encode for proteins of the RTK/PI3K/AKT signalling network pathway (McLendon et al. 2008). EGF receptor amplification, for example, is reported in 36% of primary glioblastoma (Ohgaki et al. 2004). Additionally, PI3K itself also shows alterations. More specifically, mutations in the PIK3CA gene, which encodes the p110α catalytic subunit, have been identified (Mizoguchi et al. 2004; Gallia et al. 2006). Mutations in the PIK3R1 gene encoding the regulatory subunit p85α have also been found to a lesser degree (Mizoguchi et al. 2004). Finally, in about 2% of glioblastoma AKT amplifications occur (McLendon et al. 2008). Therefore, the RTK/PI3K/AKT network seems to play an important role in the development of a glioblastoma, and some of these mutations could be driver mutations. As driver mutations in the cancer stem cell hypothesis would mainly be found in cancer stem cells, the next section will deal with cancer stem cells in glioblastoma.
1.2.2 Glioblastoma stem cells

Improving our understanding of the origins, as well as the recurrence, of glioblastoma may help in the discovery of new treatments. Scientists have often pointed to cancer stem cells as being the starting point in tumourigenesis. Moreover, the cancer stem cell hypothesis is an established concept for leukaemia. However, in terms of solid tumours, little evidence for cancer stem cells exists, with the notable exception of breast cancer (Singh et al. 2004). Interestingly, in 2002 the existence of stem-like cells in glioblastomas was postulated by Ingatova et al. (2002). The findings of the paper were also supported by studies by Singh and Hemmati, who both claimed that cells within a tumour existed which displayed stem cell-like characteristics, i.e. the ability to self-renew, to differentiate into various daughter cells, and to induce the same tumour phenotype when transplanted into an immunocompromised host (Hemmati et al. 2003; Singh et al. 2004). These so-called glioblastoma stem cells (GSCs) only make up a small percentage of the entire tumour bulk (Tabatabai and Weller 2011), but nevertheless only about 10 cells are enough to induce a new tumour (Singh et al. 2004). The GSCs share many characteristics with neural stem cells. These are stem cells in the brain which divide asymmetrically, giving rise to neuronal and glial progenitor cells (Sanai et al. 2005). Neural stem cells maintain their stem cell behaviour by upregulating the PI3K pathway (Watanabe et al. 2006). Interestingly, this pathway has also been found to play a major role in upregulating glioblastoma stem cells and thus in glioblastoma formation in general (Cheng et al. 2010). In order to maintain stemness in vivo, interactions with the microenvironment are important (Nduom et al. 2012). In an experimental system, stemness is obtained by cell-cell interactions in the absence of factors usually present. Therefore, stem cells are usually found in special stem cell niches located in perivascular and hypoxic regions within the glioblastoma bulk (Hamerlik et al. 2013). So far, it is not clear whether both niches contain the same GSCs or if different subpopulations are present in the two niches. However, what both niches have in common is that hypoxia leads to the stimulation of vascular endothelial growth factor (VEGF), inducing angiogenesis. Consequently, new perivascular regions come to exist, creating new stem cell niches (Nduom et al. 2012). In the perivascular stem cell niche, the Notch pathway plays an important role for the maintenance of stemness. Interestingly, this pathway is also involved in radioresistance of GSCs (Wang et al. 2010). Besides, GSCs are also reported to be more chemoresistant and slowly cycling than normal glioblastoma cells (Yamada et al. 2011). Both chemo- and radiotherapy mainly act by inducing apoptosis...
(Westhoff et al. 2014). However, what makes GSCs so problematic is that they possess the ability to avoid apoptosis (Yamada et al. 2011). Consequently, they are more likely to survive therapy, and are therefore likely responsible for triggering glioblastoma recurrence.

Interestingly, glioblastoma usually occurs in brain regions where neural stem cells have also been reported to exist. These regions include the subventricular white matter, the subcortical white matter and the human dentate gyrus (Nduom et al. 2012). Moreover, they both share common migration pathways and both use the same substrates for motility, namely white-matter tracts and blood-vessel basement membranes (Sanai et al. 2005). Overall, the shared location of GSC with neural stem cells, as well as shared migration routes and substrates, suggests that normal neural stem cells might give rise to the GSC; although this has so far not been proven. Alternatively, the microenvironment might induce the development of GSC.

One difficulty regarding GSC consists in the discrimination of normal glioblastoma cells from the GSC. CD133 is mostly used as a GSC marker, as it has been shown that CD133⁺ cells are able to form new tumours when transplanted into mice (Singh et al. 2004). Meanwhile, however, it has also been shown that CD133⁻ cells can also induce new tumours and that even CD133⁺ cells can emerge out of CD133⁻ ones (Wang et al. 2008). Moreover, Schneider et al. showed that new glioblastoma tumours in mice can also be induced by differentiated glioblastoma cells (Schneider et al. 2016). Therefore, it remains unclear whether GSC develop from normal neural stem cells or whether they develop by de-differentiation of mature brain cells (Galli et al. 2004). Nevertheless, regardless of GSC origin, it is important to keep in mind that cells exist within the glioblastoma that can mimic normal stem cell characteristics (i.e. self-renewal and multipotency), and that these are the cells that are likely to be responsible for glioblastoma initiation or recurrence. One has however to keep in mind that when talking about stem cells and differentiated cells, cells might in vitro behave different as in vivo. Importantly, it has been shown that established cell lines, particularly glioblastoma cell lines, only poorly mimic the gene and gene-product profile of glioblastoma in vivo (Vogel et al. 2005; Li et al. 2008a). Therefore, data obtained from cell culture experiments with established cell lines can only be understood as a hint of what might occur in the actual tumour. A better system, therefore, is cells obtained directly from clinical tumours, as used in this study.
As GSC are often held responsible for the invasiveness of glioblastoma, the next section will deal with ‘micrometastasis’ in glioblastoma.

1.2.3 ‘Micrometastasis’ in glioblastoma

Distant metastases - tumour cells that have spread from the primary tumour and formed a new tumour (Fidler 1989) - are survival-limiting factors and consequently carry a poor prognosis. Interestingly, in glioblastoma, distant (i.e. extradural) metastases do not play a large role, as glioblastoma only metastasizes in 1.2% of cases outside of the brain (Blume et al. 2013). However, glioblastoma has a highly invasive potential and so-called intradural ‘micrometastases’ are a unique feature of glioblastoma (Demuth and Berens 2004). Therefore, glioblastoma is often viewed as a systemic disease of the brain (Sahm et al. 2012), because individual or small clusters of glioblastoma cells can be found as far as 40 mm away from the actual solid tumour mass (Wilson 1992). As these ‘micrometastases’ can cause severe neurological dysfunction, they can lead to death even in the absence of a genuine glioblastoma tumour mass (Giese 2003).

Interestingly, even low-grade gliomas tend to form micrometastases, suggesting that the ability to invade surrounding tissue is acquired early in gliomagenesis (Louis 2006). Migration of glioblastoma cells does not occur randomly but instead follows preexisting structures. Migration along white matter axonal tracts and along the basement membrane of blood vessels are two such examples (Scherer 1938). As previously mentioned, these so-called ‘secondary structures of Scherer’ are also used by glial progenitors for migration during brain development. This indicates that glioblastoma cells may recapitulate the glial progenitor phenotype (Canoll and Goldman 2008).

Invasion by glioblastoma cells is dependent on a dynamic interplay between cell-cell adhesion molecules, remodeling of the extracellular matrix (ECM), and cell motility. Glioblastoma cells can activate different proteinases such as cysteine, serine and matrix metalloproteinases (MMPs). These proteinases degrade the ECM, allowing the glioblastoma cells to invade adjacent brain tissue (Louis 2006). Cell-substrate adhesions mediated by integrin receptors are also involved in invasion, as they can lead to reorganisation of the cytoskeleton (Uhm et al. 1999). Additionally, they can activate intracellular pathways (via FAK) which can directly lead to cell migration (Natarajan et al. 2003). Levels of FAK are regulated by PTEN. Loss of PTEN, a common feature of many glioblastomas, can consequently lead to increased cell motility and confers on
glioblastomas the ability to invade (Tamura et al. 1998). Moreover, FAK activates both the PI3K/AKT and the RAS/RAF/MEK/ERK pathway (Natarajan et al. 2003). Joy et al. (2003) showed that migrating glioblastoma cell lines activate the PI3K pathway, leading to high levels of phosphorylated AKT and GSK3β in the migrating cells. Inhibition of PI3K via the inhibitor LY294002 resulted in less migration and increased sensitivity to apoptosis (Joy et al. 2003). For breast cancer it has been shown that cells with a constitutively activated PI3K pathway have an increased motility. This effect is mediated by the activation of the polypeptide gene: nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB). NF-κB goes on to induce the expression and secretion of urokinase-type plasminogen activator receptor uPAR (Sliva et al. 2002). Westhoff et al. were able to show that the activation of NF-κB and thus uPAR is a common characteristic of glioblastoma cells in creating their own extracellular matrix, allowing them to migrate faster through the brain. uPAR can convert inactive plasminogen into active plasmin, resulting in the activation of MMP-2. MMP-2 can cleave fibronectin which is produced by glioblastoma cells, allowing cleaved fibronectin to act as a substrate for glioblastoma cells to create their own matrix. Glioblastoma cells can now invade the surrounding brain tissue along this self-constructed matrix (Westhoff et al. 2013b).

The highly invasive potential of glioblastoma combined with the upregulation of many survival pathways makes effective treatment of glioblastoma extremely difficult.

1.2.4 Current standard therapy for glioblastoma

Standard therapy for glioblastoma consists of maximally safe surgical resection of the tumour mass combined with radiotherapy plus concomitant and adjuvant chemotherapy with temolozomide (TMZ) (Stupp et al. 2005). Surgical resection aims to either completely remove the tumour bulk or at least to shrink it (cytoreduction) if gross total resection is not possible. Clinical benefits of surgical intervention and reduction of the tumour include reduced intracranial pressure and potential recovery of neurological functions. Resection alone extends survival by approximately 6 months, at which time glioblastoma often recur (Wilson et al. 2014). In order to delay recurrence of glioblastoma, surgical resection is combined with radio- and chemotherapy. Radiotherapy at a dose of 2 Gray (Gy) is applied for a period of 6 weeks daily from Monday to Friday.
Alongside radiotherapy, concomitant chemotherapy with TMZ is given daily at a dose of 75 mg/m² BSA/day. After this period, therapy is interrupted for 4 weeks, followed by 6 cycles of adjuvant TMZ therapy. Here, TMZ is applied at a dose of 150-200 mg/m² BSA/day for 5 days during each 28-days cycle. Following this radio- and chemotherapy protocol increases the median survival to 14.6 months (Stupp et al. 2005). Radiotherapy’s mechanism of action is the induction of single- and double-stranded breaks in the DNA, leading to chromosomal abnormalities and consequently cell death (Thompson 2012). Moreover, reactive oxygen species (ROS) are produced by the ionising radiation used in radiotherapy. Although these ROS are often pro-inflammatory and therefore tumorigenic, ROS conversely have some anti-tumorigenic effects which radiotherapy takes advantage of. For instance, high levels of ROS are associated with cell cycle arrest and therefore less cellular proliferation (Gupta et al. 2012). Moreover, ROS can lead to cell death via several mechanisms. On the one hand, ROS can directly induce apoptosis by activating proteins like Bcl-2 and Bcl-xL (Martindale and Holbrook 2002). On the other hand, ROS can effect DNA damage and therefore chromosomal abnormalities which indirectly lead to apoptosis. Additionally, the chemotherapy agent TMZ also induces DNA damage. As a derivate of the imidotetrazine class, TMZ methylates DNA at the O6 position of guanine residues as well as alkylates guanine at the N7 position and adenine at the N3 position (Newlands et al. 1997). The efficacy of TMZ depends on the enzymatic activity of O6-methylguanine-DNA-methyltransferase (MGMT). This enzyme removes the O6-methylguanine residues in the DNA, and therefore reduces the therapeutic effect of TMZ (Silber et al. 1998). Chemotherapy with TMZ is far more effective in secondary glioblastoma, as 75% of secondary glioblastomas compared to only 36% of primary glioblastomas have a methylated, i.e epigenetically silenced, MGMT promoter (Kanu et al. 2009). Aside from MGMT methylation status, low concentration of TMZ in the brain can also explain the poor effect of this chemotherapy. Every drug used in glioblastoma therapy has to be able to overcome the blood-brain barrier (BBB), a membrane that only allows ions and small molecules like glucose and hormones to diffuse into the brain tissue (Nau et al. 2010), whilst simultaneously ensuring that large molecules, like cells and pathogens, for example, stay in the vessels. Although TMZ has a 100% bioavailability, only about 20% reach and penetrate the glioblastoma cells (Ostermann et al. 2004). Consequently, since standard glioblastoma therapy is still largely ineffective, new therapies are urgently needed.
1.2.5 New approaches in glioblastoma therapy

Glioblastoma is a tumor entity with relative low mutational burden and so far uncertain driver mutation, making off-the-shelf targeted therapy an uncertain endeavor. For example, the addition of monoclonal antibodies or small molecule inhibitors to the potential therapeutic arsenal employed against glioblastoma has so far been met with mixed results.

Monoclonal antibodies are large immune molecules (about 150 kDa) that cannot penetrate the cell membrane and are used to inhibit cell surface proteins like receptor tyrosine kinases. Small molecule inhibitors, however, are smaller in size (smaller than 500 Da) and can pass through the cell membrane and inhibit specific intracellular proteins. They can therefore be used to inhibit both extracellular surface receptors as well as specific intracellular proteins (Wilson et al. 2014). In general, 4 different types of small molecule inhibitors can be distinguished: Type I small molecule inhibitors are kinase inhibitors that inhibit extracellular receptor tyrosine kinases as well as intracellular non-receptor tyrosine kinases and serin/theronine kinases and thus block the activation of important signalling cascades. Type II small molecule inhibitors act as proteasome inhibitors (Lavanya et al. 2014). The ubiquitin proteasome pathway promotes the transition from one cell cycle to the next by contributing to the proteolysis of cell cycle regulators (Bassermann et al. 2014). At the same time, the pathway prevents apoptosis by regulating the expression of caspases and inhibitor of apoptosis proteins (Wójcik 2002). Small molecule proteasome inhibitors thus lead to cell cycle arrest and sensitise cells to apoptosis. The small molecule inhibitors type IV also act in the area of apoptosis, for example by directly intervening in the apoptosis signalling pathway as Bcl-2 inhibitors. While type I,II and IV small molecule inhibitors are mainly involved in cell growth, proliferation and apoptosis, type III small molecule inhibitors prevent cell migration and invasion, for example by inhibiting MMPs. A major advantage of small molecule inhibitors over many other drugs is their oral bioavailability, which allows them to be taken daily in the form of tablets (Lavanya et al. 2014).

