The Potential and Challenges of siRNA-Based Targeted Therapy for Treatment of Patients with Glioblastoma

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1. Introduction

Brain tumor is the second leading cause of cancer-related death in children under age of 20. An estimated total of 62,930 new cases of primary brain tumors in United States (CBTRUS, 2010) and 2,600 in Canada (CCS, 2010) were expected to be diagnosed in 2010 in adults and children. This includes both malignant (23,720 in US) and non-malignant (39,210 in US) brain tumors (CBTRUS, 2010). The most common (80\%) form of malignant primary brain tumor originates from the neuroglial cells and is referred to as glioma (CBTRUS, 2010). Tumors originating from the astrocytes constitute 76\% of cases of gliomas, and glioblastoma (GBM) is the most common malignant form of glioma (53.8\%) (CBTRUS, 2010). Despite aggressive therapeutic interventions, 90\% of patients are expected to succumb to the disease within five years after diagnosis (Stupp et al., 2005). Research is desperately needed to improve our understanding of the disease and to define strategies that will increase the efficacy of our treatment options.

2. Clinical and molecular features of GBM

GBM is a highly malignant type of primary brain cancer which mainly affects patients in their fifties and older. It is classified as a grade IV tumor using the World Health Organization (WHO) grading system and is associated with a median survival of approximately 12-15 months compared to 48 months for diffuse astrocytoma (WHO grade II), its lower grade counterpart (Louis et al., 2007). Secondary GBMs progress after a period of growth from low-grade gliomas (LGGs), while \textit{de novo} GBMs arise rapidly into the most

* Corresponding Author
malignant form without the typical period of indolent growth associated with secondary GBMs. The latter preferentially affects older patients and is frequently associated with amplification of the epidermal growth factor receptor (EGFR) locus and inactivation of the phosphatase and tensin homolog (PTEN) tumor suppressor gene. Recent discovery of the recurrent R132H mutation in the isocitrate dehydrogenase 1 (IDH1) gene in a vast majority of secondary GBMs and a large percentage of LGGs further contributed to differentiate histologically similar GBM at the molecular level (Ichimura et al., 2009; Yan et al., 2009). In addition, the R132H IDH1 genotype and the mutation of TP53 are found to be frequently associated in secondary GBMs. Due to the aggressive biological phenotype of GBM, most patients present with acute headache, vomiting, occasional transient or partial blindness due to raised intracranial pressure, and local or general brain dysfunction, possibly leading to altered behavior or seizures (Brada et al., 2001; Chandana et al., 2008).

Current diagnosis and grading of gliomas are dependent on the histological analysis of biopsied or resected tumor tissue, together with immunohistochemistry and molecular testing on selected markers (Louis et al., 2007). Morphologic criteria are often arbitrary and based on the histological profile of tumor cells including 1) a high density of small and pleomorphic tumor cells that are characterized by large and elongated nuclei (Figure 1a), 2) the frequency of mitotic figures, 3) the presence of tumor necrosis that can be surrounded by dense accumulations of tumor cells (pseudopalisading necrosis, Figure 1b) and 4) the presence of microvascular proliferation (Figure 1c) (Gray, 2005; Prados M., 2002; Scherer, 1940). The presence of all these histological entities indicates an astrocytoma of WHO grade IV. Anaplastic astrocytoma, a WHO grade III tumor, does not have tumor necrosis or foci of microvascular proliferation. A feature that is common to all gliomas, regardless of grade and cell type, is the extent of local invasion and distant infiltration of the normal neuropil by the neoplastic cells. Histological correlations of this phenomenon were described by Scherer in 1940 (Scherer, 1940). Scherer described the aggregation and migration of glioma cells along normal blood vessels (perivascular satellitosis), neurons (perineuronal satellitosis), below the pial surface (subpial spread), and finally along large white matter tracts (intrafascicular growth). These “Scherer’s secondary structures” are easily identified and constitute microscopic landmarks of glioma malignancy (Holland, 2000). However, accurate pathological classification of a tumor is often confounded by an undersampling bias, especially in cases of needle biopsies of large lesions. In addition, there is significant heterogeneity among gliomas at the genetic, epigenetic, and histological level (Walker C. et al., 2003).

There is little doubt that molecular heterogeneity contributes to the clonal evolution (Gerlinger & Swanton, 2010) within a tumor, which directly impacts clinical behavior such as treatment failure and disease recurrence (Yip et al., 2009). The example of the selection of GBM tumor cells carrying inactivating mutations in the mismatch repair gene mutS homolog 6 (MSH6) during treatment illustrates well this phenomenon and will be discussed further in section 5. Moreover, one microscopic field of GBM sample taken from the same patient can appear vastly different from another area. In addition, low and high-grade gliomas from different patients, with similar morphological appearance and of the same histological grade, can behave quite differently (Frazier et al., 2009; Krex et al., 2007; Yamanaka et al., 2006). Therefore, traditional histopathology alone is not sufficient to provide information with respect to prognostic and predictive substratification. Fortunately,
molecular biomarkers are making inroads into the clinic diagnostic workup of brain tumors (Jansen et al., 2010; Yip et al., 2008).

Fig. 1. Micro- and macroscopic view of a GBM tumor. a) Astrocytomas, both low and high grade, consist of neoplastic cells with elongated nuclei in association with variable amount of eosinophilic cytoplasm often extended into coarse processes. GBM often display an exaggerated degree of morphologic heterogeneity as seen in this figure, with tumor cells with bizarre and giant nuclei. b) Pseudopalisading necrosis is a cardinal feature of GBM and is characterized by an area of tissue necrosis surrounded immediately by a rim of viable tumor cells. The microenvironment in this region is often characterized by low oxygen tension (hence the necrosis) and activation of various hypoxia-mediated molecular pathways such as HIF-1α in the tumor cells (Kaur et al., 2005; Rong et al., 2006). c) Low magnification survey of a GBM showing multiple foci of microvascular proliferation (MVP) characterized by abnormal proliferation and hypertrophy of endothelial cells as a result of an overly pro-angiogenic environment. The presence of MVP automatically “upgrades” diagnosis from an astrocytoma to a WHO grade IV GBM. Foci of MVP are often found in areas with other malignant histological features including florid mitotic activity and tumor necrosis. d) Intraoperative picture of a GBM showing significant distortion of the gross morphology of the cerebral cortex resulting in expansion and discoloration of the gyri. e) and f) Magnetic resonance imaging (MRI) of a GBM using T1 sequence in conjunction with intravenous administration of gadolinium (GAD) dye. The tumor is represented in the axial (e) and coronal (f) planes, which is essential for localization of the lesion and surgical planning. The surfeit of tumor-associated and abnormally developed blood vessels (i.e. the MVP) with a defective blood-brain barrier (BBB) allows for penetration of GAD into the tissue, and accentuates areas of BBB breakdown. This so-called “contrast- enhancing” appearance is typical of high grade gliomas.

In addition to changes in nucleotide sequences within the genetic makeup of a cancer cell, the epigenome, defined by the profile of selective methylation of CpG-islands and complex
covalent modifications of histone proteins, is equally important in defining malignant behavior (Ting et al., 2006). Epigenetic modifications result in transcriptional silencing or activation of affected gene or genes, and can have widespread consequences in the expression of genes important in survival, development, and growth regulation. Aberrant epigenetic changes in brain tumors are well documented (Fouse & Costello, 2009; Kim et al., 2006; Wu et al., 2010) and recently, The Cancer Genome Atlas (TCGA) consortium undertook a systematic large scale profiling of the GBM epigenomes (CGARN, 2008). The best-known brain tumor epigenetic biomarker is the hypermethylation of the O-6-methylguanine methyltransferase (MGMT) promoter. MGMT promoter hypermethylation correlates with an enhanced response to combined temozolomide (TMZ; Temodar®, Merck) chemotherapy and radiotherapy in patients with GBMs (Hegi et al., 2005), and predicts a better prognosis in elderly GBM patients (Gerstner et al., 2009). The discovery of the glioma-CpG Island Methylator Phenotype from epigenome-wide analysis of TCGA glioblastoma has also highlighted the potentials of the epigenome-wide approach (Noushmehr et al., 2010). The information obtained from genetic and epigenetic analyses of a tumor sample can be used to guide the decision of the treatment to offer the patient.

3. Standard of care and other therapeutic options

The current standard of care for GBM patients starts with maximal safe surgical resection. Patients might undergo repeated surgical resections to treat recurrent tumor growth (Wen & Kesari, 2008). As mentioned in the previous section, neoplastic cells from both low and high-grade glioma do share some common characteristics including the innate ability to infiltrate and invade surrounding normal brain tissue, which results in the distal spread of tumor cells at an early stage of the disease and the difficulty in clearly delineating the tumor margin. Surgical resection is followed by radiation therapy and concurrent TMZ, followed by adjuvant TMZ therapy (Clarke et al., 2010). TMZ is an alkylating agent, triggering cell death by the addition of DNA damaging alkyl groups on guanine bases (Newlands et al., 1997). The inclusion of TMZ in GBM standard of care improved the 2-year survival times from 10.4% to 26.5% (Stupp et al., 2005). However, the 5-year survival of GBM patients still remains less than 10% (CBTRUS, 2010; Stupp et al., 2009). As mentioned earlier, an important predictive factor in response to TMZ is the level of methylation of the MGMT promoter region found in the tumor. Promoter methylation of the MGMT gene prevents the expression of the MGMT enzyme capable of removing alkyl groups (Jacinto & Esteller, 2007). GBM tumors carrying a non-methylated promoter (40-55% of cases (Hegi et al., 2005; Sadones et al., 2009)) respond poorly to TMZ and there is currently no alternative treatment for these patients. At present, all patients with GBM are treated with TMZ with concurrent radiation therapy. Recurrent GBM tumors are often chemoresistant and exhibit accelerated growth rate (Cahill et al., 2007; Yip et al., 2009). Interestingly, a subset of patients with GBM who recurred after the initial treatment (surgery, radiation, TMZ) does respond to metronomic doses of TMZ. This treatment option consists of a dose-intensive daily intake of the drugs to maintain tumor suppression (Perry et al., 2010).

Before the introduction of TMZ as the standard of care for GBM patients, nitrosoureas were the foundation of GBM treatment for more than 30 years (Stupp et al., 2006). Among them, the alkylating agent carmustine (BiCNU®, Bristol-Myers Squibb) was used in conjunction with radiotherapy (Brandes et al., 2004a; Walker M. D. et al., 1978; Walker M. D. et al., 1980).
Severe pulmonary toxicity and limited efficacy of the drug due to insufficient drug delivery motivated the development of carmustine wafers (Gliadel®, Guilford Pharmaceuticals; Figure 2). Administration of carmustine using biodegradable polymer wafers provides a controlled release of the drug in the brain micro-environment while minimizing systemic toxicity (Lin & Kleinberg, 2008). However, recent trials showed that the system provides no additional benefit compared to TMZ (Adamson et al., 2009). A combination regimen consisting of the alkylating agents lomustine and procarbazine, and the microtubules destabilizer vincristine (PCV), has also been used extensively for the treatment of GBM (Kappelle et al., 2001). Yet, high incidence of hematological toxicities and inferior response rates in comparison to TMZ (Kappelle et al., 2001; Stupp et al., 2006) favored the establishment of TMZ as the current standard of care. The topoisomerase I inhibitor irinotecan (CPT-11; Camptosar®, Pharmacia & Upjohn) is a FDA-approved drug for colorectal cancer. Promising results for GBM patients in phase II trials were demonstrated with this compound, especially when used in combination with other agents such as TMZ or carmustine (Brandes et al., 2004b; Friedman et al., 2009; Turner et al., 2002; Vredenburgh et al., 2009), and it may soon be integrated among the Food and Drug Administration (FDA)-approved options for GBM patients. This compound is now recommended by the National Comprehensive Cancer Network for use in combination with bevacizumab (Avastin, Genentech/Roche) for GBM treatment (NCCN, 2011).

Fig. 2. Intraoperative picture of the placement of Gliadel® wafers into the resection cavity of a recurrent anaplastic oligoastrocytoma. The wafers, impregnated with the alkylating agent carmustine, deliver a locally concentrated dose of chemotherapeutic agent, reducing systemic toxicity.

More recently, targeted agents have been developed and showed some activity in GBM patients. Among them, a monoclonal antibody raised against the vascular endothelial growth factor (VEGF), bevacizumab, was recently approved by the FDA for the treatment of recurrent GBM, as several clinical trials demonstrated its efficacy as a single agent to prolong the progression-free survival of patients (Friedman et al., 2009; Kreisl et al., 2009).
However, results have been disappointing in terms of improvements in median survival, and a clinical trial is currently ongoing and was designed to investigate the value of combining bevacizumab with TMZ (Chinot et al., 2011). It is hoped that bevacizumab therapy can induce a normalization of the tumor vasculature (see section 10) and thus improve the homogenous delivery of the cytotoxic agent TMZ. The EGFR inhibitors gefitinib (Iressa®, AstraZeneca Canada Inc.) and erlotinib (Tarceva®, Roche) have also shown some efficacy in the treatment of malignant glioma (Raizer et al., 2010; Rich et al., 2004), but response rates have been variable and unpredictable (Stupp et al., 2006). Imatinib mesylate (Gleevec®, Novartis) was developed to specifically inhibit Bcr-Abl signal transduction in chronic myeloid leukemia, and was later shown to also inhibit c-Kit and platelet-derived growth factor receptor (PDGFR) activity. The latter finding supported clinical testing of imatinib for GBM treatment, and similarly to the EGFR inhibitors, response rates were variable but promising (Razis et al., 2009; Wen et al., 2006). Other targeted therapy compounds currently evaluated for use in GBM treatment are described in section 5.

4. Challenges

One of the major challenges of GBM chemotherapy is the achievement of adequate drug concentration within the tumor itself, and this obstacle can largely be attributed to the presence of the BBB (Clarke et al., 2010). Unlike capillaries elsewhere in the body, endothelial cells of brain capillaries have tight junctions that are highly resistant to the passage of ions or small molecules and do not exhibit trans-endothelial transport (Fawcett, 1994). Moreover, the astrocytes and pericytes play an important role in regulating this barrier through molecular cross-talk involving adhesion and tight-junction molecules, the integrins and other extra-cellular matrix (ECM) molecules (reviewed in (Wolburg et al., 2009)). The BBB in GBM tumors exhibits more frequent fenestrations, a loss of tight intercellular junctions and less developed astrocytic pericapillary sheath, all factors contributing to increasing its permeability (Engerhard et al., 1999) (Figure 3). However, this compromised BBB still acts as an obstacle for many drugs (Pardridge, 2007). Therefore, the main limitation for drug choice in the treatment of GBM is the capacity of the compound to penetrate the BBB. Two main mechanisms by which a synthetic drug molecule can cross the BBB are: 1) by transmembrane diffusion or 2) through transporters (Banks, 2009). Most drugs used for management of GBM (e.g. TMZ, carmustine) are small and lipophilic molecules that cross the BBB by transmembrane diffusion. This mechanism is a non-saturable process that depends on molecular diffusion through cell membranes. Factors influencing this process include a good balance between liposolubility (penetration through cell membrane) and hydrosolubility (to improve drug circulation and presentation to the BBB) which are influenced by chemical structure, size and charge (Banks, 2009). The capacity to escape the ATP-Binding Cassette (ABC) transporters, such as the P-glycoprotein (Begley, 2004a) responsible for brain-to-blood efflux, is also important. Saturable transport systems (i.e. ligand transporters) can be used to improve the pharmacokinetic profile of a substance and to target uptake into specific regions of the central nervous system (CNS) (Banks, 2009). Analogs of transporter ligands have been developed for various CNS pathologies (Begley, 2004b). Alternatively, influx transporters can be targeted in a “Trojan-horse” like strategy (i.e. molecules that do not cross the BBB are coupled to ligand molecules that do) but unfortunately, these chemical modifications often result in a decrease in ligand
uptake or routing of the hybrid compound to lysosome compartments for degradation (Banks, 2009).

Fig. 3. Electron micrograph showing the structure of brain capillaries from normal and tumor tissue. a) This normal brain capillary is made of two endothelial cells bound together with tight junctions (J) and forming a thin and regular layer around the lumen. Processes of pericytes (P) can be seen embedded in the basement membrane. b, c, d) Capillaries from astrocytoma tumors are lined by immature endothelial cells (E) of irregular thickness and with hyperplastic nuclei (N), discontinuous basement membrane (BM) and a decreased presence of pericytes or astrocytic processes. Abnormal intercellular junctions (AJ), fenestrations (F) and irregular slit-like lumen (L) can be seen in these micrographs. Adapted from (Deane & Lantos, 1981; Rojiani & Dorovini-Zis, 1996).