Some small molecule inhibitors have already been tested for glioblastoma. Bevacizumab, for instance, sold under the trade name Avastin®, which is a recombinant humanized antibody targeting VEGF, positively contributed to progression-free survival of GBM patients, without affecting their overall survival (Gilbert et al. 2014). Another small molecule inhibitor, Cilengitide (EMD 121974), which targets the GBM cells’ interaction with their microenvironment by blocking integrins, has shown no overall improvement
in clinical trials (Stupp et al. 2014). Interestingly, our group also investigated the effects of Venetoclax on GBM cells, a small molecule inhibitor blocking Bcl-2 and showing great promise in certain leukaemias. However, in GBM addition of this inhibitor to standard therapy had only mild effects on GBM cell viability. This is possibly due to the extreme low expression of members of the Bcl-2 protein family in GBM when compared to leukaemia (Westhoff 2019 personal communication).

However, the most promising approach for therapy with small molecule inhibitors seems to be the PI3K pathway, since the PI3K pathway is mutated in 88% of all glioblastomas and is therefore the only really relevant mutated pathway in glioblastomas. It was first attempted to target and inhibit mTORC1 by using the rapamycin analogues everolimus and temsirolimus that differ in their bioavailability, half-time and their blood metabolite patterns (Klawitter et al. 2015; Leung et al. 2018). Although mTORC1 inhibition showed promising results in pre-clinical studies, it failed to do so when carried through to the clinical trial stage (Fan et al. 2006), and worryingly increased tumour cell proliferation with some malignancies (Wen et al. 2012). The limited effectiveness of these agents was explained by the initiation of a complex negative feedback loop, leading to increased PI3K signalling when mTORC1 was inhibited (Hay 2005). To overcome this issue and consequently improve the effectiveness of this approach, multiple inhibition of the PI3K pathway at various targets has been proposed. Therefore, potent small molecule inhibitors which target several points of the PI3K pathway are currently on the market (Burris 2013). One example is the potent inhibitor pyridofuropyrimidine PI-103. At very low concentrations, PI-103 inhibits DNA-PK, p110α and mTORC1 and affects PI3-KC2β, p110δ, mTOR2, p110β and p110γ at higher concentrations (Knight et al. 2006). Fan et al. (2006) showed that PI-103 triggers a strong blockade of proliferation. This proliferative arrest was demonstrated in genetically different glioma cell lines; and, intriguingly, PTEN status did not influence these promising results. By treating xenografts of the established cell line U87 with PI-103, scientists reduced the size of experimental glioblastoma by 4-fold after 18 days compared to control. Importantly, the mice treated with PI-103 did not exhibit any obvious signs of toxicity (Fan et al. 2006). These results were supported by Guillard et al. (2009), who also achieved a reduction of tumour proliferation using PI-103 treatment. Their proliferative arrest was accompanied by a G1 cell cycle arrest, with 80% of all tumour cells in G1 phase after 24h, and a correspondingly increased expression of the negative cell cycle regulator p27 kip1 (Guillard et al. 2009).

As the PI3K/AKT cascade is an important survival pathway that might counteract
apoptosis, blocking this pathway in combination with apoptosis-inducing therapies like chemo- and radiotherapy seems to be a promising option in glioblastoma therapy. Guil-lard et al. combined PI-103 with vincristine, Bis-Chlorethyl-NitrosoUrea (BCNU) and TMZ, and showed that the overall outcome was better when PI-103 was combined with named substances, meaning that PI-103 had a synergistic effect with these substances. In U87 human xenografts, a combination of PI-103 with TMZ showed a clear therapeu-tic benefit compared to treatment with TMZ alone (Guillard et al. 2009). Opel et al. also suggested that inhibition of the PI3K/AKT cascade using a PI3K inhibitor called LY294002 could sensitisie glioblastoma cells to apoptosis triggered by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Opel et al. 2008). Westhoff et al. proposed that PI-103 could trigger apoptosis by causing DNA damage as well as re-duced DNA repair on chemosensitive glioblastoma cells. They showed that inhibition of either DNA-PK or PI3K leads to a delay of DNA repair, thus increasing apoptosis (Westhoff et al. 2009). Decreased DNA repair was also postulated to radiosensitise glioblastoma cells (Gil del Alcazar et al. 2014). By inhibiting PI3K and mTOR, they reduced the activity of DNA-PK and ataxia-telangiectasia mutated (ATM) kinases, consequently minimising the repair of radiation-induced DNA damage. Importantly, all this work was done on established glioblastoma cell lines. To date, these promis-ing results have not been replicated in a clinical setting, as several clinical trials have failed to show the benefit of blocking the PI3K/AKT network (Akhavan et al. 2010; Cloughesy et al. 2014; Mendiburu-Elicabe et al. 2014).

1.3 Aim of thesis

There is a strong discrepancy between successfully inhibiting the PI3K pathway in established glioblastoma cell lines in the laboratory versus actually achieving similar results in human clinical trials. This study aims to use PI-103 to investigate the effects of inhibiting the PI3K pathway in patient-derived glioblastoma cells. These cells are more representative of the actual gene and protein expression profiles exhibited by real glioblastoma tumours, in contrast to established cell lines cultured in long-term culture. Therefore, this thesis aimed to explore the effects of inhibition of the PI3K pathway on glioblastoma cell proliferation, survival, cell differentiation and migration. Culture conditions were optimised for glioblastoma SCs as well as for development of short-term differentiated cells into primary cells (DCs). The similarities and differences in
the response of these SCs and DCs were examined. Furthermore, PI-103 was combined with different chemotherapeutics and small molecule inhibitors in order to investigate the effectiveness of combination therapy. In addition, inhibition of the PI3K pathway will be compared to inhibition of the RAS/RAF/MEK/ERK pathway by UO126 in single and combination treatment.
2 Materials and Methods

2.1 Materials

2.1.1 Cell lines

G 35 DC  differentiated cell
G 35 SC  stem cell
G 38 DC  differentiated cell
G 38 SC  stem cell
G 40 DC  differentiated cell
G 40 SC  stem cell

2.1.2 Cell culture reagents

DMEM  Life Technologies, Paisley, UK
DMEM/F-12 (1:1)  Life Technologies
FCS  Life Technologies, Grand Island, USA
Fungizone  Life Technologies
Penicillin/streptomycin  Life Technologies
Phosphate-buffered saline  Biochrom, Berlin, Germany
Trypsin/EDTA solution  Biochrom
TrypsinLE Express  Life Technologies
B27 supplement without vitamin A  Life Technologies
EGF  Biomol GmbH, Hamburg, Germany
bFGF  Miltenyi Biotec GmbH, Bergisch Gladbach, Germany
2.1.3 Pharmacological inhibitors

PI-103 Cayman Chemicals, Ann Arbor, USA
Temozolomide Sigma-Aldrich, Steinheim, Germany
Irinotecan Tocris, Wiesbaden, Germany
UO126 Cell Signaling, Frankfurt, Germany
SB 415286 Selleckchem, Munich, Germany

2.1.4 Casyton Analysis

CASYton solution Roche Roche, Mannheim, Germany
CASYton tubes Innovatis, Reutlingen, Germany
CASYton clean Innovatis
CASYton DT Innovatis

2.1.5 MTT Analysis

MTT solution Sigma-Aldrich
DMEM (1X) with 4.5 g/l D-glucose, L-glutamine, 25 nM HEPES without sodium pyruvate, phenol red Life Technologies
Isopropyl alcohol VWR, Leuven, Belgium
2.1.6 FACS Analysis

Propidium iodide  Sigma-Aldrich, Hamburg, Germany
Triton X-100   Sigma-Aldrich
Sodium citrate-dihydrat   AppliChem, Darmstadt, Germany
PBS   Biochrom
FACS Scan   Beckton Dickinson, Heidelberg, Germany
FACS tubes   Sarstedt, Nürnberg, Germany
FACS Flow   BD Biosciences, San José, USA
FACS Clean   BD Biosciences
FACS Rinse   BD Biosciences

2.1.7 Time-lapse photography

Microscope CK40   Olympus, Hamburg, Germany
Triton X-100   Sigma-Aldrich
CAMEDIA C-4040ZOOM digital camera   Olympus
### 2.1.8 Protein Extraction and SDS-Page

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<td>TEMED</td>
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<td>Tris</td>
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<td>PBS</td>
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<td>H$_2$O</td>
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<td>BCA Protein Assay Reagent Kit</td>
<td>Thermo Scientific, Schwerte, Germany</td>
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<td>Roth</td>
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<td>Glycine</td>
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<td>Bromphenolblue</td>
<td>Merck</td>
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<td>Protein Marker Page Ruler</td>
<td>Fermentas, St. Leon Rot, Germany</td>
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### 2.1.9 Western Blot Analysis

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<td>Glycine</td>
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<td>Tris</td>
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<td>Hybond-ECL Nitrocellulose membrane</td>
<td>GE Healthcare, Munich, Germany</td>
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<td>Blotting paper</td>
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<td>BSA</td>
<td>Serva</td>
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<td>Whatman blotting paper</td>
<td>VWR GmbH, Darmstadt, Germany</td>
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<td>ECL Western Blotting Substrate</td>
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<td>Amersham Hyperfilm ECL</td>
<td>GE Healthcare, Munich, Germany</td>
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<td>Power supply</td>
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### Primary Antibodies

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<td>Cell Signaling, Frankfurt, Germany</td>
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<td>Rabbit anti-phospho-AKT (Thr 308)</td>
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<td>Cell Signaling</td>
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<tr>
<td>Mouse anti-AKT</td>
<td>1:1000</td>
<td>BD Bioscience, Heidelberg, Germany</td>
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<td>Rabbit anti-phospho-S6</td>
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<td>Cell Signaling</td>
</tr>
<tr>
<td>Mouse anti-S6</td>
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</tr>
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<td>Mouse anti-phospho-Tyr (PY20)</td>
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<td>Santa Cruz Biotechnology, Santa Cruz, USA</td>
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<tr>
<td>Monoclonal mouse anti-rabbit glyceraldehyde-3-phosphate dehydrogenase</td>
<td>1:1000</td>
<td>Intelligate, Turku, Finland</td>
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### Secondary Antibodies

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<td>Goat anti-rabbit IgG conjugated to horseradish peroxidase</td>
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<td>Santa Cruz Biotechnology</td>
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<tr>
<td>Goat anti-mouse IgG conjugated to horseradish peroxidase</td>
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### 2.1.10 Plasticware

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<td>Cell strainer (70 µM)</td>
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### 2.1.11 Hardware

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<td>Optimax Developer</td>
<td>Protec, Oberstenfeld, Germany</td>
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<td>EPC-9 amplifier</td>
<td>HEKA, Lambrecht, Germany</td>
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<td>Waterbath W 12</td>
<td>Medingen, Arnsdorf, Germany</td>
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2.1.12 Software

CellR

Olympus Soft Imaging Solutions
GmbH, Münster, Germany, 1986-2009

ImageJ

Rasband, W.S., ImageJ, U.S.
National Institutes of Health,
Bethesda, Maryland, USA,

CELLQuest Software

Becton, Dickinson and Company,
Franklin Lakes, New York, USA

GraphPad Prism version 5.00 for Windows

GraphPad Software, San Diego, USA

Inkscape 0.91

Free Software Foundation, Boston,
USA

Excel 2013

Microsoft Cooperation, Redmond,
USA

Texmaker 4.45

http://www.xm1math.net/texmaker/

2.1.13 Stock Solutions and Buffers

Cell Culture Medium

Differentiation medium

Dulbeco’s modified Eagle’s medium (DMEM)
10 % FCS
1 % penicillin/streptomycin

Suspension medium

Dulbeco’s modified Eagle’s medium (DMEM)/F-12 (1:1)
0.02 % B27 supplemented without vitamin A
0.0001 % EGF
0.0004 % bFGF
FACS Measurement

*Nicoletti Buffer*

- 50 µg/ml propidium iodide
- 0.1 % sodium citrate dihydrate
- 0.4 % Triton X-100

Protein Extraction

*Lysis Buffer*

- 30 mM Tris-HCl (pH 7.5)
- 150 mM NaCl
- 1 % Triton X-100
- 10 % glycerol
- Protease inhibitor cocktail

SDS-Polyacrylamide Gel Electrophoresis (SDS PAGE)

*6x SDS loading buffer*

- 60 mM Tris-HCl (pH 6.8)
- 1 % SDS
- 5 % Glycerol
- 0.01 mg/ml bromphenolblue
- 0.34 M β-mercaptoethanol

*Resolving gel buffer - 15 % buffer*

- 3.45 ml H2O
- 3.75 ml 1.5 M Tris-HCl (pH 8.8)
- 150 µl 10 % SDS
- 7.5 ml 30 % polyacrylamid rotiphorese
- 150 µl 10 % APS
- 6 µl TEMED
Stacking gel buffer - 5%

- 2.75 ml H₂O
- 1.25 ml 1.5 M Tris-HCl (pH 6.8)
- 50 µl 10% SDS
- 850 µl 30% polyacrylamid rotiphorese
- 50 µl 10% APS
- 5 µl TEMED

Running Buffer

- 125 mM Tris-HCl (pH 6.8)
- 1.25 mM glycine
- 0.1% SDS

Western Blot

PBS/T

- 0.1% Tween 20 in PBS

Blotting buffer

- 48 mM Tris-HCl (pH 6.8)
- 39 mM glycine
- 0.037% SDS
- 20% methanol

Milk buffer

- 5% milk powder in PBS
2.2 Methods

2.2.1 Cell Culture

G 35, G 38 and G 40 SCs and DCs were derived from 3 different patients with WHO grade IV astrocytoma. The Ulm University Hospital Ethics Committee approved the study (file reference: 162/10 - CL/Sta) and all patients agreed with the study in a written informed consent form. Patients underwent surgical resection of their tumour at the Department of Neurosurgery, Ulm University Medical Center. The tumour specimens were then kept in DMEM/F-12. Cells were mechanically disaggregated as soon as possible and put in ice-cold PBS. Afterwards they were centrifuged at room temperature at 1300 rpm for 5 min. The liquid supernatant was removed and 5 ml TrypLE was added. After 5 min of incubation at room temperature, cells were filtered by means of a 70µM cell strainer. Cells were then cultured in T75 non-tissue culture flasks with DMEM/F-12 supplemented with EGF, bFGF and B27 in a water-saturated atmosphere at 37°C, 5% CO₂ and 21% O₂. After approximately two weeks, cells formed free-floating SC spheres. These cells were split twice a week in a ratio between 1:2 and 1:5 depending on the confluency of the cells. For it the contents of the T75 non-tissue culture flasks were transferred to 50 ml tubes and centrifuged at 1300 rpm for 5 min at room temperature. After the supernatant was removed, 1 ml of new cell culture medium as described above was added and cells were resuspended. Depending on the number of cells of the stock, approximately 0.5 ml was then transferred to a new non-tissue cell culture flask containing 15 ml of the described ‘stem cell medium’. The stem cell culture was also used to generate differentiated glioblastoma cells. For that purpose, SCs were transferred to 50 ml tubes, centrifuged (1300 rpm, 5 min, room temperature) and then resuspended in DMEM, supplemented with 10% FCS and penicillin/streptomycin. Cells were cultured in T75 tissue culture flasks and split twice a week. For that, cell medium was removed and cells were incubated with 3 ml trypsin/EDTA at 37°C for 5 min. After addition of 7 ml of the appropriate medium, cells were transferred to 50 ml tubes and centrifuged (1300 rpm, 5 min, room temperature). After the supernatant was removed, 1 ml of the new appropriate cell culture medium as described above was added and cells were resuspended. Depending on the number of cells in the stock, approximately 0.2-0.5 ml was then transferred to a new non-tissue cell culture flask containing 20 ml of the new appropriate medium. Differentiated cell populations were maintained for less than 10 weeks.
U87 and A172 cell lines were obtained from ATCC and cultured in DMEM, supplemented with 10% FCS, glutamine and penicillin/streptomycin. They were also split twice a week as described for differentiated cells.