5. The targets and therapeutic effects

In the case where therapeutic agents capable of crossing the BBB, such as TMZ or irinotecan, are available and do exhibit considerable anticancer activity, the most significant problem for GBM cancer patients is repopulation of malignant cells following treatment, causing inevitable relapse (CBTRUS, 2010; Stupp et al., 2005). For example, recent identification of the somatic inactivation of the mismatch repair gene $\text{MSH6}$ in a subgroup of recurrent GBM has highlighted the constant evolution of malignant glioma cells in the presence of selective pressure, in this case alkylator chemotherapy and concurrent radiotherapy. This initial discovery, achieved through whole kinome sequencing of two recurrent GBM, was bolstered by corroborative findings from the TCGA project studying a much larger number of GBM samples (CGARN, 2008; Hunter et al., 2006). Subsequent studies have also highlighted the importance of somatic $\text{MSH6}$ integrity on the $\text{in vivo}$ and $\text{in vitro}$ growth of
GBM with direct impact on patient survival (Cahill et al., 2007; Yip et al., 2009). In fact, a careful evaluation of the TCGA data of the recurrent GBM cohort shows that a majority of the recurrent tumors with the MSH6 hypermutation phenotype, a genetic signature of defects in the cellular mismatch repair (MMR) system, also have somatic mutations and epigenetic silencing affecting other members of the MMR family. This highlights a novel mechanism of tumor survival during alkylator therapy and is especially relevant in a cancer for which alkylators, such as TMZ, are used as the therapeutic mainstay. One possible explanation for this phenomenon is that a small number of GBM cells, prior to therapy, harbor mutations in the MMR genes and these cells are positively selected during therapy, leading to clonal expansion. Alternatively, and equally plausible, random mutations inactivating MMR genes during therapy could lead to outgrowth of a resistant clone of tumor cells which exhibits therapeutic resistance.

Although it is acknowledged that advances in GBM treatment will continue to rely on conventional treatment approaches (surgery, radiation and/or chemotherapy), the value of combining standard chemotherapy with targeted agents that increase tumor drug sensitivity is now being recognized (Krakstad & Chekenya, 2010). The goal of this effort is to target survival and/or proliferation-promoting proteins which are overexpressed, or have upregulated activity, in cancer cells. This approach has the additional benefit of overcoming resistance mechanisms due to the activation of single pathways (e.g. MGMT). Moreover, many trials have indicated that groups of cancer patients treated similarly on the basis of a histopathology classification (e.g. WHO II astrocytoma, WHO III anaplastic astrocytoma, and WHO IV glioblastoma) exhibit very different responses to the same treatment (Stupp et al., 2006), implying that various gene expression patterns underlying the disease phenotype may play a more important role in treatment outcomes. This observation highlights the necessity of developing tumor-targeted gene therapy treatments that would be personalized for the specific molecular lesions present in a patient’s tumor.

Cancer is believed to be a genetic disease, arising as a result of genetic mutations that endow the cell with many specific functional capabilities (Vogelstein & Kinzler, 1993) such as cell proliferation, survival, invasion and metastasis (Hanahan & Weinberg, 2000). Recent advances in molecular genetics have enabled the development of methods to specifically target gene expression (small interfering RNA; siRNA or antisense oligonucleotides; ASO) or the activity of proteins (small molecule inhibitors; SMI). Our current understanding of cancer biology has made it clear that gene targeting therapies will provide an effective strategy to treat cancer. The next paragraphs will present an overview of the approaches used in targeted therapy for the treatment of malignant glioma. The targets and potential therapeutic effects will be discussed based on the six essential alterations in cell physiology that dictate malignant growth as described elsewhere (Hanahan & Weinberg, 2000,2011) and consist of the capacity for (I) sustaining proliferative signals and II) evading growth suppressors; (III) resisting cell death; (IV) inducing angiogenesis; (V) activating invasion and metastasis and (VI) enabling replicative immortality. Table 1 and 2 present a list of genes or associated protein targets that have been inhibited by means of non-viral gene silencing agents in orthotopic GBM pre-clinical or clinical studies (Table 1), or by SMI in GBM clinical trials phase II/III (Table 2). It is interesting to note that very few of these studies have assessed the efficacy of the targeted therapy agent in combination with standard chemotherapy or radiation.
<table>
<thead>
<tr>
<th>Target</th>
<th>Experimental design</th>
<th>Agent</th>
<th>Benefit</th>
<th>Ref</th>
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<tbody>
<tr>
<td><strong>Self-sufficiency in growth signals and insensitivity to growth suppressors</strong></td>
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<tr>
<td>EGFR</td>
<td>i.cr U87 line in mice</td>
<td>shRNA in PEGylated immunolip. (h-Insuline R and m-Transferrin R) i.v.</td>
<td>Tumor growth inhibition and increased survival</td>
<td>(Zhang et al., 2004)</td>
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<tr>
<td></td>
<td>i.cr U87 line in mice</td>
<td>ASO plasmid in PEGylated immunolip. (h-Insuline R and m-Transferrin R) i.v.</td>
<td>Tumor growth inhibition and increased survival</td>
<td>(Zhang et al., 2002)</td>
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<tr>
<td>PKC family</td>
<td>Phase II CTr</td>
<td>ASO i.v. (Aprinocarsen)</td>
<td>Minimal benefit</td>
<td>(Grossman et al., 2005)</td>
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<tr>
<td>UPA/UPAR</td>
<td>4910 line i.cr. in mice</td>
<td>shRNA i.p.</td>
<td>Tumor growth inhibition and increased survival</td>
<td>(Gondi et al., 2007)</td>
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<td><strong>Evasion of cell death</strong></td>
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<td>Bcl-2</td>
<td>U251 line i.cr. in mice</td>
<td>siRNA cDNA i.p.</td>
<td>Enhanced efficacy of taxol on tumor growth inhibition</td>
<td>(George et al., 2008)</td>
</tr>
<tr>
<td>Survivin</td>
<td>U87 line i.cr. in mice</td>
<td>PEI-complexed siRNA i.t.</td>
<td>Increased survival</td>
<td>(Hendruschk et al., 2011)</td>
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<td><strong>Induction of angiogenesis</strong></td>
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<td>Pleitrophin</td>
<td>U87 line i.cr. in mice</td>
<td>PEI-siRNA i.t.</td>
<td>Tumor growth inhibition</td>
<td>(Grzelinski et al., 2006)</td>
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<td><strong>Invasion and metastasis</strong></td>
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<tr>
<td>Cathepsin B</td>
<td>SNB19 line i.cr. in mice</td>
<td>shRNA i.t.</td>
<td>Tumor growth inhibition</td>
<td>(Gondi et al., 2004)</td>
</tr>
<tr>
<td>MMPs</td>
<td>SNB19 or U251 line i.cr. in mice</td>
<td>shRNA i.t.</td>
<td>Tumor growth inhibition and enhanced sensitivity to radiation</td>
<td>(Badiga et al., 2011; Lakka et al., 2005)</td>
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<td>Tenascin-C</td>
<td>Phase I CTr</td>
<td>Long RNA molecule i.t.</td>
<td>Increased survival</td>
<td>(Wyszko et al., 2008; Zukiel et al., 2006)</td>
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<td><strong>Replicative immortality</strong></td>
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<td>hTERT</td>
<td>SNB-19 and LN-18 lines i.cr. in mice</td>
<td>ASO in cat. lip.</td>
<td>Tumor growth inhibition. More active when combined with IFN-γ injections</td>
<td>(George et al., 2009)</td>
</tr>
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<td></td>
<td>U373 line i.cr. in mice</td>
<td>ASO i.t.</td>
<td>Increased survival</td>
<td>(Mukai et al., 2000)</td>
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<tr>
<td></td>
<td>U251MG i.cr. in rats</td>
<td>GRN183 ASO i.n.</td>
<td>Tumor growth inhibition and increased survival</td>
<td>(Hashizume et al., 2008)</td>
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<tr>
<td><strong>Other function</strong></td>
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<tr>
<td>TGF-β</td>
<td>F98 line i.cr. in rats</td>
<td>Nanoparticle-complexed ASO i.p.</td>
<td>Increased survival when given with immunization</td>
<td>(Schneider et al., 2008)</td>
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Table 1. Protein targets that have been inhibited by means of non-viral gene silencing agents in orthotopic GBM pre-clinical studies or clinical trials (CTr) (definition of abbreviations is given at the beginning of this chapter)
<table>
<thead>
<tr>
<th>Target</th>
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<th>Benefit</th>
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<tr>
<td>EGFR</td>
<td>Phase II CTr</td>
<td>SMI Erlotinib (Tarceva®, Gefitinib (Iressa®) i.v.)</td>
<td>Minimal as single-agent. Some efficacy with TMZ</td>
<td>(Franceschi et al., 2007; Prados M. D. et al., 2009; Raizer et al., 2010)</td>
</tr>
<tr>
<td>PKC family</td>
<td>Phase II/III CTr</td>
<td>SMI Enzastaurin</td>
<td>Minimal benefit</td>
<td>(Kreisl et al., 2010; Wick et al., 2010)</td>
</tr>
<tr>
<td>mTOR</td>
<td>Phase II CTr</td>
<td>SMI Temsirolimus (i.v)</td>
<td>Disease stabilization and increased survival</td>
<td>(Chang et al., 2005; Galanis et al., 2005)</td>
</tr>
<tr>
<td>Growth factors</td>
<td>Phase II CTr</td>
<td>SMI Suramin i.v.</td>
<td>No benefit with radiotherapy</td>
<td>(Laterra et al., 2004)</td>
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<tr>
<td>Farnesyl-transferase</td>
<td>Phase II CTr</td>
<td>SMI Tipifarnib (i.v)</td>
<td>No benefit</td>
<td>(Cloughesy et al., 2006; Lustig et al., 2008)</td>
</tr>
<tr>
<td>HDAC</td>
<td>Phase II CTr</td>
<td>SMI Vorinostat (i.v)</td>
<td>Modest activity</td>
<td>(Galanis et al., 2009)</td>
</tr>
<tr>
<td>PDGF R</td>
<td>Phase II CTr</td>
<td>SMI Imatinib (Gleevec®) (i.v)</td>
<td>Variable response</td>
<td>(Razis et al., 2009; Wen et al., 2006)</td>
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<tr>
<td><strong>Induction of angiogenesis</strong></td>
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<tr>
<td>VEGF</td>
<td>Phase II/III CTr</td>
<td>mAb Bevacizumab (Avastin) (i.v.)</td>
<td>Active, with and w/o irinotecan or TMZ and FDA-approved for recurrent disease</td>
<td>(Friedman et al., 2009; Kreisl et al., 2009; Lai et al., 2011)</td>
</tr>
<tr>
<td>Fibroblast growth factors</td>
<td>Phase II CTr</td>
<td>Thalidomide (i.v.)</td>
<td>Minimal benefit as a single agent, some benefit with carmustine or irinotecan</td>
<td>(Fadul et al., 2008; Fine et al., 2003; Marx et al., 2001)</td>
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<tr>
<td><strong>Invasion and metastasis</strong></td>
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<tr>
<td>Integrins</td>
<td>Phase I/II CTr</td>
<td>SMI Cilengitide (i.v.)</td>
<td>Some activity with TMZ</td>
<td>(Stupp et al., 2010)</td>
</tr>
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Table 2. Protein targets that have been inhibited by means of SMI or mAb in GBM clinical trials phase II/III (definition of abbreviations is given at the beginning of this chapter)