2.2.2 MTT

The MTT assay, a colorimetric assay, measures metabolic activity, which was used as a readout for cell viability in this doctoral thesis. Tetrazolinum dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced by viable cells to a dark purple, water-insoluble formazan. The released formazan of solubilized cells could be measured spectrophotometrically. G35, G38, G40 SCs and DCs as well as U87 and A172 were seeded in a 96-well plate at a density of $4.65 \times 10^4$ cells/cm$^2$ in 100 µl of the appropriate medium (= 1500 cells/well/100 µl) for SCs and $7.81 \times 10^4$ cells/cm$^2$ in 100 µl of the appropriate medium (= 2500 cells/well/100 µl) for DCs. After 24 hrs, the cells were treated with indicated concentrations of PI-103, TMZ, irinotecan and UO126, a MEK inhibitor, or with a combination of TMZ and PI-103, irinotecan and PI-103, TMZ and UO126 or irinotecan and UO126. The established cell lines U87 and A172 were treated either with PI-103, TMZ and irinotecan alone or with a combination of TMZ and PI-103 or irinotecan and PI-103. After indicated times, cell viability was measured. For SCs, the 96-well plate was centrifuged for 5 min at 13000 rpm at room temperature. Afterwards, medium was replaced by 100 µl phenol red-free medium containing 1 mg/ml of MTT salt. For DCs as well as for the established cell lines, medium was replaced directly without centrifuging the plates beforehand. The plates were then incubated at 37°C for approximately 3 hrs. Afterwards, the reaction was halted by adding 100 µl isopropanolol. After another 30 min of incubation at room temperature, optical density was analysed by the microplate reader EL800 at $\lambda = 54$ nm.

2.2.3 Changes in Cell Numbers

Cells were seeded on 24-well plates at a density of $4.65 \times 10^4$ cells/cm$^2$ in 500 µl of the appropriate medium per well for SCs and $7.81 \times 10^4$ cells/cm$^2$ in 500 µl of the appropriate medium per well for DCs. After 24 hrs, cells were treated with indicated concentrations of PI-103, UO126 and SB, a GSK3β inhibitor, or treated with DMSO as control. Then cells were allowed to proliferate for indicated times. Afterwards, medium
was replaced by a trypsin/EDTA solution (for DCs), and spheres were dispersed mechanically (for SCs). The cell suspensions were diluted 1:100 in CASYton solution, and cell numbers were determined using CASY1 DT.

2.2.4 Apoptosis Measurement and Cell Cycle Analysis

Fluorescence-activated cell sorting analysis, which measures DNA fragmentation of propidium iodide-stained nuclei, was used as a readout for apoptosis and cell cycle analysis. As cells in different phases of the cell cycle contain a different amount of DNA (apoptotic cells < G0/G1 < S < G2/M), the cell cycle can be analysed, and apoptotic cells can be detected. Therefore, cells were seeded on 24-well plates at a density of 4.65 × 10^4 cells/cm² in 500µl of the appropriate medium per well for SCs and 7.81 × 10^4 cells/cm² in 500µl of the appropriate medium per well for DCs. After 24 hrs, cells were treated with indicated concentrations of PI-103 and UO126 or DMSO as control and incubated. After indicated times, SCs were directly transferred into FACS tubes. For DCs, medium was transferred into FACS tubes and cells were then incubated with 200µl trypsin/EDTA for 5 min at 37°C. After addition of 400µl PBS, the solution was also transferred to corresponding FACS tubes. The tubes were then centrifuged (SCs: 1300 rpm, 4°C, 5 min; DCs: 1800 rpm, 4°C, 5 min). The supernatants were discarded and the pelleted cells were resuspended in 50µl of hypotonic Nicoletti buffer. All steps were performed on ice. For measurements, a FACSCalibur Flow Cytometer was used, which measured the hypodiploid (sub-G1) DNA content. Analysis was done by using the computer software CELLQuest. 10000 cells were detected. The percentage of specific apoptosis was calculated as follows:

\[
\text{specific apoptosis } [%] = \frac{\text{experimental apoptosis } [%] - \text{spontaneous apoptosis } [%]}{100 \% - \text{spontaneous apoptosis } [%]} \times 100
\]

For cell cycle analysis, cells in G0/G1 phase, in S phase and in G2/M phase were gated by the FL2 detector. These three populations were summed and set as the total amount of living cells (100 %). Then the percentage of cells in each cell cycle phase was calculated.
Cells were seeded on 6-well plates at a density of $0.3 \times 10^4$ cells/cm$^2$ in 2 ml of the appropriate medium and incubated for 24 hrs at 37°C. They were treated with set concentrations of PI-103 and allowed to proliferate for indicated times. All following steps were then carried out on ice. SCs were directly transferred to 50 ml tubes and centrifuged (1800 rpm, 4°C, 5 min). For DCs, medium was transferred to 50 ml tubes, and 500 µl of ice-cold PBS was added to each well. Adherent cells were scraped from the bottom of the wells and transferred to the corresponding tubes. After centrifugation (1800 rpm, 4°C, 5 min) the supernatants were discarded and pellets were resuspended in 1 ml of ice-cold PBS and transferred to an Eppendorf tube. Centrifugation was repeated and the supernatants were again discarded. For lysis, 30 µl of lysis buffer was added, followed by incubation on ice of 15 min. Samples were then centrifuged (14000 rpm, 20 min, 4°C) and the supernatants were transferred to new Eppendorf tubes. The pellets were discarded. The protein concentrations of the lysates were determined using the BCA Protein Assay Reagent Kit according to the manufacturer’s instructions. Absorbance was measured with the plate reader EL800 at $\lambda = 550$ nm. 50 µg protein lysates of each sample were prepared and the samples were filled-up to an equal volume with water. 5 µl of 6 × SDS loading buffer were added and lysates then denatured for 5 min at 96°C and centrifuged at 13000 rpm for 1 min. For separation of the extracted proteins according to their molecular weight, protein samples were loaded to a 10% SDS PAGE gel. The first and the last pocket of each gel were loaded with 5 µl of pre-stained protein marker as a protein size standard. The remaining pockets were loaded with 5 µl of the probes. The lysates were concentrated in the 5% stacking gel for 30 min at 100 V and 400 mA and separated in the 1% resolving gel for approximately 5 hours at 120 V and 400 mA. Proteins were blotted onto hypond ECL nitrocellulose membranes by a semi-dry blotting system: three sheets of Whatman paper, a nitrocellulose membrane, the gel and another three sheets of Whatman paper were stacked on top of each other and air bubbles were removed. The stack was placed into the Trans-Blot Semi-Dry Transfer Cell and blotted for 1 hour at 2 V and 130 mA. The nitrocellulose membrane was blocked in 5% milk for 1 hour in order to reduce unspecific protein binding and afterwards washed with PBS/T by agitating the membrane on a rotor for 3 × 10 min. In order to detect the proteins, the membranes were incubated overnight at 4°C with the appropriate primary antibody, which had been diluted in 0.1% Tween, 0.02% sodium acid and 2% BSA. This was again followed by three periods of washing with PBS/T for
about 10 min and by another period of incubation with the corresponding secondary antibody (diluted in milk buffer) at room temperature for 1 hr. Three additional periods of washing with PBS/T for each 10 min followed. For visualization of the proteins, incubation for 1 min with self-made enhanced chemiluminescence (ECL) solution was carried out. Chemiluminescence was captured by an autoradiography hyperfilm ECL and developed in an Optimax developer to visualize the protein bands. In order to perform various antibody examinations, the membranes were used several times, each time being washed and reincubated with a new antibody.

2.2.6 Differentiation

Firstly, to determine when cells become adherent, SCs were seeded on 24-well cell culture plates at a density of $4.65 \times 10^4$ cells/cm² in 500 µl of DMEM supplemented with 10 % FCS and penicillin/streptomycin. Cells were then allowed to become adherent and differentiate some for 24 hrs or for 72 hrs respectively. After the indicated times, cells that had become adherent and cells still in suspension were counted as described above (see Chapter 2.2.3). Secondly, to determine if treatment influences differentiation, SCs were seeded on 24-well cell culture plates at a density of $4.65 \times 10^4$ cells/cm in 500 µl of DMEM supplemented with 10 % FCS and penicillin/streptomycin. Cells were then incubated for 24 hours at 37°C and treated with the indicated concentrations of PI-103, UO126 and SB as well as DMSO control. After 72 hrs cells that had become adherent and had differentiated were counted as described above (see Chapter 2.2.3).

2.2.7 Scratch Assay

A scratch assay was performed to investigate semi-directed migration by observing the migration of cells from a confluent environment into an unoccupied space. Cells were grown to approximately 70 % confluence. They were then treated with PI-103 and incubated for 1 hr at 37°C. Afterwards, a scratch was inflicted to the cell layer using a 10 µl pipet tip. Either directly after scratching or 18 hours after scratching, cells were stained, fixed in 4 % PFA and stained with Giemsa.
2.2.8 Time-lapse Photography

In order to investigate non-directed motility, time-lapse photography was performed. DCs were seeded on a 12-well plate at a density of $7.81 \times 10^4$ cells/cm$^2$ in 1500 µl of the appropriate medium per well. After 24 hrs of incubation at 37°C, cells were treated with PI-103 and then again incubated at 37°C for one more hour. Then, over a time period of 24 hrs, a picture of the cell was taken every 20 min using a CK40 microscope with a CAMEDIA C-4040ZOOM digital camera. Further analysis of the pictures was done using ImageJ software. The 24 hrs were divided into three eight-hour intervals. In each interval, ten cells were tracked. Cells leaving the microscopic field as well as cells undergoing apoptosis were excluded from further analysis. Finally, the velocity of the cells as well as their migration path was analysed using a chemotaxis tool within the ImageJ software.

2.2.9 Statistical analysis

The statistical significance was determined using the student’s t-test. $p < 0.05$ was considered statistically significant.
3 Results

3.1 The effects of PI-103 on cell viability

In order to characterise the effects of PI-103 on the PI3K/AKT pathway, the cellular behaviour of three paired sets of SCs and DCs, namely G35, G38, and G40, was examined after their exposure to PI-103. Firstly, the ideal concentration of PI-103 had to be found. For this purpose, cellular metabolic activity was analysed after 24 and 72 hrs of treatment with various concentrations of PI-103 by MTT assays (see Figure 5). In SCs, maximal effects were achieved using \( \sim 0.6 \mu g/ml \) PI-103 at both time points. Higher concentrations did not induce greater effects as metabolic activity plateaued or increased again. Intriguingly, slightly higher concentrations were needed in DCs to achieve the same effects as in SCs, meaning that the metabolic activity of SCs is even more sensitive to PI-103 than that of DCs. However, in DCs, higher concentrations did not induce an increase in metabolic activity. Moreover, differences in the three cell lines were observed. Among the SCs, G35 are more sensitive than G38 or G40, whereas among the DCs, G40 seem to be the most sensitive and G35 the least sensitive ones. Furthermore, there is a biphasic curve progression of cell viability from low to high concentrations of PI-103. A more detailed analysis of this phenomenon can be found in the appendix.
Figure 5: Effects of PI-103 on cell viability

Three distinct glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three corresponding distinct differentiated glioblastoma cell lines (G 35 DC, G 38 DC, G 40 DC – right side) were treated either with DMSO (controls) or with the indicated concentrations of PI-103. After 24 hrs (black bars) as well as after 72 hrs (grey bars), MTT assays assessing the cell populations’ viability were performed. The controls were defined as 100 %. Results after 24 hrs are illustrated in black, results after 72 hrs in grey.

The figure shows the mean and standard deviation of three independent experiments. Each of them was performed in triplicate.

MTT = tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;
DMSO = dimethyl sulfoxide; SC = stem cell; DC = differentiated cell.

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3.2 The effects of PI-103 on protein signalling

More differences between SCs and DCs were found when focusing on the effects of PI-103 on protein signalling. In order to investigate this relationship, AKT and S6 were used as surrogate read-outs for the activity of PI3K and mTORC1, respectively, after 24 hrs of PI-103 treatment (see Figure 6). The concentrations of PI-103 (namely 0.15µg/ml, 0.3µg/ml and 0.6µg/ml) were selected, based on observed effects in the MTT experiments.

**Figure 6: Effects of PI-103 on protein signalling of different glioblastoma cells**

Three distinct glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three corresponding distinct differentiated DC lines (G 35 DC, G 38 DC, G 40 DC – right side) were treated either with DMSO (controls) or with the indicated concentrations of PI-103. After 24 hrs, protein expression levels and phosphorylation status of AKT and S6 ribosomal protein served as surrogate read-outs for PI3K and mTOR activity, respectively, and were analysed by Western blotting. GAPDH served as loading control.

The figure shows a representative example of two independent replicates.