5.1 Self-sufficiency in growth signals and insensitivity to growth suppressors

Tumor cells generate many of their own growth signals, thereby reducing their dependence on stimulation from the normal tissue microenvironment (Hanahan & Weinberg, 2000,2011). Molecular strategies for achieving autonomy involve: I) alterations in extracellular growth signals, II) alterations in transducers of those signals, III) alterations in components enabling or preventing the cell to enter cell cycle (Hanahan & Weinberg, 2000). For example, protein overexpression of EGFR, involved in growth signal transduction, was reported in 60% of
GBM cases; EGFR gene amplification was reported in 40% of cases; EGFR truncated transcript encoding for a constitutive activity of the receptor was reported in 20% of cases and mutations of EGFR extracellular domain was reported in 15% of cases (Nicholas et al., 2006; Ohgaki et al., 2004). These mutations are quite often combined in the same tumor cell, leading to overactivation of EGFR pathways (Ekstrand et al., 1991; Idbaih et al., 2008). ASO- (Zhang et al., 2002) and RNA interference- (Zhang et al., 2004) mediated inhibition of EGFR has been shown to induce a strong reduction in tumor growth and increased the survival of orthotopic GBM tumor bearing mice. However, the inhibition of EGFR using SMI in clinical phase II trials was not representative of this success. As discussed earlier, erlotinib (Prados M. D. et al., 2009; Raizer et al., 2010) or gefitinib (Franceschi et al., 2007; Rich et al., 2004) SMI showed variable activity in GBM patients although better efficacy was seen when used in combination with TMZ. The constitutive activation of the PI3K/AKT pathway associated with the mutation of PTEN (60% of GBM cases) (Haas-Kogan et al., 1998; Knobbe et al., 2002) has also received considerable attention as it is generally associated with aggressive disease and poor prognosis (Ermoian et al., 2002; Rasheed et al., 1997; Schmidt et al., 1999).

The mammalian target of rapamycin (mTOR) was identified as a major downstream effector in this pathway (Manning & Cantley, 2003). Phase II clinical trials using the mTOR SMI Temsirolimus (CCI-779) induced disease stabilization and an increase in survival in some GBM patients of phase II clinical trials (Chang et al., 2005; Galanis et al., 2005).

5.2 Evasion of cell death

The ability of tumor cell populations to expand in number is determined not only by the rate of cell proliferation but also by the rate of cell death. Acquired resistance toward programmed cell death, apoptosis, is a hallmark of most and perhaps all types of cancer (Hanahan & Weinberg, 2000,2011), causing the tumor to resist conditions that would normally kill cells. In this context, Bcl-2 targeted therapy has attracted the most attention with studies both in pre-clinical cancer models and patients (ASO Oblimersen, SMI AT-101). Although the potential of Bcl-2 targeting has not yet been demonstrated in GBM patients, one report showed that siRNA-mediated inhibition of Bcl-2 can increase the efficacy of taxol in a pre-clinical orthotopic model of GBM (George et al., 2008).

5.3 Induction of angiogenesis

Strong evidence indicates that the growth of GBM depends on angiogenesis (Jain et al., 2007; Norden et al., 2009). However, experimental models of GBM have shown that the resulting vessels are poorly organized and poorly functional, and it is believed that high levels of angiogenesis in GBM are associated with increased hypoxia and interstitial fluid pressure, which contribute to the disease malignancy and resistance to treatments (Blasberg et al., 1983; Groothuis et al., 1983). This aspect of GBM vasculature will be discussed further in section 10. Members of the VEGF family have emerged as prime mediators of angiogenesis in GBM (Jain et al., 2007). As already noted, several clinical trials have demonstrated the efficacy of bevacizumab, a monoclonal antibody (mAb) against VEGF as a single agent and in combination with TMZ or irinotecan to prolong the survival of patients (Friedman et al., 2009; Kreisl et al., 2009; Lai et al., 2011). Bevacizumab was recently approved by the FDA for the treatment of recurrent GBM and is showing promising efficacy in clinical trials for newly
diagnosed GBM patients (Lai et al., 2008). Many other VEGF or VEGFR inhibitors are currently being tested in the clinic (Norden et al., 2009).

5.4 Invasion and metastasis

Proteins responsible for tissue invasion and metastatic behavior are often effectors allowing the cell to grow in the absence of ECM adhesion signals. The most obvious example is the integrins family, which is involved in ECM anchorage-independent growth of tumor cells, and provides the traction necessary for cell motility and invasion (reviewed in (Desgrosellier & Cheresh, 2010)). An integrin SMI, cilengitide, has shown some promising activity in GBM clinical trial phase I/II in combination with TMZ (Stupp et al., 2010), and is now moving to phase III (Carter, 2010). In addition, enzymes that are involved in the degradation of the ECM will allow cancer cells to invade surrounding brain tissue. Matrix-metalloproteinases (MMPs) were shown to play a central role in the proteolysis necessary for this process (Nakada et al., 2003). Intratumoral administration of a shRNA against MMP-9 inhibited tumor growth in an orthotopic GBM mouse model (Lakka et al., 2005). To date, no MMP inhibitors have made their way to a phase II clinical trial for GBM treatment. Moreover, the clinical evaluation of MMP inhibitors as single agents in cancers other than GBM has not been associated with significant anti-tumor responses (Brinker et al., 2008; Chu et al., 2007) and they will most likely show better efficacy in a combination setting.

5.5 Replicative immortality

Some studies suggest that at a given point during the course of tumor progression, evolving premalignant cell populations acquire the capacity to breach the mortality barrier (Hanahan & Weinberg, 2000); they become capable of unlimited replicative cycles. Overexpression of telomerase reverse transcriptase (hTERT), a unique ribonucleoprotein enzyme responsible for adding telomeric repeats onto 3’ ends of chromosomes (Holt & Shay, 1999), could play an important role in the development of cellular immortality and oncogenesis. Telomerase activity has been detected in 89% of GBM cases and correlates with tumor grade (Le et al., 1998), whereas low expression of hTERT was shown to be associated with a better prognosis (Wager et al., 2008). Pre-clinical investigation of hTERT targeted therapy illustrates that downregulation of this gene results in tumor regression and increased survival in orthotopic GBM murine models (George et al., 2009; Mukai et al., 2000).

6. The potential of targeting multiple pathways

Hallmarks of cancer cell malignancy include upregulation or dysregulation of multiple pathways, with deregulations increasing in number as the cancer progresses. In contrast to this observation, the vast majority of clinical trials to date have focused on a single agent that targets a single molecular aberration. It is expected that a therapeutic modality targeting one of these dysregulated pathways will only result in modest benefits to patients in terms of disease-free survival time. Cellular proliferation, growth and death are regulated by an intricate network of cellular functions, and it is very likely that disturbances in the balance between these pathways will lead to the activation of compensating mechanisms in normal cells as well as cancer cells. While it is well understood that a combination of chemotherapeutic agents inclusive of drugs with differing mechanisms of action is generally
more efficacious than single agent chemotherapy in the treatment of aggressive cancers, clinicians and scientists are now beginning to realize the benefits of combining agents targeting different biological pathways in order to effectively silence as many cancer phenotypes as possible.

The therapeutic value of targeting two different pathways is exemplified by some research data obtained by our laboratory (Verreault et al., 2011a). One of the most commonly reported molecular defects in GBM is the aberrant activation of the PI3K/AKT pathway, which is associated with increased proliferation rate, invasion, metastasis and poor prognosis (Ermoian et al., 2002; Haas-Kogan et al., 1998; Li X. Y. et al., 2010). Rictor, the rapamycin-insensitive companion of mTOR, is a protein member of the mTOR Complex 2 (mTORC2), and can activate AKT through direct phosphorylation at its serine 473 site (Sarbassov et al., 2004; Sarbassov et al., 2005; Sparks & Guertin, 2010). Elevated levels of Rictor were found in human GBM tumor tissue samples and cell lines when compared to normal brain tissue (Masri et al., 2007). Rictor and EGFR proteins were silenced alone and in combination by siRNA in vitro transfection in a panel of three human GBM lines (U251MG, U118MG and LN229). It was found that the co-silencing of Rictor and EGFR exerted effects on cell migration and sensitivity to chemotherapeutic drugs that were not observed by the single silencing of either target (Verreault et al., 2011a). The most striking evidence of the validity of this combined silencing came from the in vivo aspect of this study, which was done by intracranial inoculation in mice brains of U251MG cells expressing small hairpin RNA (shRNA) specific to each target. Silencing of EGFR or Rictor alone had no significant effect on tumor growth, but the dual silencing resulted in the eradication of the tumor (Verreault et al., 2011a). Also, tumor growth block in response to the combined suppression of EGFR and PI3K pathway was reported previously using the SMIs gefitinib and LY294002 in a GBM xenograft model (Fan et al., 2003), while monotherapy of each inhibitor had no impact on tumor burden. Taken together, these studies strongly support the value of inhibiting both EGFR and PI3K/mTORC2/Rictor/AKT pathways to achieve therapeutic effects that may not be observed by the single inhibition of either pathway, and provide compelling evidences of the potential of targeting multiple pro-oncogenic pathways in GBM.

7. Drug delivery to brain tumors

The idea of targeting gene expression at the level of transcription or translation has been mirrored by the emergence of gene therapy as a strategy to specifically silence the activity of any defective or overactive gene without the limiting step of SMI availability (Bumcrot et al., 2006). Since tumor cells have a different pattern of gene expression in comparison with normal cells, gene silencing can theoretically be used to specifically target tumor-associated genes or mutated genes without altering gene expression of normal cells (Helene, 1994). The most commonly used strategies employed to achieve gene silencing involve administration of ASOs or siRNAs that can inhibit the expression of specific proteins. However, the therapeutic value of this technology is proving difficult to establish, in part because of the lack of pharmaceutically viable products that can be administered orally, intravenously or intraperitoneally, and that can deliver the gene silencing agent to tumor cell populations in sufficient quantity to achieve target knockdown. Despite the fact that we are still learning to navigate the technology of RNAi in order to achieve optimal benefits with minimal side-
effects, the high specificity and potency of siRNAs, together with the unlimited possibility of designs for siRNAs against any genes, make this technology an attractive option for targeted therapy. Once a viable option for safe and effective delivery to the tumor is defined, RNAi will be regarded as the most powerful tool for designing personalized treatment strategies. Some of the strategies that have been developed and explored pre-clinically for gene silencing agent delivery to brain tumors are discussed below, with references to successful achievements in the clinic for chemotherapeutic agent delivery.

7.1 Bypassing the blood-brain barrier

As discussed earlier, the BBB constitutes one of the main barriers to the development of new therapeutics for GBM treatment. Hence, a great deal of research has been focused on defining strategies aimed at bypassing the BBB and increasing delivery of therapeutics. One of these strategies consists of a direct intratumoral (i.t.) injection, and has been successfully used in pre-clinical brain tumor models for delivery of gene silencing agents (George et al., 2009; Gondi et al., 2004; Lakka et al., 2005; Thakker et al., 2005), and in the clinic for chemotherapeutic agents (Boiardi et al., 2005; Patchell et al., 2002). The only clinical trial testing the efficacy of RNAi in GBM was done by local delivery of a 146 nucleotides long RNA molecule (ATN-RNA) targeting Tenascin-C mRNA (Wyszko et al., 2008; Zukiel et al., 2006). Although its role in GBM pathology is still unclear, the expression of the ECM glycoprotein Tenascin-C was found to correlate with tumor grade (Pas et al., 2006). Treatment with ATN-RNA was associated with increased survival in GBM patients and these results constitute the first demonstration of a potential clinical application for RNAi in the treatment of GBM. Convention-enhanced delivery (CED) is another technique tested in the clinic for local delivery, and consists of placing catheters into the surgical cavity after the resection procedure and to deliver antineoplastic agents through the catheters using positive pressure (0.5 to 15.0 μl/min). This technique was shown to increase the anti-tumor efficacy of paclitaxel (Lidar et al., 2004) and of the recombinant protein Cintredekin besudotox (Kunwar et al., 2007) in GBM patients. CED was also used to deliver siRNAs to the CNS of non-human primates, and resulted in a durable and specific silencing of the selected target (Querbes et al., 2009). Carmustine wafers (Gliadel) are currently used in the clinic and represent a good example of local delivery (Figure 2). In this system, carmustine is incorporated in a hydrophobic matrix made of a polyanhydride polymer that protects the agent from hydrolysis (Brem et al., 1994; Grossman et al., 1992). After tumor resection, the wafer discs are implanted at the surface of the resection cavity and the drug is slowly released for a period of three weeks (Brem et al., 1991). Although wafers have shown some promising efficacy when combined with TMZ (Gururangan et al., 2001), it is not indicated for patients with infiltrative or multifocal tumors. It is believed that local administration does not allow access to infiltrative cancer cells that are a predominant hallmark of GBM, and this is especially true for macro-molecules such as ASOs or siRNAs.