DMSO = dimethyl sulfoxide; AKT = protein kinase B; pAKT = phosphorylated AKT; thr = threonin; ser = serine; S6 = ribosomal protein S6; pS6 = phosphorylated S6; GAPDH = Glycerinaldehyd-3-phosphat-Dehydrogenase; mTOR = mammalian target of rapamycin; SC = stem cell; DC = differentiated cell.

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In SCs as well as in DCs, 0.3µg/ml and 0.6µg/ml of PI-103 both strongly inhibited phosphorylation of S6, whereas 0.6µg/ml had a more potent effect on AKT phosphory-
lation than 0.3µg/ml. Interestingly, inhibition of S6 seemed to be more potent in G 35 than in G 38 and G 40, preferably in DCs. Phosphorylation of the two phosphorylation sites of AKT, namely threonin 308 and serin 473, differed between SCs and DCs. In SCs, phosphorylation of serin 473, is preferably inhibited, whereas in DCs phosphorylation of threonin 308 is reduced. Within each group of glioblastoma cells (SCs or DCs) and with regards to the particular phosphorylation site (threonin 308 and serin 473, respectively) of AKT more differences between G 35, G 38 and G 40 were observed. In SCs, pAKT<sub>ser 473</sub> seemed to be slightly more inhibited in G 35 than in G 38 and G 40, matching the results of the MTT assays, where more inhibition of the PI3K pathway led to less relative viability. In contrast, the generally less inhibited pAKT<sub>thr 308</sub> in SCs seems to be more active in G 35 than in G 38 and G 40. Consequently, pAKT<sub>thr 308</sub>, phosphorylation in the three SC cell lines is inverse to the corresponding metabolic activity in the MTT assay. Similar results can be seen in DCs, where – comparing the three cell lines - the less phosphorylated pAKT<sub>ser 473</sub> is least inhibited in G 40, the cell line with the least relative viability upon treatment and consequently the best response to PI-103. In summary, for the respective cell types (SCs and DCs), the results of the Western Blots correlate with the MTT results after PI-103 administration for the more strongly inhibited binding site of AKT, while they are reciprocal for the less strongly inhibited binding site of AKT.

After the identification of the most effective concentrations of PI-103, the effect of length of incubation time with PI-103 on protein signalling was analysed. Generally, when using both 0.3µg/ml (see Figure 26, Appendix) as well as 0.6µg/ml PI-103 (see Figure 7), SCs’ and DCs’, inhibition of the PI3K signalling pathway is rapid and long-lasting and persists for at least 72 hrs. However, clear differences between SCs and DCs can be seen; for example, phosphorylation of S6 is stronger and more rapid in DCs than in SCs. A more detailed analysis of the differences between SCs and DCs can also be found in the Appendix.

Taken together, all these data suggest that DCs and SCs significantly differ in the role and regulation of the PI3K signalling pathway.
Figure 7: Effects of prolonged exposure of glioblastoma cells to 0.6\(\mu\)g/ml PI-103 on protein signalling

Three distinct glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three corresponding distinct glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – right side) were treated either with DMSO (controls) or with 0.6\(\mu\)g/ml PI-103 for the indicated times. Protein expression levels and phosphorylation status of AKT and S6 ribosomal protein served as surrogate read-outs for PI3K and mTOR activity respectively, and were analysed by Western blotting. GAPDH served as loading control.

The figure shows a representative example of two independent replicates.

DMSO = dimethyl sulfoxide; AKT = protein kinase B; pAKT = phosphorylated AKT; thr = threonin; ser = serine; S6 = ribosomal protein S6; pS6 = phosphorylated S6; GAPDH = Glycerinaldehyd-3-phosphat-Dehydrogenase; mTOR = mammalian target of rapamycine; SC = stem cell; DC = differentiated cell.

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3.3 The effects of PI-103 on cell numbers and apoptosis

Next, the effects of blocking the PI3K signalling pathway on cellular behaviour were investigated by looking at changes in cell numbers as a marker for proliferation and at DNA fragmentation as a surrogate readout for apoptosis. In general, DCs proliferate more rapidly than SCs, as in 120 hours DCs were at least quintupling, whereas SCs were only approximately doubling. Consequently, addition of PI-103 had a more potent effect on cell numbers in DCs than in SCs. While 0.3 \( \mu \text{g/ml} \) of PI-103 already affected cell numbers (see Figure 27, Appendix), almost any proliferation was blocked for 72 hrs and at least retarded for 120 hrs by using 0.6 \( \mu \text{g/ml} \) PI-103 (see Figure 8). Interestingly, this effect seemed to be caused mainly by a block in proliferation, i.e. a cytostatic effect, as no apoptosis, indicated by DNA fragmentation, could be observed for 120 hrs using either 0.3 \( \mu \text{g/ml} \) (see Figure 28, Appendix) or 0.6 \( \mu \text{g/ml} \) of PI-103 (see Figure 9). Therefore, it is clear that reduced increase of cell numbers after addition of PI-103 is caused by less proliferation of the treated cells.

As this inhibition of proliferation by PI-103 is often caused by a block in the G0/G1 phase of the cell cycle (Bagci-Onder et al. 2011), the cell cycle was analysed after 24 hrs (see Figure 10). Intriguingly, a cell cycle block in G0/G1 phase could not be demonstrated meaning that this could not be responsible for the block in proliferation, suggesting the possibility that cells are actually arrested in their actual cell cycle phase when encountering PI-103.
Figure 8: Cellular proliferation after prolonged exposure to 0.6 µg/ml PI-103

Three different glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three distinct corresponding glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – right side) were treated either with DMSO (controls) or with 0.6 µg/ml PI-103. Cells were counted every 24 hrs for a total of 120 hrs. The figure shows the mean and standard deviation of three independent replicates. Each of them was performed in triplicate. Significant results ($p < 0.05$) are asterisked. Significance was tested by a two-sided Student’s t-test.

DMSO = dimethyl sulfoxide; SC = stem cell; DC = differentiated cell.

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Figure 9: Effect of 0.6µg/ml PI-103 on specific DNA fragmentation in glioblastoma cells

Three different glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three corresponding distinct glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – right side) were treated either with DMSO (controls) or with 0.6µg/ml PI-103. After the indicated times, FACS analysis of DNA fragmentation of propidium iodide-stained nuclei was performed. PI-103-induced DNA fragmentation is shown relative to spontaneous DNA fragmentation of DMSO treated cells.

The figure shows the mean and standard deviation of three independent replicates. Each of them was performed in triplicate. Significant results ($p < 0.05$) are asterisked. Significance was tested by a two-sided Student’s t-test.

DMSO = dimethyl sulfoxide; SC = stem cell; DC = differentiated cell.

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3.4 The effects of PI-103 in combination with chemotherapy

As combination treatment with chemotherapeutics and PI3K inhibitors has been shown to be effective against glioblastoma cell lines (Opel et al. 2008; Westhoff et al. 2009), these results should be verified in a more relevant cellular system, while simultaneously investigating differences between SCs and DCs. The current standard chemotherapeutic TMZ was used for combination therapy. Firstly, an effective concentration of TMZ in SCs and DCs was determined. As can be seen in Figure 11, even with very high concentrations, TMZ had rather weak effects on cell viability, so that no IC\textsubscript{50} could effectively be determined. Again, the hypothesis that SCs are more resistant to chemotherapy (Grotenhuis et al. 2012) could not be confirmed, as the slowly proliferating SCs were clearly more sensitive to TMZ treatment than were their corresponding DCs.
Figure 11: Effects of TMZ on glioblastoma cell viability

Three distinct glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three corresponding distinct glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – right side) were treated either with DMSO (controls) or with the indicated concentrations of TMZ [µM]. After 120 hrs, MTT assays measuring the cell population’s viability were performed. The controls were defined as 100%.

The figure shows the mean and standard deviation of three independent replicates. Each of them was performed in triplicate.

MTT = tertazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO = dimethyl sulfoxide; TMZ = temozolomide; SC = stem cell; DC = differentiated cell.

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In order to determine the effectiveness of the combination therapy, cell viability in general (covering changes in cell numbers as well as death) was analysed by performing MTT assays. The effects on cell viability of the substances alone as well as in combination were analysed simultaneously. On combining 100µM of TMZ with 0.3µg/ml (see Figure 29, Appendix) or with 0.6µg/ml of PI-103 respectively (see Figure 12), yielded no therapeutic benefit, as combination turned out to be antagonistic in all cases.

Interestingly, there was no difference in the effectiveness of the combination therapy regardless of whether 0.3µg/ml PI-103 or 0.6µg/ml PI-103 were used. In most cases, combination therapy was approximately as effective as PI-103 alone. Comparing SCs and DCs and TMZ and PI-103, in DCs PI-103 is more potent than TMZ, whereas in SCs TMZ reduced cellular viability almost as strongly as did PI-103. As TMZ acts by methylating and alkylating DNA (Newlands et al. 1997), consequently requiring cell proliferation for full potency, the inhibition of cell proliferation after PI-103 treatment could be the basis of an antagonistic effect. Therefore, the experiment was repeated using irinotecan, a chemotherapeutic that has also already shown some effect in glioblastoma therapy (Vredenburgh et al. 2009). Again, effective concentrations of irinotecan were determined by MTT assays (see Figure 13).
Figure 12: Effects of combination treatment of 100µM TMZ and 0.6µg/ml PI-103 on glioblastoma cell viability

Three different glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three corresponding distinct glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – right side) were treated either with 100µM TMZ or 0.6µg/ml PI-103 alone or in combination. After 72 hrs and after 120 hrs, respectively, cell viability was measured by MTT analysis. Control cell number was treated with DMSO and was defined as 100%.

The figure shows the mean and standard deviation of three independent replicates. Each of them was performed in triplicate. The mean of an additive effect determined by Bliss analysis is marked by a red bar.

MTT = tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;
DMSO = dimethyl sulfoxide; TMZ = temozolomide; SC = stem cell; DC = differentiated cell.

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Figure 13: Effects of irinotecan on glioblastoma cell viability

Three distinct glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three corresponding distinct glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – right side) were treated either with DMSO (control cell number) or with the indicated concentrations of Irinotecan [nM]. After 48 hrs (black bars) and after 72 hrs (grey bars), MTT assays measuring the cell population’s viability were performed. The controls were defined as 100 %. The results after 48 hrs are illustrated by black bars, results after 72 hrs by grey ones.

The figure is the mean and standard deviation of three independent replicates. Each of them was performed in triplicate.

MTT = tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;
DMSO = dimethyl sulfoxide; SC = stem cell; DC = differentiated cell.

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Longer incubation with irinotecan resulted in less cellular viability. Moreover, DCs in general were more sensitive to irinotecan treatment. While in DCs the effect of irinotecan on viability increases with higher concentrations, in SCs the relative viability plateaus and concentrations higher than 5 nM cannot induce stronger effects. Additionally, in DCs, no clear differences between G 35, G 38 and G 40 can be seen, whereas in SCs the G 38 cells are clearly more sensitive to irinotecan than the other two.

Intriguingly, combination of 10 nM Irinotecan with 0.3 µg/ml PI-103 or 0.6 µg/ml PI-103 was still antagonistic in SCs, but did cause synergistic effects in DCs (see Figure 30, Appendix and Figure 14). Similar to the experiments with TMZ, it made no difference whether 0.3 µg/ml or 0.6 µg/ml PI-103 were used. However, in this set of experiments, reaction of DC G 38 to combination therapy is remarkable. Comparing the three different types of DCs, G 38 was the only cell line in which the combination treatment yielded no synergistic effect when irinotecan was used, whereas in the experiment with TMZ, G 38 was the only cell line that displayed at least an additive effect. These results achieved on patient-derived cell lines were in contrast to the results achieved in established cell lines (see Figure 15). Using U7 and A172 cell lines, combination treatment with TMZ and PI-103 for 96 hrs had a synergistic effect on both cell lines. Irinotecan combined with PI-103 for 48 as well as for 72 hrs, however, was less effective, resulting in only additive effects.
Figure 14: Effects of combination treatment with 10 nM irinotecan and 0.6 µg/ml PI-103 on glioblastoma cell viability

Three different glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three corresponding distinct glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – right side) were treated either with 10 nM irinotecan or 0.6 µg/ml PI-103 alone or with a combination of both. After 48 hrs and after 72 hrs, cell viability was measured by MTT assays. Control cell number was treated with DMSO and was defined as 100%.

The figure shows is the mean and standard deviation of three independent replicates. Each of them was performed in triplicate. The mean of an additive effect determined by Bliss analysis is marked by a red bar.

MTT = tertazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO = dimethyl sulfoxide; TMZ = temolozomide; SC = stem cell; DC = differentiated cell.

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Figure 15: Effects of combination treatment with TMZ/PI-103 and irinotecan/PI-103 on established glioblastoma cell lines

Two different established glioblastoma cell lines, U87 and A172, were treated either with 100 µM TMZ, 0.6 µg/ml PI-103 or a combination of both or with 10 nM irinotecan, 0.6 µg/ml PI-103 or a combination of both. At the indicated time points cell viability was measured by MTT analysis. Control cell number was treated with DMSO and was defined as 100%.

The figure shows the mean and standard deviation of three independent replicates. Each of them was performed in triplicate. The mean of an additive effect calculated by the Bliss analysis is marked by a red bar.

MTT = tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;
DMSO = dimethyl sulfoxide; TMZ = temozolomide.

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As chemotherapeutics yielded rather disappointing results in combination therapy, combinations with inhibitors of other central cellular pathways with PI-103 were tested. The RAS/RAF/MEK/ERK pathway is such a pathway and its activity is often intrin-
sically linked to PI3K-mediated signalling (Steelman et al. 2011). UO126 itself had a
stronger effect on cell numbers than PI-103, especially in SCs (see Figure 16 A).

![Comparison of the effects of UO126 and PI-103 on glioblastoma cell viability and specific DNA fragmentation](image)

**Figure 16: Comparison of the effects of UO126 and PI-103 on A) glioblastoma cell viability and B) specific DNA fragmentation in glioblastoma cells**

A) Three distinct glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three corresponding distinct glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – right side) were treated either with 0.6 µg/ml PI-103 or with 50 µM UO126. After 72 hrs, cell viability was measured by MTT assays. Control cell number was treated with DMSO and was defined as 100%.

B) Three distinct glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three corresponding distinct glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – right side) were treated either with 0.6 µg/ml PI-103 or with 50 µM UO126. After 72 hrs, FACS analysis of DNA fragmentation of propidium iodide-stained nuclei was performed. PI-103- and UO126-induced DNA fragmentation is shown relative to spontaneous DNA fragmentation of DMSO-treated cells.