In order to overcome the limitations of direct i.t. administration of agents, other routes of administration have been explored, including systemic administration (intraperitoneal (i.p.) and intravenous (i.v.)), some of which integrating the use of delivery systems (Table 1). Challenges and opportunities for these strategies will be discussed in the following section. Interestingly, the intranasal (i.n.) route of delivery was recently shown to promote a rapid and efficient delivery of molecules that do not cross the BBB to the brain (Thorne et al.,
1995). The i.n. technique allows for a noninvasive bypass of the BBB via the nasal mucosa, through the olfactory and trigeminal nerves, directly to the brain and cerebrospinal fluid (Thorne et al., 2004). The ASO GRN163 specific to hTERT was successfully delivered to preclinical orthotopic tumors using this technique (Hashizume et al., 2008). The i.n. technique was also used in a phase I/II clinical trial to administer perillyl alcohol, a Ras inhibitor, and results suggested some antitumor activity without any toxicity in GBM patients (da Fonseca et al., 2008). These studies, together with other pre-clinical reports (Sakane et al., 1999; Thorne et al., 2004; Wang D. et al., 2006; Wang F. et al., 2003), suggest that the i.n. route of administration may be of great therapeutic value for treatment of brain tumors and could be part of the solution to the issue of polynucleotide therapeutics delivery. It should be noted, however, that material delivery in tissues other than the brain (liver, kidney, heart, muscles) was also detected following i.n. administration (Thorne et al., 2004), suggesting the need for combining this administration route with delivery systems that would improve specificity to the tumor. The currently accepted mechanisms of transport following intranasal administration are the intraneuronal transport following endocytosis or an extracellular diffusion along the nerves (Thorne et al., 1995). Thus it is clear that the size limitation imposed by these routes may restrict the possible delivery systems that could be used.

7.2 Limitation to systemic administration

Strategies used to administer agents through systemic administration include simple infusion as well as more sophisticated delivery systems designed to promote intracellular delivery. In this context, the success of gene silencing therapy for cancer depends in large part on stable and tumor-specific delivery, which can be achieved only if therapeutic molecules can survive as active agents as they cross various biological barriers. These barriers are i) degradation in the blood or uptake by the liver, ii) passage from the circulation across the BBB and into the extravascular space within the tumor, ii) passage into cytoplasm of target cells, iv) release from the carrier and/or the endosomes if associated with a carrier system or internalized via endocytosis, v) escape from nucleases in tumor cell’s cytoplasm and vi) binding to target mRNA. As described earlier, the BBB consists of endothelial cells, pericytes, astrocytes endfeet and neuronal cells that are organized in such way to confer a unique selective permeability to the CNS vascular network (Rubin & Staddon, 1999), restricting the passive transport of most therapeutic molecules (Pardridge, 2007). Some success in delivering molecules across the BBB has been made with long circulating carrier systems (see section 8) that can take advantage of the fact that tumor-associated BBB consists of poorly formed vascular endothelium that is more permeable to circulating macromolecules than the normal BBB (Patel et al., 2009). Strategies to open the BBB have also been explored and include osmotic disruption (Bellavance et al., 2008), the use of vasomodulators to increase permeability (Ningaraj et al., 2003), or the use of potassium channel agonists to increase the formation of transport vesicles (Ningaraj, 2006). Although it has been shown that siRNAs are more stable in cells than a single-stranded antisense molecule (Bertrand et al., 2002), naked RNA sequences injected in vivo are rapidly eliminated and have a short duration of effect (Khan et al., 2004). Pre-clinical studies suggest that this can be overcome by use of multiple i.v. or i.p. injections of naked siRNAs (Fillieur et al., 2003), and can lead to successful downregulation of the target in intracranial tumor (George et al., 2008). However, other studies reported very little accumulation of siRNA in
the brain following i.v. administration, and preferential accumulation was observed in the liver and the kidneys (Braasch et al., 2004; De Paula et al., 2007; Santel et al., 2006). Other reports show that a high-pressure delivery technique could increase delivery of siRNAs given i.v. (Lewis et al., 2002; McCaffrey et al., 2002; Song et al., 2003) or i.p (Heidel et al., 2004), but no evidence of delivery to the brain was shown (Lewis et al., 2002). Moreover, this technique is not relevant to human therapies as it involves high pressure and massive volume delivery schemes to generate transiently high local intravascular pressure (Lieberman et al., 2003; Shuey et al., 2002). It is now widely recognized that if siRNAs are to be used in the clinic for GBM patients, they will have to be formulated with a delivery system strategy in order to increase the agent’s half-life and tumor specific delivery.

8. Lipid nanoparticle delivery systems

Studies over the last few decades have established that liposomal nanoparticle (LNP) formulations of selected antineoplastic agents can be more effective than a drug administered in its free form, due to their capacity to increase drug circulation time. Further, increased tumor delivery is observed due to the increased permeability of blood vessels in the tumor environment, a process referred to as the “enhanced permeability and retention effect” (EPR) (Maeda et al., 2009). Conventional LNPs, which consist of bilayer lipid vesicles, are prepared with phospholipids (e.g. phosphatidylcholine or phosphatidylglycerol) (Storm & Crommelin, 1998). The incorporation of cholesterol in these formulations influences the mechanical strength and permeability of LNP membranes (Ohvo-Rekila et al., 2002). Stealth LNP can also be made by coating the LNP surface with the hydrophilic polymer polyethylene glycol (PEG), which provides a barrier against interactions with molecular and cellular components in the plasma compartment (Storm & Crommelin, 1998) and can engender remarkable increases in plasma longevity of the carrier (Park et al., 2004). The FDA-approved and commercially available doxorubicin LNP formulation (Caelyx®, Schering-Plough or Doxil®, Centocor Ortho Biotech Inc) is an example of a PEG-coated formulation (Barenholz, 2007).

Liposomal formulations have shown some success in the delivery of drugs to brain tumors. Caelyx® was reported to be less toxic in the clinic than the unencapsulated form (Judson et al., 2001; O’Brien et al., 2004; Porter & Rifkin, 2007); when used to treat malignant glioma, stabilization of the disease (reduction of tumor volume of < 50% or a < 25% increase in tumor volume for more than 8 weeks) was observed (Fabel et al., 2001; Hau et al., 2004). An orthotopic GBM pre-clinical study showed anti-vascular activity of doxorubicin when encapsulated in LNPs, and these effects were not observed with the free form of the drug or in normal brain tissue (Zhou et al., 2002). Other pre-clinical studies showed that liposomal formulations of irinotecan are more efficacious than the unencapsulated form in brain tumors (Krauze et al., 2007; Noble et al., 2006; Verreault et al., 2011b) and in colorectal and adenocarcinoma tumors (Hattori et al., 2009; Messerer et al., 2004; Ramsay et al., 2008). More specifically, our laboratory has established that Irinophore C™ (IrC™), a LPN formulation of irinotecan, exhibits improved anti-cancer efficacy compared to the free drug in a GBM orthotopic model (Verreault et al., 2011b). We demonstrated that the presence in the brain of irinotecan and its active metabolite SN-38 is extended following administration of IrC™ compared to irinotecan. At equivalent doses (50 mg/kg), the average survival of GBM-tumor bearing animals was improved of 17% compared to the free drug-treated group (49.1% compared to untreated animals). Further, a repeated dose tolerability study showed
that IrC™ is much better tolerated than the free drug. This increase in tolerability permitted the administration of 100mg/kg IrC™ doses, which provided an increase in average survival of 83% compared to the untreated group. These studies demonstrate the potential of LNP delivery systems to improve chemotherapeutic drug delivery to brain tumors and consequently, to increase drug therapeutic effects. Interestingly, very little effort has been made in the development of SMI that could be administered in LNPs. It appears that if the expertise and knowledge that was gained over the last decades in the field of lipid-based delivery systems was directed towards improving SMI delivery to brain tumors, potential therapeutic success could have already been achieved.