The figure shows the mean and standard deviation of three independent replicates. Each of them was performed in triplicate.

MTT = tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;
DMSO = dimethyl sulfoxide; SC = stem cell; DC = differentiated cell.

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In contrast to PI-103, UO126 caused some DNA fragmentation, mainly in DCs (see Figure 16 B). Nevertheless, DNA fragmentation contributed little to the reduction in cell numbers, so that again reduced cell numbers were mainly due to inhibition of cell proliferation. Using UO126 in combination therapy, however, also provided disappointing results, as combination of UO126 with TMZ as well as with irinotecan yielded mostly antagonistic effects (see Figure 17).
Figure 17: Effects of combination treatment with either 10 nM irinotecan and 50 µM UO126 or 100 µM TMZ and 50 µM UO126 on glioblastoma cell viability

Three different glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three corresponding distinct glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – right side) were treated either with 10 nM irinotecan, 50 µM UO126 or a combination of both or with 100 µM TMZ, 50 µM UO126 or a combination of both. After 72 hrs, cell viability was measured by MTT analysis. Control cell number was treated with DMSO and was defined as 100%.

The figure shows the mean and standard deviation of three independent replicates. Each of them was performed in triplicate. The mean of an additive effect determined by Bliss analysis is marked by a red line.

MTT = tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO = dimethyl sulfoxide; TMZ = temolozomide; SC = stem cell; DC = differentiated cell.

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Interestingly, when using UO126 and TMZ, differences between the three cell lines were more obvious than differences between SCs and DCs. While in G 35 additive (DCs) or even synergistic (SCs) effects were observed, in G 40 combination therapy was even less effective than either UO126 or TMZ alone (drug antagonism). Taken all these data together, one must conclude that combination therapy with inhibitors of key survival singalling pathways and different chemotherapeutics did not lead to biological effects suggestive of a potential therapeutic benefit.

3.5 The effects of PI-103 on cell differentiation

Inhibition of the PI3K signalling pathway has been suggested to also influence differentiation of glioblastoma SCs (Sunayama et al. 2011; Tian et al. 2012). As adhesion might be a prerequisite for differentiation, adhesion of cells was first analysed. As can be seen in Figure 18, cellular adhesion occurs rapidly during the first 24 hrs. The fraction of cells remaining in suspension (either dead or undifferentiated) was still consistently low and unchanged in number after 24 hrs and after 72 hrs.
Figure 18: Adhesion of glioblastoma stem cells seeded on non-coated cell culture-plasticware

Three different glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC) were seeded on cell culture plasticware in the presence of differentiation medium (DMEM + 10% FCS + 1% penicillin/streptomycin). After 24 hrs and after 72 hrs, cells that had become adherent (a surrogate for differentiation) and cells that were still in suspension (either dead or still not differentiated) were counted.

The figure shows the mean and standard deviation of three independent replicates. Each of them was performed in triplicate.

DMEM = Dulbecco’s modified Eagle’s medium; FCS = fetal calf serum; SC = stem cell.

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In order to investigate this hypothesis SCs were cultured in cell culture plasticware with ‘differentiation’ medium and allowed to differentiate into DCs in the absence or presence of PI-103. The addition of PI-103 led to a remarkable reduction in the number of differentiated cells after 72 hrs (see Figure 19A). This reduction could be explained by the effect PI-103 had on cell proliferation. In G 35 cells, for example, cell numbers were reduced to approximately 70% for SCs and to 42% for DCs in the presence of PI-103 compared to controls (see Figure 19B, C and D). A fraction of 59.48% differentiated cells compared to controls can be found with PI-103 treatment. Consequently, the reduction in differentiation does not exceed the reduction in cell numbers, and the reduction in cell numbers could explain the effects seen on differentiation. This context plausible for G 35 may also apply to G 38 and G 40 (see Figure 19D).
Figure 19: Effect of PI-103 on the differentiation of glioblastoma stem cells

A) Three different glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC) were seeded on cell culture plasticware in the presence of ‘differentiation’ medium and were treated with the indicated concentrations of PI-103. Controls were treated with corresponding amounts of DMSO. After 72 hrs, the numbers of adherent cells (a surrogate for differentiation) were counted. Control cell numbers were set 100 %.

B) Three different glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC) were cultured under ‘stem cell conditions’ and treated with the indicated concentrations of PI-103. After 72 hrs, cell numbers were counted. Controls were treated with corresponding amounts of DMSO and control cell number was set 100 %.

C) Three different glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC) were cultured under adherent cell culture conditions and treated with the indicated concentrations of PI-103. After 72 hrs, cell numbers were counted. Controls were treated with corresponding amounts of DMSO and control cell number was set 100 %.

D) *) shows the effect in [%] on proliferation and differentiation of treating G 35, G 38 and G 40 with 0.6 µg/ml PI-103 for 72 hrs. The number 70.55 indicates that after 72 hrs in the presence of 0.6 µg/ml PI-103, 70.55% of G 35 SCs were counted compared to control cells (100 %) cultured in the absence of PI-103. The same applies to the numbers shown for the DCs as well as for G 38 and G 40 SCs and DCs. 59.48 means that after 72 hrs 59.48% of the SCs are differentiated to DCs.

A), B) and C) show the mean and standard deviations of three independent replicates. Each of them was performed in triplicate. Significant results (p < 0.05) are asterisked. Significance was tested by a two-sided Student’s t-test.

DMSO = dimethyl sulfoxide; SC = stem cell; DC = differentiated cell.

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Consequently, no effect of PI-103 on differentiation was observed under the experimental conditions used. In the literature, GSK3β has been associated with the differentiation of glioblastoma SCs (Tian et al. 2012). Therefore, this set of experiments was repeated using SB 415286, an inhibitor of GSK3β. In contrast to the published literature, differentiation was reduced by inhibition of GSK3β. However, similar to PI-103, reduction in cell differentiation is paralleled by reduction in cell numbers (see Figure 20).

In contrast to all the other inhibitors used within the experimental framework of this thesis, SB 415286 affected cell numbers in SCs at least as much as in DCs. Moreover, as the MEK/ERK signalling cascade is important in glioblastoma, the set of experiments was also conducted with UO126 and delivered similar results as PI-103 and SB 415286, namely that reduction in proliferation explains the reduction differentiation. This is an important finding as it demonstrates that when studying differentiation the antiproliferative effects of the inhibitors used always have to be considered.

### 3.6 The effects of PI-103 on cell motility

A major problem in treating glioblastoma is its highly migrating and invasive phenotype, which renders this tumour inoperable and, moreover, is strongly associated with tumour recurrence after treatment (Demuth and Berens 2004). Inhibition of cell motility in glioblastoma would consequently be an important milestone towards better treatment. Consequently, the effect of PI-103 on cell motility was tested, firstly by treating a monolayer of primary differentiated cells. After one hour of addition of PI-103, a linear defect was applied to the layer and its repair was followed for 18 hrs (see Figure 21).
Figure 20: Effects of SB 415286 and UO126 on differentiation of glioblastoma stem cells

A) Three different glioblastoma SC lines (G35 SC, G38 SC, G40 SC) were seeded on cell culture plasticware in the presence of differentiation medium. They were treated either with 10 µM SB or 50 µM UO126. Controls were treated with corresponding amounts of DMSO. After 72 hrs, the numbers of adherent cells (a surrogate for differentiation) were counted. Control cell numbers were defined as 100 %.

B) Three different glioblastoma SC lines (G35 SC, G38 SC, G40 SC) were cultured under ‘stem cell conditions’ and treated either with 10 µM SB 415286 or 50 µM UO126. After 72 hrs, cell numbers were counted. Controls were treated with corresponding amounts of DMSO and control cell numbers were defined as 100 %.

C) Three distinct glioblastoma DC lines (G35 DC, G38 DC, G40 DC) were cultured under cell culture conditions and treated either with 10 µM SB or 50 µM UO126. After 72 hrs, cell numbers were counted. Controls were treated with corresponding amounts of DMSO and control cell numbers were defined as 100 %.

A), B) and C) show the mean and standard deviations of three independent technical replicates. Each of them was performed in triplicate. Significant results (p < 0.05) are asterisked. Significance was tested by a two-sided Student’s t-test.

DMSO = dimethyl sulfoxide; SC = stem cell; DC = differentiated cell.

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The figure shows the mean and standard deviation of three independent replicates. Each of them was performed in triplicate.

DMSO = dimethyl sulfoxide; DC = differentiated cell.

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After 18 hrs PI-103 led to a retardation of wound healing, but was not able to block it completely. The same results with an even stronger retardation were achieved in established cell lines. To better quantify this effect, time lapse analysis was performed following random motility of DCs for 6 hrs (see Figure 22).
Figure 22: Effects of PI-103 on cell motility as determined by time-lapse microscopy

Three distinct differentiated glioblastoma cell lines (G 35 DC, G 38 DC, G 40 DC) as well as two different established glioblastoma cell lines (A172 and U87) were cultured under cell culture conditions and either treated with 1.8μM PI-103 or left untreated (addition of DMSO). Random motility was measured by time-lapse microscopy over a time frame of 6 hrs.

Two independent experiments are summarized. In each experiment 10 cells were tracked. The symbols mark the average motility of both experiments, whereas the mean is indicated by the black line. Significant results ($p < 0.05$) are asterisked. Significance was tested by a two-sided Student’s t-test.

DMSO = dimethyl sulfoxide; DC = differentiated cell.

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This data set reveals two interesting findings - firstly, the average ‘spread’ of differentiated cells exceeds that of the two established cell lines and secondly, in each case motility was significantly inhibited - with one exception - by the addition of PI-103. This is an important finding as it indicates that the PI3K signalling pathway seems to be closely involved in cell motility. Blocking the PI3K pathway might therefore drastically reduce the invasive nature of glioblastoma and consequently be of great therapeutic benefit.
4 Discussion

This work investigates the therapeutic potential of PI-103, a dual kinase inhibitor, on glioblastoma stem cells and on differentiated glioblastoma cells (both early-passage). While PI-103 does not evoke apoptosis per se, it has a clear effect on proliferation. Interestingly, in contrast to the strong effect combination therapy with chemotherapeutics has on glioblastoma cell lines, it does not provide any therapeutic benefit when primary glioblastoma cells are used. Finally, a potent effect of PI-103 on motility can be demonstrated.

4.1 The role of PI3K signalling in glioblastoma biology

The PI3K pathway is an important survival pathway in general. It is activated in 88% of all glioblastomas, usually due to mutation of PTEN or loss of heterozygosity leading to reduced PTEN expression and consequently permanent PI3K signalling. Glioblastomas in general harbour relatively few genomic transformations. Apart from IDH mutations, MGMT promoter hypermethylation and PI3K activation only some signature alterations are found. Therefore, the PI3K pathway must play a crucial role in glioblastoma.

This was indeed verified in terms of proliferation, as PI-103 led to significantly reduced levels of cell numbers compared to controls (see Figure 8). The importance of the PI3K pathway for proliferation has been demonstrated for several tumours, like breast cancer (Papломата and O'Regan 2014), ovarian cancer (Dobbin and Landen 2013), lymphoma (Tabe et al. 2014), leukemia (Fransecky et al. 2015) and others as well as for glioblastoma (Fan et al. 2006), and consequently, the results of this study are in line with published data. This is an expected result, as downstream effectors of AKT are frequently involved in proliferation (Chang et al. 2003). As many substrates of AKT - like CDK2, Wee1 and p27 as well as p21 and cyclin D1 (Liang and Slingerland 2003) - directly control the cell cycle, more potent inhibition of AKT naturally results in less proliferation. A cell cycle stop after inhibition of AKT consequently seems evident. This was shown by Westhoff et al. (2013) for neuroblastoma and by Georgakis et al. (2006) for Hodgkin’s lymphoma, where in both cases inhibition of the PI3K pathway led to an arrest in the G0/G1 phase of the cell cycle. This effect was communicated by cyclin D1 and MDM2. However, cell cycle arrest in G0/G1 phase could not be confirmed in
this thesis. The lack of ability to synchronize glioblastoma cell proliferation in order to allow maximal efficacy of DNA-damaging treatment options might at least in part explain why PI3K inhibition has had such limited success in clinical settings. Cells already arrested in G0/G1 phase are more susceptible to further DNA damage. For example, it has been shown that these cells are more sensitive to apoptosis induction by TRAIL (Jin et al. 2002). If there are however many cells that reside in other phases of the cell cycle like S or M phase, they will pass checkpoints of the cell cycle where DNA damage might be repaired. As mentioned above, AKT has many downstream effectors that influence the cell cycle at different points. Inhibition of GSK3β and consequently increased cyclin D1 regulates G1/S cell cycle progression. Moreover, the PI3K pathway might influence G2/M transition (Liang and Slingerland 2003). Thus, after inhibition of AKT cell cycle progression might be interrupted at various phases so that no clear arrest in one phase can be detected and synchronisation of glioblastoma cells is not possible. Moreover, although an effect of PI-103 on cell proliferation has been demonstrated, this effect was even stronger when ERK was inhibited by UO126 (see Figure 20 B and C). This suggests that although the PI3K pathway seems to be involved in proliferation it does not play the key role.

Furthermore, an activated PI3K pathway is not necessary for cell survival in glioblastoma, as no apoptosis is triggered solely by PI3K pathway inhibition (see Figure 9). This is a quite surprising result, as on the one hand many downstream effectors of AKT inhibit apoptosis and consequently, inhibition of AKT should induce apoptosis (Cheng et al. 2009), and on the other hand apoptosis has been induced by inhibition of the PI3K pathway in many other tumours such as leukaemia (Fransecky et al. 2015), Hodgkin’s lymphoma (Georgakis et al. 2006), colorectal carcinoma (Itoh et al. 2002) and others. However, for glioblastoma the results are in line with published data, where also no apoptosis could be induced (Fan et al. 2006; Cheng et al. 2009). Regarding the PI3K pathway, these results imply that in glioblastoma no oncogene addiction exists, which is quite surprising as the PI3K pathway is so frequently mutated. Hence, the PI3K pathway might play a role in oncogenesis and tumour formation rather than in tumour survival, as will be discussed later on in Chapter 4.6.