In developing lipid-based delivery systems for gene silencing agents, the goal is to design a system that simultaneously achieves high efficiency (defined by delivery and release of the agent to the disease site), prolonged effects and low toxicity (Lundstrom & Boulikas, 2003). Small cationic LNPs can interact with negatively charged DNA or RNA, leading to the formation of complexes with a prolonged half-life in the circulation (Cattel et al., 2004) and capable of promoting cellular internalization (Storm & Crommelin, 1998). A report showed that hTERT-targeted ASO delivery using cationic LNPs resulted in increased survival of intracranial tumor bearing mice (Mukai et al., 2000). Numerous other studies have been done using i.v. or i.p. injections of siRNAs (Aigner, 2008; Sioud & Sorensen, 2003; Sorensen D. R. et al., 2003) or ASOs (Shoji & Nakashima, 2004) complexed with cationic LNPs in cancer models other than brain tumor. These techniques led to good silencing efficiency with no significant signs of toxicity. It is important to mention that some studies have demonstrated immune system activation induced by common cationic lipids following systemic administration (Freimark et al., 1998; Li Š. et al., 1999; Scheule et al., 1997). Furthermore, another limitation to the therapeutic use of positively charged complexes is that they are cleared rapidly following intravenous administration (Nishikawa et al., 1998) as they bind to proteins in the plasma and form aggregates which are eliminated by non-target cells (Ogris et al., 1999). Since neutralized complexes have proven to be less efficient, cationic complexes possessing hydrophilic steric barriers, achieved through the use of surface-grafted polymers like PEG, have been pursued to address the problem of plasma protein binding and rapid elimination (Allen et al., 2002). PEG-immunoliposomes (made by adding antibodies at the surface of LNPs, see section 9) are able to efficiently deliver ASO (Zhang et al., 2002) and siRNAs (Zhang et al., 2004) to orthotopic brain tumors following systemic administration. Stable nucleic-acid-lipid particles (SNALP) consist of lipid bilayer particles prepared with a mixture of cationic and fusogenic lipids, and have been shown to exhibit the stability, small size, low surface charge and low toxicity required for in vivo administration (Morrissey et al., 2005), and to promote efficient siRNA cellular uptake (Heyes et al., 2005). The lipid particles are coated with PEG molecules which dissociates from the SNALP rapidly after administration, thus transforming the carrier into a transfection-competent entity (Ambegia et al., 2005). SNALP-formulated siRNA have shown improved circulation time and increased downregulation efficacy in mice and nonhuman primates liver (Judge et al., 2009; Morrissey et al., 2005; Zimmermann et al., 2006), and recent modifications to the lipid composition have engendered a substantial 10-fold improvement in activity in vivo (Semple et al., 2010). Alternatives to cationic lipids complexed with therapeutic polynucleotides are also being explored to overcome the limitations observed with current formulations. In particular, reductions in in vivo toxicity and targeting efficiency for a certain cell population are a main focus. These options are discussed further in a previously published review (Verreault et al., 2006).
9. Targeted delivery

The concept of targeted delivery has been suggested by many to be the solution to the obstacle of siRNA delivery to brain tumors (Lichota et al., 2009; Prakash et al., 2010). An efficient targeted delivery strategy should promote specific crossing of the therapeutic material across tumor-associated BBB, passage through cancer cell membranes, and prevention of accumulation in healthy tissue. Antibody-coupled liposomes (immunoliposomes) combine the capacity of LNPs to increase nucleic acid half-life in the blood compartment with specific targeting to tumor sites. One of the first attempts to deliver material to the brain using immunoliposomes was done by coupling the monoclonal antibody OX26 specific against the transferrin receptor (Huwyler et al., 1996) to PEGylated liposomes made with DSPE lipids. The transferrin receptor is present at the surface of normal brain capillary endothelial cells and is upregulated in brain tumor tissue (Recht et al., 1990). Following i.v. injection of OX26 coupled-DSPE-PEG immunoliposomes encapsulating the chemotherapeutic agent daunomycin, an average of 0.03% of the injected dose of daunomycin was measured in the brain of rats after 60 min, while only 0.008% of injected daunomycin dose was measured following administration of free daunomycin or non-OX26-conjugated daunomycin DSPE-PEG carrier (Huwyler et al., 1996). The use of mouse transferrin receptor-targeted immunoliposomes has also shown success in the delivery of bigger molecules such as DNA plasmids (Shi et al., 2001) or siRNAs (Pirollo et al., 2006) to brain tumors.

It can be speculated that many other types of antibodies specific against GBM cells or micro-environment antigens could also be used to produce immunoliposomes that would increase delivery of nucleic acid sequences to the tumor tissue. For example, the arginine-glycine-aspartic acid (RGD) motif of fibronectin has been used to target delivery of siRNAs in a s.c. model of neuroblastoma (Schiffelers et al., 2004). RGD binds to integrins that are expressed on activated endothelial cells found in tumor vasculature of many advanced cancers including GBM (Gladson & Cheresh, 1991). In vivo studies demonstrated the accumulation of CY5.5-RGD in cells and vessels of orthotopic GBM tumors following i.v. injection (Hsu et al., 2006), supporting its potential use for siRNA targeted delivery to GBM tumors. Tumor-associated endothelial cells in GBM have higher levels of VEGFR2 than normal endothelial cells (Charalambous et al., 2006). Targeted delivery specific to the VEGFR2 receptor could also be used to specifically deliver material across the brain tumor-associated BBB. CD44 is a surface receptor overexpressed in GBM tumor cells (Axelsen et al., 2007) and is another example of GBM-specific marker that could be used for immunoliposome targeting. Further, antigens found at the surface of brain tumor initiating cells (BTIC) (e.g. CD133) (Altaner, 2008; Guo et al., 2006) could potentially allow for specific delivery of silencing agents against defective genes in these cells. BTIC are a sub-population of cells that have the ability to reconstitute the overall tumor cell population and are typically more resistant to chemotherapy and radiation than the rest of the tumor cell population (Hadjipanayis & Van Meir, 2009). It is now believed that treatment resistance and eventual relapse result in part from a failure to eliminate BTICs (Xie & Chin, 2008). It is important to note that CD133 is also expressed in normal stem cells (Tarnok et al., 2009) and use of this antigen for targeted delivery could negatively impact healthy CD133+ cells. Therefore, it appears necessary to combine several targeted delivery strategies to achieve both efficacy and specificity. For example, use of immunoliposomes specific to human insulin receptor and mouse transferrin receptor was more effective at delivering nucleic acid molecules to brain tumors cells than a
carrier specific to mouse transferrin receptor only in a human xenograft murine model (Zhang et al., 2003). This dual receptor targeting strategy was used to deliver ASOs or shRNA plasmids to orthotopic brain tumors (Zhang et al., 2004; Zhang et al., 2002) and resulted in 88-100% increase in animals’ lifespan when compared to untreated animals. Thus, a treatment aimed at targeting BTIC could include a proportion of transferrin and insulin targeting immunoliposomes that would also incorporate antibodies against the CD133 marker. Such system could increase the likelihood that all targeted CD133+ cells are part of the tumor tissue. The complex and heterogeneous nature of brain tumors seems to require multivalency of delivery systems in order to achieve highly specific and efficient siRNA delivery. Targeted delivery systems will also have to be versatile, allowing the encapsulation of diverse combinations of siRNA (e.g. against EGFR and Rictor) selected based on the patient’s tumor genetic profile.

Interestingly, the capacity of exogenous neural stem cells (NSC) administered directly into the brain parenchyma, the cerebral ventricles or even in the systemic circulation to migrate great distances into sites of intracranial pathology has triggered the interest of using these cells as anti-cancer therapeutics vehicles (reviewed in (Yip et al., 2006)). Indeed, exogenous NSCs were shown to be able to accumulate around brain tumors and to track tumor cells migrating into the surrounding tissue (Aboody et al., 2000). This unique tropism of NSCs for gliomas has motivated the development “genetically-armed” NSC to target cancer cells through the delivery of a variety of therapeutic gene products. NSCs have been produced to express cytokines to enhance the immune response against the tumor (Benedetti et al., 2000; Ehtesham et al., 2002b), the proapoptotic protein TRAIL (Ehtesham et al., 2002a) or the pro-drug converting enzyme cytosine deaminase (Aboody et al., 2000). Given the potential of NSCs to deliver therapeutic agents in a specific and sustained manner to brain tumors, it will be interesting to evaluate whether shRNA-expressing NSCs could be used to secrete and deliver siRNAs in the vicinity of tumors and invading tumor cells. These therapeutic NSCs can also be designed to express bioluminescence or red fluorescence (Shah et al., 2005; Yip & Shah, 2008). Hence, it is hoped that therapeutic NSCs could be used as biological, motile and dynamic diagnostic tools as well as specific delivery systems for therapeutic agents in gliomas, especially for infiltrating tumor cells in the close vicinity to normal CNS structures and therefore not remediable by traditional therapy (Shah et al., 2005; Yip & Shah, 2008). However, it is not sure whether therapeutic NSCs have the ability to transgress the abnormal tumor-associated vasculature, and research into the underlying molecular mechanism and clinical utility of these cells is active and ongoing.