By this reasoning a glioblastoma treatment inhibiting only the PI3K pathway is thus a cytostatic one. While tumour therapy generally focuses on cytotoxicity, prolonged cytostasis can be a reasonable aim of therapy (Rixe and Fojo 2007). If there are no side effects caused by the tumour mass itself, patients would certainly benefit by a cytostatic therapy that prevents tumour growth and metastasis and consequently chronify
4.2 The role of PI3K in glioblastoma therapy resistance

If the PI3K pathway is particularly important neither for survival \textit{per se} nor for apoptosis in particular, one may wonder if inhibition of the PI3K pathway is more successful under additional stress, e.g. in combination of chemotherapy and PI3K pathway inhibition. Both chemotherapy itself and chemotherapy in combination with radiotherapy have yielded disappointing results in glioblastoma. It may be speculated that cancer cells under radiotherapy or under chemotherapy upregulate survival pathways to avoid apoptosis. For ovarian cancer, for example, it has been shown that treatment with doxorubicin induces HER3-PI3K-AKT signalling that, in turn, causes chemoresistance (Bezler et al. 2012). This observation suggests that inhibition of important survival pathways may sensitize cancer cells for radio- and chemotherapy-induced apoptosis. Such combination therapies with inhibition of the PI3K pathway as one of the most important cellular survival pathways of a cell and a chemotherapeutic agent or radiotherapy have already been successfully tested in different studies: Gil del Alcazar (2013) showed that the repair of radiotherapy-induced double-strand breaks could be fore-stalled by PI3K and mTOR inhibition, making the combination of radiotherapy with dual PI3K pathway inhibition a more effective treatment. Effectiveness of chemotherapy could also be clearly improved by simultaneously blocking the PI3K pathway (Opel et al. 2008; Westhoff et al. 2009; Prasad et al. 2011; Choi et al. 2014). Surprisingly, however, in the work for this doctoral thesis, the combination of TMZ or irinotecan with PI-103 has only occasionally achieved additive or synergistic effects (see Figure 12 and 14). However, Westhoff et al. (2009a) and Opel (2008) only looked at apoptosis in their studies, whereas the experiments of this thesis investigated metabolic activity as a surrogate of viability. As for tumour control \textit{in vivo} it is not only apoptosis that is relevant but the whole viability of the glioblastoma cells, my experimental approach seems to be more comprehensive for the investigation of combination therapy. Moreover, the contradictory results of the studies cited may have several causes. Firstly, it has to be taken into account that most chemotherapeutics, such as the ones used in this study, i.e. TMZ, exert their effects on highly proliferating cells (Moiseeva et al. 2016). As PI-103 strongly reduces proliferation, this might impair the effect of some
chemotherapeutics. Secondly, the study of Opel et al. (2008) was done under low-serum conditions, meaning that cells were stressed additionally as nutrition factors were limited; and it may be that only the combination of all three components (low nutrition factors, chemotherapy and PI3K inhibition) leads to a good therapeutic effect. Thirdly, most studies (Opel et al. 2008; Westhoff et al. 2009, 2013b; Choi et al. 2014) were done on glioblastoma cell lines. It has often been shown that established glioblastoma cell lines do not reflect gene expression patterns of genuine glioblastoma tumours. Li et al. (2008) and Vogel et al. (2005) have both shown that gene and protein expressions differ considerably between established cell lines and primary tumours, and that consequently glioblastoma cell lines poorly represent the biology of clinical glioblastomas. The experiments in this study confirm this finding, as combination therapy done on the cell lines A172 and U87 sensitized these cells against chemotherapeutic drugs (see Figure 15) whereas combination therapy on primary glioblastoma cells did not. Fourthly, TMZ uses a different mode of action than other chemotherapeutics, which often intercalate DNA and induce double-strand breaks. Temozolomide (TMZ), however, methylates the guanine and adenine bases and consequently leads to faulty base pairs. Ataxia-teleangiecstasia mutated (ATM) and ATR attempt to repair the faulty base pairs by removing the wrongly incorporated base. As the methylated base still exists, a faulty base will again be incorporated and will again be removed by ATM and ATR and so on. Ataxia-teleangiecstasia mutated (ATM) and ATR require energy in the form of ATP, which results in ATP depletion (Zhang et al. 2012; Eich et al. 2013). Moreover, TMZ induces autophagy in glioma cells (Kanzawa et al. 2004) and thereby limits its cytotoxic effect (Kanzawa et al. 2004; Lin et al. 2012).

Autophagy is also an effect that is induced by PI3K inhibitors in glioblastoma (Cheng et al. 2009; Fan and Weiss 2012). Consequently, if PI3K inhibition, for example by PI-103, causes further autophagy, the cytotoxic effect of TMZ might be reduced instead of enhanced. Hence, simply combining an inhibitor of the PI3K pathway with chemotherapy might not be a generally advisable strategy for cancer therapy.

Furthermore, it must be kept in mind that the right timing of combination treatment is essential. For radiosensitization, for example, different combination schedules achieve different efficacy (Kuger et al. 2013). This has also been proved for combination therapy with chemotherapeutic agents, where simply changing the timing of a treatment combination altered the therapeutic effect from antagonistic to synergistic (Westhoff et al. 2013a).

Besides the right scheduling it is certainly also important to combine suitable sub-
stances to achieve optimal effects. Nonnenmacher et al. (2015) showed for their RIST therapy (combination therapy of rapamycin, irinotecan, sunitinib, temozolomide) that by replacing rapamycin, a relatively downstream inhibitor of the PI3K pathway, with GDC-0941, a more upstream inhibitor, a highly effective therapy in vivo became a poorly effective one, whereas in vitro combination with GDC-0941 was more effective than combination with rapamycin. This was explained by the modulation of blood vessels by GDC-0941, which in combination with the anti-angiogenic effect of sunitinib leads to decreased microvessel density and consequently to a reduced concentration of chemotherapeutics. In summary, a great deal of further investigation has to be done regarding combination therapy to determine which substances should be combined in which schedule.

Generally, however, it might be a misconception to believe that inhibiting one ‘critical’ growth pathway at several points is enough to induce a proliferation stop as well as apoptosis. Kast et al. (2014) compared different intracellular pathways with the distributaries of the Nile. If one distributary is blocked, the unblocked distributaries will carry excess water and at the end the same amount of water will reach the sea. It is assumed that something similar occurs with the different intracellular pathways, which are tightly linked to each other, so that inhibition of one pathway might not be enough. Inhibition of the PI3K pathway, for example, can result in upregulation of the RAS/RAF/MEK/ERK pathway (Albert et al. 2009). This can be explained by the inhibition of negative feedback loops within the PI3K pathway that then lead to increased insulin/IGF signalling or EGFR activation, which in turn lead to overactivation of MEK (Rozengurt et al. 2014). It seems that inhibiting the PI3K pathway results in increased activity of the RAS/RAF/MEK/ERK pathway and vice versa so that one pathway serves as a backup for the other pathway (Bloxham 2013). Will et al. (2014), for example, were able to show that for the induction of apoptosis in glioblastoma, inhibition of the RAS/RAF/MEK/ERK pathway is necessary in addition to the inhibition of the PI3K pathway. Interestingly, simultaneous inhibition of the PI3K pathway and the RAS/RAF/MEK/ERK pathway also resulted in significantly increased inhibition of cell proliferation and migration (Bloxham 2013).

As in several clinical trials (Galanis et al. 2005; Cloughesy et al. 2006) only one pathway was blocked, this could at least in part explain why these trials achieved only limited success. Hence, wise combination therapy using different substances could be an important component of cancer therapy, as blocking not only one but all substantial pathway distributaries within a cancer cell could lead to the prevention of tumour cell
growth or even to tumour death. A great deal of further investigation is needed to substantiate these hypotheses.

4.3 The role of PI3K in differentiation

Although the statistical significance of aberrant PI3K pathway activation has already been shown for glioblastoma, one would still question why this pathway is mutated so often in glioblastoma if the above-named biological effects of PI3K inhibition are the only ones. Following the demonstration that pluripotency of embryonic stem cells is dependent on active PI3K/AKT signalling (Yu and Cui 2016), it was assumed that the PI3K pathway played a role in differentiation. Several studies found that the up-regulation of the PI3K pathway plays an important role in inhibiting differentiation, thus keeping the tumour in an undifferentiated state: Gont et al. (2013) showed that PTEN loss results in inhibition of differentiation whereas inhibition of the PI3K pathway by using the pan inhibitor LY294002 induces differentiation (Tian et al. 2012). Several other authors have demonstrated that downstream effectors of AKT play an important role in differentiation. Constantly active GSK3β, for example, initiates differentiation of U87 glioblastoma cells (Li et al. 2010). Moreover, active, meaning non-phosphorylated, FOXO3a leads to differentiation of glioblastoma cells (Sunayama et al. 2011). Both GSK3β and FOXO3a are normally inhibited by AKT (Hay 2011; Hermida et al. 2017). Hence, inhibited AKT should lead to less inhibition of GSK3β/FOXO3a and consequently induce differentiation. However, this expected phenomenon could not be confirmed by the data generated in this thesis. In my experiments, stem cells were cultured in medium and treated with PI-103. After 24 hrs, the number of cells becoming adherent was checked; and after 72 hrs, adherent, differentiated, cells were counted again. The number of cells that became adherent after 24 hrs of PI3K pathway inhibition by PI-103 was comparable to the control. After 72 hrs of inhibition of the PI3K pathway by PI-103, reduced numbers of differentiated cells were found. However, this effect could be explained by the effect PI-103 has on proliferation. When repeating the experiments using the ERK inhibitor UO126, the same results as for PI-103 were found again, contradicting published studies where inhibition of the MAP/ERK pathway induced differentiation (Sunayama et al. 2010, 2011). To make sure that the observed difference was not merely due to different inhibition of the PI3K pathway because of using another inhibitor than in the published studies, GSK3β was blocked directly with
the expectation of obtaining less differentiation than previously published (Li et al. 2010). Again, after 24 hrs the same numbers of cells became adherent compared to control, but after 72 hrs fewer differentiated cells were present in the treated group. This finding is therefore in line with published data. However, in all differentiation experiments in this work, the smaller number of differentiated cells after 72 hrs could easily be explained by the effect of PI-103 on cell proliferation rather than on the effect on differentiation itself. The aforementioned publications did not consider to this possibility; i.e. my data are not in contrast with published studies but offer an alternative explanation. Moreover, some of the previous studies were done in cell lines (Li et al. 2010; Sunayama et al. 2010) and cannot, as discussed above, be directly compared to patient-derived glioblastoma cells. Consequently, for more precise findings regarding differentiation effects of PI-103, this treatment should be applied to patient-derived glioblastoma cells, examining both differentiation detected by staining differentiation markers and determining cancer stem cell gene expression as well as other effects of blocking the PI3K pathway, like proliferation or cell death, that could also influence differentiation.

4.4 PI3K mediates motility of a PI3K subpopulation

Interestingly, the PI3K pathway is almost always active and glioblastoma is generally invasive. Consequently, it is suggestive that there might be a link between PI3K activation and invasion. The highly invasive phenotype of glioblastoma represents one of the major therapeutic problems as at the time of diagnosis large areas of the brain are already infiltrated. This makes surgical resection of the entirety of tumour cells impossible. Systemic chemotherapy as well as radiation of the whole brain have also failed to address cells that are remote from the tumour bulk (Giese 2003).

Invasion is a complex interaction of adhesion turnover on the one hand and degradation of extracellular matrix proteins and active cell migration on the other hand (Demuth et al. 2000). Invasion in vivo can be reproduced by motility in vitro to some extent (Mariani et al. 2001). Already during embryological development the PI3K pathway is activated for epithelial-mesenchymal transition and consequently confers increased motility (Xue and Hemmings 2013); the PI3K pathway seems, therefore, to be a promising target for the inhibition of cell invasion. Consequently, motility of glioblastoma cells under treatment with PI-103 was investigated. Adding PI-103 resulted in a significantly
decreased cellular mobility. This is in line with several other publications. Many different agents that decrease cell motility operate by the inhibition of the PI3K pathway (Chandrasekar et al. 2003; Yang et al. 2016). Consequently, several downstream effectors of the PI3K pathway have been shown to be involved in motility by various mechanisms. In squamous cell carcinomas, AKT confers epithelial-mesenchymal transition that is associated with increased cellular motility (Grille et al. 2003). In addition, AKT increases MMP-2 and MMP-9 activity, enhancing the proteolytic properties of the tumour. Matrix metalloproteinases activity is also regulated by PTEN (Paw et al. 2015). Berven et al. (2004) demonstrated that S6K regulates the actin cytoskeleton and consequently cell motility (Berven et al. 2004). By blocking mTORC1, which is upstream of S6K, motility and invasion in several cell types, like arterial smooth muscle (Sakakibara et al. 2005), ovarian cancer cells (Zhou and Wong 2006) and hepatocellular carcinoma (Chen et al. 2009), are reduced. Gulati et al. (2009) were able to show that in glioblastoma, however, mTOR inhibition via rapamycin is insufficient and PI3K has to be blocked at the same time in order to achieve a meaningful biological effect. This indicates, as described above, that many effectors of the PI3K pathway are involved in glioblastoma cell invasion and that for effective treatment the whole pathway or at least fairly apical member molecules have to be blocked. In contrast, Caino et al. (2015) arrived at the conclusion that inhibition of the PI3K pathway resulted in increased cell motility and enhanced invasion of glioblastoma cells. They held mitochondrial repositioning to the cortical cytoskeleton responsible for this phenomenon. Interestingly, organotypic cultures, which imitate in vivo conditions more closely, were used in this study. This again indicates that in vitro experiments often do not adequately reproduce conditions in vivo. Besides, most of the studies cited and the time-lapse experiments in this thesis have also only investigated random motility of cells, disregarding directed migration, although one can assume that in vivo cell motility is mostly directional (Demuth et al. 2000). Unfortunately, when using PI3K pathway inhibitors, random motility is more effectively blocked than directional migration (Gulati et al. 2009). Hence, no definite statement can be made that PI-103, for example, is also effective when cell migration does not occur randomly, although this is of primary interest. Therefore, further investigation using transwell assays, for example, should be carried out. Moreover, this doctoral thesis only investigated motility of differentiated cells and cell lines under treatment, whereas stem cells were not investigated because the typical stem cell model with stem cells as floating spheres was used and consequently did not allow investigation of migration. As, however, stem cells are the population that
is mainly considered to give rise to new tumour formations (Cheng et al. 2010), stem cell motility is of particular interest. Therefore, our group proceeded to elucidate the initial observation by using a modified stem cell culture system and by investigating the role of PI3K signalling in the motility of stem cells. Surprisingly, inhibition of the PI3K pathway only reduced the velocity of differentiated cells, not the velocity of stem cells (Langhans et al. 2017). However, even without treatment, stem cells are about 10-20\% faster than differentiated cells, and increased velocity is often claimed to be a consequence of reduced proliferation, which is also called the ‘go or grow’ dogma (Hatzikirou et al. 2012). Although ‘go or grow’ are often considered mutually exclusive, they might rather be the two ends of a continuum in which the cells exist and depending on the microenvironmental situation will either go or grow. Under adverse conditions like hypoxia or inflammatory stress, epithelial-to-mesenchymal transition is provoked, inducing the cell to ‘go’ (Jing et al. 2011), whereas under favorable conditions tumour growth might be promoted by the secretion of cytokines and growth factors within the microenvironment (Quail and Joyce 2013). Hence, a combination therapy might again be advisable, using drugs that stress the cell, inhibit growth and simultaneously block motility.

4.5 Differences between distinct glioblastoma populations of identical genetic origin

One of the key observations emerging from the data acquired for this thesis is that depending on the differentiation status of the cell (and not its genetic identity) the PI3K pathway seems to be differently regulated in SCs and DCs. Generally, disregarding the PI3K pathway, different characteristics and different behaviour under treatment have been ascribed to these two kinds of cells in the literature. Therefore, in this doctoral thesis, glioblastoma SCs and DCs are treated separately. In accordance with the literature, SCs were cultured as neurospheres in serum-free medium in the presence of FGF and EGF (Günther et al. 2008). For further differentiation between stem cells and differentiated cells many stem cell markers, like CD133, CD15 (also known as SSEA-1), CD44, Nestin, Sox 2 and A2B5, have been described in the literature (Gilbert and Ross 2009; Kyurkchiev et al. 2014); however, the underlying concept is not universally accepted. CD133 was initially referred to as one of the most important stem cell markers in glioblastoma and only CD133\(^+\) cells were considered to be tumorigenic (Singh et al. 2000).
2004); however, newer studies have demonstrated stem cell-like behaviour in CD133− cells (Beier et al. 2007; Joo et al. 2008; Ogden et al. 2008). When CD15 is used, the situation is similar to CD133, as 8 % of all glioblastoma stem cells (identified as being highly tumourigenic in vivo) examined by Son and colleagues were negative for CD15 (Son et al. 2009). Similarly, in a study by Jin et al., glioblastoma stem cells were found that were negative for CD133 and CD15. Unlike CD133 and CD15, nestin could be detected in all glioblastoma stem cells examined (Jin et al. 2013). Consequently, nestin is considered the best stem cell marker presently known. Glial fibrillary acidic protein (GFAP), in contrast, is widely accepted as an astrocyte differentiation marker (Gomes et al. 1999). Accordingly, stem cells used in this thesis were positive for nestin and negative for GFAP. In summary, however, markers alone are not an absolute method of distinguishing stem cells from differentiated cells. Therefore, the different behaviours of these two kinds of cells have also been taken into account. Meanwhile, this approach can be considered a reasonable standard after Schneider et al. have shown that for the characterization of the cells their behaviour is more important than markers and that the characteristics of cells can change under different conditions (Schneider et al. 2016). If one considers the characteristics of the examined cells in this thesis, one can state that in principle two different cell groups can be distinguished. Although tumour cells from three different patients were studied and these cells were genetically different, SCs and DCs, respectively, of all three patients were more similar to each other in keys of their biological behaviour than the two cell types of each individual patient. In conformity with a previously released study (Stupp and Hegi 2007) it was shown that independently of treatment the increase in cell numbers over time is considerably lower in SCs than in DCs. As was also demonstrated (see control group Figures 8 and 9), this is mainly due to the decreased extent of proliferation in SCs and not due to apoptosis. This observation is in line with published work (Altaner 2008). Regarding the behaviour of stem cells under chemotherapy, this study yields results that are both in line with as well as contradicting the literature. Increased resistance to apoptosis of SCs under chemotherapy compared to DCs (Stupp and Hegi 2007; Yamada et al. 2011) has been confirmed when using TMZ, whereas with irinotecan no differences in apoptosis sensitivity between SCs and DCs were observed. However, if metabolic activity is considered as a surrogate of viability - i.e. if the observed effects can be caused by either less proliferation, less metabolic activity, or more apoptosis - SCs were even more sensitive to treatment with TMZ than DCs. When using irinotecan, however, things changed and DCs were again more sensitive. Although apoptosis resistance is
considered to be a direct consequence of low SC proliferations, results with irinotecan indicate that there is no linear relationship between proliferation and apoptosis (Mitchison 2012).

Intriguingly, SCs and DCs use different pathways to survive under treatment and consequently different chemotherapeutics that act via different mechanisms yield different therapeutic efficacies. One clue supporting this theory is our recently published data, by which we have shown, for example, that the expression of apoptosis markers is distinct in SCs and DCs (Schneider et al. 2016). This idea is further supported by the findings presented in this thesis where different reactions of SCs and DCs to PI3K inhibition are demonstrated. While in SCs AKTser is inhibited above all, in DCs it is AKTthr. Moreover, S6 inhibition occurs earlier in DCs, whereas in SCs AKT is first inhibited. Consequently, different roles of the PI3K pathway in SCs and DCs seem obvious. In many stem cell systems, the PI3K pathway is essential for maintaining ‘stemness’ (Kimura and Nakano 2009) and might also be involved in tumorigenesis as well as in escaping therapy (Jhanwar-Uniyal et al. 2015). This brings up the question as to the general role of the PI3K pathway and whether there is a future role for PI3K inhibition in glioblastoma therapy. Since no definite significance of PI3K signalling for glioblastoma survival has been demonstrated, it remains possible that the PI3K pathway plays a role in establishing glioblastoma, a hypothesis that will be discussed in the next section.

4.6 The role of the PI3K pathway in general oncology

In chicken embryo fibroblasts, mutations in PIK3CA are highly tumorigenic (Kang et al. 2005). In humans, mutations in p110α and p110β promote tumorigenic transformation (Zhao et al. 2006; Jia et al. 2008). Consequently, mutations in the PI3K pathway could primarily be responsible for the initial oncogenic transformation and then play only a minor role in tumour survival, as, for example, the PI3K pathway could be responsible for an initial growth advantage while subsequent new mutations would ascertain tumour survival. In embryonic stem cells, for example, PI3K deficiency does neither affect embryonic stem cell proliferation nor survival, but is responsible for maintenance of pluripotency (Yu and Cui 2016).

In glioblastoma the role of the PI3K pathway is complex and far from completely understood. There are too many mediators, feedback loops, cross talks between different
pathways etc. to be able to understand the function of each mediator in the current setting of knowledge and technology. This might be the principal reason why clinical studies (Galanis et al. 2005) have not been able to confirm the efficiency of PI3K inhibition in vitro. As already pointed out, various pathways in a cell are tightly linked to each other. The limited success of clinical trials with PI3K pathway inhibitors might be explained by activation of other pathways, like the RAS/RAF/MEK/ERK pathway (Rozengurt et al. 2014). Furthermore, it has been shown that cancer cells develop drug resistance under PI3K inhibition. On the one hand, prolonged treatment leads to translocation of FOXO to the nucleus, inducing transcription of several tyrosine kinase receptors (Rozengurt et al. 2014). On the other hand, cells under PI3K pathway inhibition become resistant to treatment by metabolic reprogramming (Ghosh et al. 2015). In the cited study, metabolic reprogramming was inhibited by combination therapy with Gamitrinib, a HSP90 inhibitor. Combination therapy therefore holds potential for effective therapy. It ‘just’ has to be worked out which drugs have to be combined and how. In doing so, it has to be kept in mind that substances that are best as single agents are not necessarily best as a part of combination therapy. Single treatment with GDC-0941 alone, for example, is superior to treatment with rapamycin, whereas combination therapy of rapamycin with other drugs outperforms combinations of theses drugs with GDC-0941 in vivo (Nonnenmacher et al. 2015). When combining different drugs, their side effects and interactions always have to be taken into account as well, as these might crucially interfere with successful therapy. One promising model of combination therapy is the CUSP9v3 (Comprehensive Undermining of Survival Path with 9 repurposed drugs, version 3) project, where aprepitant, celecoxib, auranofin, captopril, minocyclin, disulfiram, itraconazole, ritonavir and sertraline are combined with low-dose metronomic TMZ. All these drugs are already used effectively in the treatment of non-oncologic diseases and known to be relatively well tolerated without great side effects and to have good pharmacological effects (Kast et al. 2013, 2014). They are all likely to inhibit native growth-promoting pathways in glioblastoma as well as pathways that are compensatorily up-regulated as a result of exposure to cytotoxic drugs, like AKT, mTOR, hedgehog, MMP2 and MMP9. In vitro the combination of the CUSP9 drugs elicits cell death in a highly effective manner at concentrations that are clinically achievable in plasma. CUSP9v3 is currently evaluated in a prospective clinical trial conducted in the Department of Neurosurgery Ulm University Hospital and has shown promising results on interim analysis (Halatsch et al. 2018).
4.7 Limitations of the experimental approach used

When discussing the data presented here, it has to be taken into account that all the results have been obtained under standard cell culture conditions, meaning that there is no adequate modelling of the in vivo microenvironment and that substances are added directly to the cells meaning that no brain blood barrier has to be crossed. Both facts are a general problem with experiments done in cell culture and could be one possible explanation why treatments that are effective in vitro do not work similarly well in vivo. Most therapies act by treating the malignant cells themselves, like, for example, all therapies examined in this doctoral thesis. Westhoff et al. (2014) however, showed that when in contact with their microenvironment, cancer cells can avoid therapy-induced apoptosis by survival signals through adhesion contacts – a process called adhesion-mediated apoptosis resistance (AMAR). Consequently, if only the malignant cell is treated, treatment-induced apoptosis may be counteracted by glioblastoma cell interactions with their microenvironment. Interestingly, the PI3K pathway seems to play a crucial role in AMAR, as adhesions like adherens junctions or focal adhesions often mediate their survival signals by using the PI3K and/or RAS/RAF/MEK/ERK pathways (Westhoff et al. 2014). Moreover, microglia can be ‘enslaved’ by gliomas into tumour-supporting phenotypes (Wei et al. 2013). There is evidence that upregulation of the PI3K pathway is necessary to convert microglia into a tumour-supportive state (Crespo et al. 2016). Therefore, by treating the PI3K pathway, on the one hand the malignant cell itself is treated, on the other hand communication between the malignant cell itself and its environment can be modulated.

In the process, the plasticity of tumour cells should not be disregarded. It is not clear if the cells named stem cells in this doctoral thesis are really identical with the ‘true’ glioblastoma stem cells or if the cells investigated merely mark different end points of a continuum of differentiation within a tumour mass (Schneider et al. 2016). Indeed, whether a true glioblastoma stem cell exists at all is open to debate. In addition, statements about differentiation are difficult to make in cell culture. Differentiation not only depends on intrinsic pathways that are amenable to analysis in cell culture but also on environmental factors. Hypoxia, for example, helps to preserve the undifferentiated state of glioblastoma stem cells (Li et al. 2009; Seidel et al. 2010). Moreover, endothelial cells have been shown to promote self-renewal of stem cells (Shen et al. 2004). This exemplifies that experiments investigating stem cells are hard to carry out under in vitro conditions. ‘Real’ stem cells need their stem cell niche for ‘real’ stem cell
behaviour (Plaks et al. 2015). On the other hand, recent investigations suggest that
differentiated cells can de-differentiate. Schneider et al. (2016) were able to show that in
mice, tumours could be induced not only by injecting stem cells but also by injecting
differentiated cells intracerebrally. De-differentiation of cells might also explain why
glioblastoma rapidly reappear after surgical resection. If one accepts the three-layer
concentric model of glioblastoma (Persano et al. 2011), stem cells are localized in the
middle of the tumour and should consequently be removed by surgical resection. If
differentiated glioblastoma cells at the border of the tumour mass, or indeed among
the invading cells that are not surgically removed, could de-differentiate, this could
explain the reappearance of the tumour. Another theory of gliomagenesis, which is not
without controversy, points in the same direction: it postulates that de-differentiation
of neurons and astrocytes is responsible for the formation of a new tumour (Friedmann-
Morvinski et al. 2012).

Furthermore, in the experimental work for this doctoral thesis, therapeutic agents have
been administered directly to the cells. In the process, important aspects like bioavail-
ability could not be modelled: PI-103, for example, is rapidly metabolized, does not
cross the brain blood barrier and, therefore, can only be used for experiments in cell cul-
ture but is not suitable for the treatment of patients. However, by using this substance
in cell culture, important findings about the PI3K pathway can be made (Wen et al.
2012). Fortunately, as replacement for the early relatively nonspecific inhibitors like
wortmanin or LY294002, which not only have significant limitations regarding their
bioavailability but also reveal considerable side effects and toxicity (McNamara and
Degterev 2011), more selective and, above all, orally administrable, better-tolerated
substances have been found (Workman et al. 2010). However, it has to be taken into
account that different substances act differently and that a different inhibition of the
PI3K pathway could have great effects on therapeutic efficiency. All PI3K isoforms
play a unique role in cell physiology and, therefore, selective inhibition of different
PI3Ks has different effects. Inhibition of p110δ, for example, leads to less effective
inhibition of phosphorylation of AKT than inhibition of p110α or p110β (Fan et al.
2006). But even when comparing only inhibitors that most potently target p110α (for
example PIK-64, PIK-85, PIK93), great differences in their efficacy of blocking AKT
phosphorylation become apparent (Fan et al. 2006). Moreover, the PI3K pathway can
be blocked at different stages of signalling. Inhibition of PI3K, for example, can also
inhibit the RAS/RAF/MEK/ERK pathway whereas inhibition of AKT does not and
hence is less effective (Will et al. 2014). In terms of efficacy in glioblastoma treatment, simultaneous inhibition of PI3K and mTOR has been shown to be especially effective (McNamara and Degterev 2011). As the dual inhibitor PI-103, which is frequently used for in vitro experiments, is not suitable for clinical therapy, some dual inhibitors of PI3K and mTOR have been developed for clinical use, like NVP-BEZ235, GSK2126458, PF-04691502 or DS7423 (McNamara and Degterev 2011; Koul et al. 2017). DS7423, for example, is orally-available, crosses the brain blood barrier and potently suppresses PI3K-associated biomarkers (Koul et al. 2017).