10. Opportunity for improving tumor drug delivery by vascular normalization

Pre-clinical models showed that GBM tumors are poorly perfused (Blasberg et al., 1983; Groothuis et al., 1983) due to factors such as reduced blood flow rates, elevated hematocrit and interstitial fluid pressure, and an increase in geometric resistance (Baish et al., 1996; Vajkoczy & Menger, 2000; Vajkoczy et al., 1998; Yuan et al., 1994). The microvasculature of GBM was characterized as tortuous and fenestrated vessels with diameters that are larger than normal (Vajkoczy & Menger, 2004) and discontinuous basement membrane which rarely encloses pericytes (Deane & Lantos, 1981). The poorly organized architecture of GBM vessels, illustrated in figure 3, impedes vascular function and reduces drug delivery to the tumor tissue. In glioma (Kamoun et al., 2009; Sorensen A. G. et al., 2009; Winkler et al., 2004), tumor vascular normalization has been described as the specific activity of an agent
(e.g. antiangiogenic therapy) against proliferating vasculature, which results in the growth inhibition of new vessels, the pruning of immature and inefficient tumor vessels, and the normalization of surviving vasculature by increasing the fraction of pericyte-covered vessels, restoring the abnormally thick and irregular basement membrane and reducing the high vascular permeability of these vessels (Baffert et al., 2006; Jain, 2001). The normalization of tumor vessels appears to be transient in nature, but was suggested to create a window where blood flow is improved, leading to an opportunity to improve delivery of other drugs (Jain, 2005). In GBM patients, a “vascular normalization index”, defined by changes in vascular permeability ($K_{\text{trans}}$ values), microvessel volume and circulating collagen IV, was found to be closely associated with overall survival and progression-free survival in response to Cediranib, a pan-VEGFR inhibitor (Sorensen A. G. et al., 2009). Pre-clinically, the delivery of TMZ in an intracranial model of glioma was increased after treatment with the angiogenesis inhibitor SU5416. It was suggested that SU5416 restored capillary architecture and decreased interstitial fluid pressure (Ma et al., 2003), allowing for an increase in TMZ delivery to the tumor tissue. Our laboratory has recently reported that IrCTM therapy (once weekly for three weeks) can lead to normalization of GBM blood vessel structure and function (Verreault et al., 2011c). IrCTM treatment restored the basement membrane architecture of the tumor vasculature, reduced blood vessel diameters and reduced vessel permeability to the fluorescent dye Hoechst 33342, suggesting a restoration of the vessel architecture and function to a more normal state (Verreault et al., 2011c). Treatment also increased the quantity of vessel in the center of tumors, suggesting a more homogeneous distribution of blood across the entire tumor (Verreault et al., 2011c). Further, IrCTM significantly reduced $K_{\text{trans}}$ values calculated from Dynamic Contrast Enhanced Magnetic Resonance Imaging (DCE-MRI) studies (Verreault et al., 2011c), which was also suggestive of a decrease in vessel permeability (O’Connor et al., 2007). Taken together, these observations strongly suggested an improvement in vascular function: the tumor blood vessels in tumors from animals treated with IrCTM were behaving more like vessels in the normal brain. Thus, IrCTM exerts a dual mechanism of action in GBM tumors. As described in section 8, the therapeutic activity of irinotecan is improved by the extended exposure of tumor cells to the drug provided by the drug carrier (Verreault et al., 2011b). Moreover, the effects of the formulation on the tumor micro-environment may increase the delivery and efficacy of a second agent (Verreault et al., 2011c). This dual mechanism may provide an opportunity for designing a therapy which would encompass the cytotoxic activity of an optimized chemotherapeutic agent together with the increase in tumor delivery of an antibody-coupled carrier encapsulating siRNAs specific to pro-malignancy genes. It can be expected that such therapy could lead to significant therapeutic benefits for GBM patients. Studies designed to evaluate the capacity of IrCTM therapy to increase delivery and efficacy of TMZ in GBM are currently ongoing.

11. Conclusion

It is widely recognized that there is a tremendous potential in the use of targeted therapy agents to treat GBM and, importantly, to become the therapeutic modality of choice when developing target-specific personalized treatment options. The following three main areas of investigation could lead to improved treatment outcome in GBM: (i) Use of targeted agents in combination with conventional treatment options: the capacity of SMIs or siRNAs to enhance the activity of chemotherapeutic agents or radiation should be tested in established
siRNA-Based Therapy for Glioblastoma Patients

GBM orthotopic models and in clinical studies. (ii) Use of targeted agents customized therapy: the anti-tumor efficacy of SMIs or siRNAs should be tested using GBM tumors arising from orthotopic inoculation of tumor cells isolated from patient tumor biopsies for which a list of genetic defects (e.g. EGFR amplification, PTEN mutation) is available. (iii) Use of delivery systems to circumvent the obstacles of delivery to brain tumors and improve efficacy of targeted agents and chemotherapeutics: antibody-coupled carriers could be designed to improve delivery to the tumor, and the ligand specificity of such a carrier could be made based on immunohistopathology analysis of each individual tumor.

Chapter summary

- The most significant problem for GBM cancer patients is repopulation of malignant cells following treatment, causing inevitable relapse. This is thought to be the result of genetic mutations that endow the cell with many specific functional capabilities such as cell proliferation, survival, invasion and metastasis. Although it is acknowledged that advances in GBM treatment will continue to rely on conventional treatment approaches (surgery, radiation and/or chemotherapy), the value of combining standard chemotherapy with targeted agents that increase tumor drug sensitivity is now being recognized.
- Several targeted therapy approaches are currently being evaluated in GBM pre-clinical and clinical studies using SMI, ASOs and RNAi-based compounds (siRNA or shRNA), and the targets include proteins involved in sustaining proliferative signals, evading growth suppressors, resisting cell death, inducing angiogenesis, activating invasion and metastasis and enabling replicative immortality.
- The potential benefits of combining agents targeting different biological pathways in order to effectively silence as many cancer phenotypes as possible is also being recognized.
- Another major challenge of GBM treatment is the achievement of adequate concentration of the therapeutic agent within the tumor itself, and this obstacle can largely be attributed to the presence of the BBB. Strategies that are currently being tested to overcome this obstacle include the bypass of the BBB using alternative delivery routes (i.t., CED, i.n., “genetically-armed” NSCs) or the use of delivery systems (LNPs, immunoliposomes) which have been shown to promote a more efficient and specific delivery of therapeutics to brain tumors following intravenous administration.
- The poorly organized architecture of GBM vessels is thought to impede vascular function and to reduce drug delivery to the tumor tissue, and the normalization of GBM vasculature has been approached in an attempt to improve drug delivery to brain tumors.

It has become clear that delivery systems, whether they are lipid-based, polymer-based or antibody-conjugated, can have a significant benefit in enhancing stability of drugs, facilitating delivery to tumor sites and perhaps delivery to the intracellular compartments containing the molecular targets. Moreover, the reduced toxicity profile associated with many liposomal drug formulations compared to the free form of the drug (Mayer et al., 1995; O’Brien et al., 2004) could be used to administer higher doses that would lead to
increased drug delivery to brain tumors. To date, the full potential of this technology has not been explored in GBM and may constitute a significant opportunity for delivery of targeted therapeutic approaches that encompass the use of multiple therapeutics all designed to inhibit phenotypes of GBM that contribute to its aggressive behavior.

It is now obvious that conventional treatment approaches for patients affected by GBM must change if improved treatment outcomes are going to be achieved. One important avenue would be to determine how many treatment agents must be included in order to achieve GBM cure. While most combination clinical trials will typically test 2 or 3 agents, it may be necessary to consider using 5 or even 10 different compounds that will block or eradicate all tumorigenic phenotypes of a cancer. Obviously, the design complexity of these trials may be a limiting factor. However, pre-clinical approaches where animals are inoculated orthotopically with tumor cells from patient samples could allow for testing several combination therapy options in a model that is more representative of the clinical reality than conventional models using commercially available cell lines. Moreover, non-invasive imaging using cancer cell lines expressing fluorescent proteins (e.g. mKate2 or mCherry proteins (Verreault et al., 2011d)) or bioluminescence (Maes et al., 2009) will facilitate the use of such models by providing immediate information on treatment response. It can be expected that the future of GBM treatment will incorporate information acquired from pre-clinical models obtained from orthotopic inoculation of patients’ tumor samples to guide treatment decisions for these particular patients. We can be hopeful that the personalization of therapy options will improve treatment outcomes for individuals diagnosed with this devastating cancer.

12. References


Novel Therapeutic Concepts for Targeting Glioma offers a comprehensive collection of current information and the upcoming possibilities for designing new therapies for Glioma by an array of experts ranging from Cell Biologists to Oncologists and Neurosurgeons. A variety of topics cover therapeutic strategies based on Cell Signaling, Gene Therapy, Drug Therapy and Surgical methods providing the reader with a unique opportunity to expand and advance his knowledge of the field.

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