4.8 Outlook/Future directions

Signalling in glioblastoma cells has proved to be complex, and we are far from comprehensively understanding the different intracellular processes and signalling crosstalks in a glioblastoma cancer cell. It is often claimed that the future of cancer therapy will be personalised therapy (Shirai and Chakravarti 2011; Singh et al. 2018). Already the differences between the three cell lines G 35, G 38 and G 40 used in this doctoral thesis demonstrate that tumour cells of different patients behave differently under the same treatment (e.g., different inhibition of AKT and S6 after addition of PI-103). With current sequencing techniques, it is already possible to determine the driver gene mutations of each patient’s tumour. However, Le Tourneau et al. (2015) showed in their SHIVA study that personalised therapy produced no better results than standard therapy (Le Tourneau et al. 2015). This study used only single agents for therapy without paying attention to the fact that patients with mutations in the PI3K pathway often also harbour alterations in the RAS/RAF/MEK/ERK pathway. Therefore, the results of the SHIVA study should not be interpreted to imply that personalised medicine is not effective, but that monotherapy of agents is not advisable (Tsimberidou and Kurzrock 2015). However, it may be reasonable to determine individually which pathways are upregulated in each patient and then to specifically inhibit them in a combination therapy. Consequently, it is likely that in future cancer therapy, different drugs will be combined in order to improve efficacy, to avoid the development of drug resistance and to reduce side effects, just to name some of the advantages of combination therapy in general. Certainly, the inhibition of the PI3K pathway will play a role in future combination therapies as it has been shown that inhibition of the PI3K pathway can significantly reduce cellular proliferation and motility.
5 Summary

Glioblastoma is the most frequently occurring primary malignant brain tumour in adults. With a median survival of approximately 16-18 months under current standard treatment with safe maximal resection and radiochemotherapy it remains one of the most aggressive and deadly tumour entities. The reason for this is mainly rooted in rapid tumour recurrence. As radio- and chemotherapy achieve limited effects, new therapeutic possibilities are urgently investigated. In this context it has been demonstrated that the Phosphatidylinositol-3-kinase (PI3K) pathway is activated in up to 88% of all glioblastomas. This pathway is central to every cell, involved in cell growth, proliferation, apoptosis, maintenance of stemness and migration. Consequently, this study aimed to investigate the effect of inhibiting the PI3K pathway by means of PI-103, a dual inhibitor of PI3K and Mammalian target of rapamycin complex (mTOR), a kinase downstream of PI3K that also regulates cell proliferation, cell growth and cell survival. For this purpose, primary glioblastoma stem cells as well as ‘differentiated’ primary glioblastoma cells from three different patients were treated with PI-103 and their biological behaviour, like proliferation, apoptosis, differentiation and migration, was observed compared to untreated controls. Moreover, it was tested whether PI-103 can sensitize glioblastoma for chemotherapy. This study shows that PI3K inhibition leads to a significant reduction in proliferation but is unable to induce apoptosis and consequently has a cytostatic rather than a cytotoxic effect. While there was no effect on differentiation, migration was strongly inhibited. In contrast to established cell lines, the primary glioblastoma cells used could not be sensitized to chemotherapy-induced apoptosis by PI-103. Nevertheless, with its significance for proliferation and migration, inhibition of the PI3K pathway may still remain a significant aspect of future glioblastoma therapy, for example in combination with the inhibition of other important signalling pathways. To achieve this, it has to be determined which substances have to be combined in which doses, sequence and temporal spread, an inquiry that so far has proven complex and difficult in glioblastoma.
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# References

1. Adamson C, Kanu OO, Mehta AI, Di C, Lin N, Mattox AK, Bigner DD: Glioblastoma multiforme: a review of where we have been and where we are going. Expert Opin Investig Drugs 18: 1061–1083 (2009)


breast cancer cells to chemotherapy. Journal of the National Cancer Institute 100: 672–679 (2008b)


173. Sakakibara K, Liu B, Hollenbeck S, Kent KC: Rapamycin inhibits fibronectin-induced migration of the human arterial smooth muscle line (E47) through the


221. Westhoff MA: (2019 personal communication)


8 Appendix

8.1 Biphasic progression of MTT results

The MTT assays reveal that different concentrations of PI-103 have interesting effects on metabolic activity: intriguingly, after 24 hrs of treatment induction in SC G35, very low concentrations of PI-103 reduced metabolic activity to a level above that induced by 0.6µg/ml PI-103. Very high concentrations, however, had almost no effect on metabolic activity. This biphasic curve shape seen in G35 SC is weaker in G40 SC and can only be guessed at in G38 SC. Moreover, after 72 hrs, this phenomenon was only slightly detectable in SC G35 and SC G40, but a clear biphasic process was not present. In DCs, the curve shape at low concentrations is similar to that in SCs, but the biphasic shape is visible especially after 72 hrs and not after 24 hrs. Consequently, while reduction of metabolic activity at very low concentrations of PI-103 diminishes after some time in SCs, longer incubation with PI-103 appears to induce this phenomenon in DCs. However, in DCs metabolic activity did not increase again at high concentrations. In order to investigate this phenomenon more precisely, the effect on protein signalling evoked by the very low and very high concentrations of PI-103 used in the MTT assays was examined by Western Blotting (Figure 23).
Figure 23: Effects of very high and very low concentrations of PI-103 on protein signalling in glioblastoma cells

Three different glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – upper row) and three corresponding distinct glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – lower row) were treated either with the indicated concentrations of PI-103 or with corresponding amounts of DMSO (controls). After 24 hrs, protein expression levels and phosphorylation status of AKT and S6 ribosomal protein served as surrogate read-outs for PI3K and mTOR activity, respectively, and were analysed by Western blotting. GAPDH expression served as a loading control.

The figure shows a representative example of two independent replicates.

DMSO = dimethyl sulfoxide; AKT = protein kinase B; pAKT = phosphorylated AKT; thr = threonin; ser = serine; S6 = ribosomal protein S6; pS6 = phosphorylated S6; GAPDH = glycerinaldehyde-3-phosphat-dehydrogenase; mTOR = mammalian target of rapamycin; SC = stem cell; DC = differentiated cell.
In SCs, low concentrations did not inhibit (0.01 µg/ml) or only slightly inhibit (0.04 µg/ml) phosphorylation of AKT and S6, whereas 5 µg/ml as well as 20 µg/ml almost completely prevented phosphorylation of AKT and S6. In DCs, 0.01 µg/ml and 0.04 µg/ml had no effect on protein signalling. High concentrations, however, strongly inhibited phosphorylation of S6 and pAKT\text{ser} 473 simultaneously, inducing double bands in pAKT\text{thr} 308. As changes in phosphorylation of AKT and S6 did not explain the biphasic curve shape seen in the MTT assays, the phosphorylation state of proteins at tyrosine residues (ptyr) was examined 24 hrs after addition of the inhibitor (Figure 24).

In SC G 35 – the cell line with the most pronounced biphasic curve shape – the ptyr status reflected this biphasic shape, as 0.01 µg/ml PI-103, for example, led to a stronger reduction in overall ptyr than 0.04 µg/ml (see Figure 25). To a lesser extent, this was also observed in SC G 38 and SC G 40. In DCs, the ptyr status did not correlate with the MTT results. However, it has to be considered that biphasic shapes in the MTTs of DCs were only visible after 72 hrs of addition of PI-103, whereas ptyr Western Blots were completed after 24 hrs.
Figure 24: Effects of very high and very low concentrations of PI-103 on phosphorylation of tyrosine residues in glioblastoma cells

Three different glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – upper row) and three corresponding different glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – lower row) were treated either with the indicated concentrations of PI-103 or with corresponding amounts of DMSO (controls). After 24 hrs, phosphorylation status of tyrosine residues was analysed by Western blotting. GAPDH expression served as a loading control.

The figure shows a representative example of two independent replicates.

DMSO = dimethyl sulfoxide; ptyr = phosphorylation of tyrosine residues; GAPDH = glycerinaldehyde-3-phosphat-dehydrogenase; mTOR = mammalian target of rapamycine; SC = stem cell; DC = differentiated cell.
Figure 25: Comparison of glioblastoma cell viability and phosphorylation of tyrosine residues after addition of PI-103

Three different glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three corresponding different glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – right side) were treated either with the indicated concentrations of PI-103 or with corresponding amounts of DMSO (controls). After 24 hrs, MTT assays measuring the cell population’s viability were performed. Western Blot bands of phosphorylated tyrosine residues of the corresponding concentrations of PI-103 (see Figure 24) are also illustrated.

The bar chart shows the mean and standard deviations of three replicates. Each of them was performed in triplicates.

Western Blot bands are representative results of two independent replicates.

MTT = tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;
DMSO = dimethyl sulfoxide; SC = stem cell; DC = differentiated cell.

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8.2 Differences between SCs and DCs in protein signalling

In SCs, a similar biphasic effect as in the dose-response kinetic could also be seen in the time-response kinetic, especially when using 0.3 µg/ml PI-103 (see Figure 26), as inhibition declined for up to 24 hrs and then increases again. Moreover, differences between SCs and DCs were detectable again: using 0.3 µg/ml PI-103 in SCs, phosphorylation of S6 was mainly inhibited after 48 and 72 hrs, whereas in DCs maximal inhibition was reached after 24 hrs. Furthermore, in DCs S6 is upregulated for 4 to 72 hrs after addition of 0.3 µg/ml PI-103. When 0.6 µg/ml PI-103 was used, this phenomenon did not occur. However, 0.6 µg/ml PI-103 had a more potent effect, leading to a stronger and more rapid inhibition of phosphorylation of S6 in DCs. In SCs, inhibition was more pronounced, starting earlier but also reaching its maximum after 72 hrs. Regarding AKT in SCs, inhibition started rapidly after addition of 0.3 µg/ml PI-103 with maximal inhibition already after 1 hr of incubation, whereas in DCs inhibition did not begin until 24 hrs after addition of the inhibitor, and almost no inhibition was seen after 1 and 4 hrs. When using 0.6 µg/ml PI-103, however, inhibition of phosphorylation of AKT reversed in DCs, with maximal inhibition after 1 and 4 hrs and reduced inhibition of phosphorylation after 24, 48 and 72 hrs. In SCs, time kinetic stayed the same as with 0.3 µg/ml PI-103, but 0.6 µg/ml PI-103 had a more potent effect.
Figure 26: Effects of prolonged exposure to 0.3 µg/ml PI-103 on protein signalling in glioblastoma cells

Three different glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three corresponding different glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – right side) were either treated with 0.3 µg/ml PI-103 or with corresponding amounts of DMSO (controls) for the indicated time periods. Protein expression levels and phosphorylation status of AKT and S6 ribosomal protein served as surrogate read-outs for PI3K and mTOR activity, respectively, and were analysed by Western blotting. GAPDH expression served as a loading control. The figure shows a representative result of two independent replicates.

DMSO = dimethyl sulfoxide; AKT = protein kinase B; pAKT = phosphorylated AKT; thr = threonin; ser = serine; S6 = ribosomal protein S6; pS6 = phosphorylated S6; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; mTOR = mammalian target of rapamycin; SC = stem cell; DC = differentiated cell.

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8.3 Supplementary figures

Figure 27: Evolution of glioblastoma cell numbers after prolonged exposure to 0.3 µg/ml PI-103

Three different glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three corresponding different glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – right side) were treated either with 0.3 µg/ml PI-103 or with corresponding amounts of DMSO (controls). Cells were counted every 24 hrs for a total of 120 hrs.

The figure shows the mean and standard deviations of three independent replicates. Each of them was performed in triplicate. Statistically significant results (two-sided Student’s t-test; \( p < 0.05 \)) are asterisked.

DMSO = dimethyl sulfoxide; SC = stem cell; DC = differentiated cell.

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Figure 28: Effects of 0.3µg/ml PI-103 on specific DNA fragmentation in glioblastoma cells

Three different glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three corresponding different glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – right side) were treated either with 0.3µg/ml PI-103 or with corresponding amounts of DMSO (controls). After the indicated time periods, FACS analysis of DNA fragmentation within propidium iodide-stained nuclei was performed. PI-103-induced DNA fragmentation is shown relative to spontaneous DNA fragmentation of DMSO-treated cells.

The figure shows the mean and standard deviations of three independent replicates. Each of them was performed in triplicate. Statistically significant results (two-sided Student’s t-test; p < 0.05) are asterisked.

DMSO = dimethyl sulfoxide; SC = stem cell; DC = differentiated cell.

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Figure 29: Effects of combination treatment with 100µM TMZ and 0.3µg/ml PI-103 on glioblastoma cell viability

Three different glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three corresponding different glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – right side) were treated either with 100µM TMZ or 0.3µg/ml PI-103 alone or with a combination of both. After 72 hrs as well as after 120 hrs, cell viability was measured by MTT analysis. Controls were treated with corresponding amounts of DMSO and control cell numbers were defined as 100%.

The figure shows the mean and standard deviations of three independent replicates. Each of them was performed in triplicate. The mean of an additive effect as determined by Bliss analysis is marked by a red line.

MTT = tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO = dimethyl sulfoxide; TMZ = temozolomide; SC = stem cell; DC = differentiated cell.

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Figure 30: Effects of combination treatment with 10 nM irinotecan and 0.3 µg/ml PI-103 on glioblastoma cell viability

Three different glioblastoma SC lines (G 35 SC, G 38 SC, G 40 SC – left side) and three corresponding different glioblastoma DC lines (G 35 DC, G 38 DC, G 40 DC – right side) were treated either with 10 nM irinotecan or 0.3 µg/ml PI-103 alone or with a combination of both. After 72 hrs as well as after 120 hrs, cell viability was measured by MTT analysis. Controls were treated with corresponding amounts of DMSO and control cell numbers were defined as 100%.

The figure shows the mean and standard deviations of three independent replicates. Each of them was performed in triplicate. The mean of an additive effect determined by Bliss analysis is marked by a red line.

MTT = tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;
DMSO = dimethyl sulfoxide; TMZ = temolozomide; SC = stem cell; DC = differentiated cell.

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9 Statutory declaration

Hereby I declare that I wrote the present dissertation, entitled:

“The effects of the dual kinase inhibitor PI-103 on glioblastoma cells”,

independently and used no other aids that those cited. In each individual case, I have clearly identified the source of the passages that are taken word for word or paraphrased from other works.

Furthermore, I hereby declare that I have carried out my scientific work according to the principles of good scientific practice in accordance with the current “Satzung der Universität Ulm zur Sicherung guter wissenschaftlicher Praxis” [Rules of the University of Ulm for the Ensuring of Good Scientific Practice].

Ulm, 22.02.2019

Stephanie Ströbele
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11 Curriculum Vitae

Curriculum vitae removed for privacy reasons.
12 Publications

