

Antisense Oligonucleotides Therapy in the Treatment of Cerebral Gliomas: A Review

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Abstract

Patients affected by cerebral gliomas, despite classical strategies adopted, show a very poor prognosis. Current treatment consists of regimens that include surgical debulking, radiation therapy, and systemic chemotherapy. However, the median survival after surgery and radiation therapy alone is 9 months, and systemic chemotherapy is minimally effective.

Advances in molecular biology have better depicted the mechanisms involved in the genesis of cerebral gliomas and identified specific gene sequences to be targeted in the malignant cell genome. Gene expression can be blocked using various strategies. The concept of antisense-mediated gene inhibition has now emerged as a potentially powerful alternative or adjunct to conventional cancer chemotherapy. This strategy is able to block selectively glioma cells which interfere to gliomagenesis molecular pathways. The antisense molecules, delivered inside the brain, penetrate into glioma cells blocking specific genetic functions. Antisense oligonucleotides are complementary to the target mRNA and this bind cause the block and/or the reduction of the encoded protein synthesis. Genes coding for growth factors and their receptors, proto-oncogenes, cellular proteases, kinases, and proteins important in cell cycle control and apoptosis represent ideal target for antisense oligonucleotides treatment.

In this study, we report the most relevant findings of antisense oligonucleotides application in gliomas treatment.

Keywords: Antisense; Brain delivery; Glioma; Glioblastoma; Gliomagenesis; Oligonucleotides

Abbreviations: AKT: Protein Kinase B; AON: Antisense Oligonucleotide; BBB: Blood-Brain Barrier; CNS: Central Nervous System; CpG: Cytosine-Phosphorous-Guanine; dsRNA: Double-Stranded RNA; ECM: Extracellular Matrix; EGF: Epidermal Growth Factor; EGFR: Epidermal Growth Factor Receptor; GBM: Glioblastoma Multiforme; GKR: Gamma Knife Radiosurgery; GFAP: Glial Fibrillary Acidic Protein; HGF: Hepatocyte Growth Factor; HIF: Hypoxia-Inducible Factor; hTERT: Human Telomerase Reverse Transcriptase; IAP: Inhibitors of Apoptosis; IGF-1: Insulin-Like Growth Factor-1; IGF-1R: Insulin-Like Growth Factor-1 Receptor; IL: Interleukin; ILK: Integrin-Linked Kinase; LNA: Locked Nucleic Acids; MDM2: Mouse Double Minute 2; mTOR: Mammalian Target of Rapamycin; NSCLC: Non-small-Cell Lung Carcinoma; PAMAM: Poly(amidoamine); PDGF: Platelet-Derived Growth Factor; PEG: Propylene Glycol; PI3K: Phosphoinositide-3 Kinase; PNA: Peptic Nucleic Acid; PTEN: Phosphatase and Tensin Homolog; RNase: Ribonuclease; RISC: RNA-Induced Silencing Complex; RNAi: RNA Interference; siRNA: Small Interfering RNA; TfR: Transferring Receptor; TNF: Tumor Necrosis Factor; TGF-beta: Transforming Growth Factor-beta; VEGF: Vascular Endothelial Growth Factor; VEGFR: Vascular Endothelial Growth Factor Receptor; WHO: World Health Organization

Introduction

Gliomas account for about 45% of all primary CNS tumors and 77% of all malignant primary CNS tumors [1]. Although brain tumors constitute only a small proportion of overall human malignancies, they carry high rates of morbidity and mortality. The current treatment is a multimodal approach combining neurosurgery, fractionated radiation therapy and chemotherapy with the DNA methylating agent temozolomide [2]. However, mortality is still close to 100% and the average survival of patients with GBM is less than 1 year [2-5].

Gliomas are divided into different subtypes based on cell line

from which they originate. The 4-level grading system proposed by the WHO is the most widely accepted and widespread [6]. Grade I gliomas are benign with a slow proliferation rate and include pilocytic astrocytoma. Grade II gliomas are characterized by an high degree of cellular differentiation and are prone to malignant progression. They include astrocytoma, oligodendroglioma and oligoastrocytoma. Grade III lesions include anaplastic astrocytoma, anaplastic oligoastrocytoma and anaplastic oligodendroglioma. These tumors show a higher cellular density and a notable presence of atypia and mitotic cells. Grade IV tumors are the most malignant and the most frequent and include GBM and gliosarcoma. These tumors presented microvascular proliferations and pseudopalisading necrosis.

Gliomagenesis is characterized by several biological events, such as activated growth factor receptor signaling pathways, down-regulation of apoptotic mechanisms and unbalance among pro-angiogenic and anti-angiogenic factors. The cellular and molecular events that initiate and promote malignant gliomas development are, however, not completely understood. Tumor cells invasion is a multifactorial process, consisting of cell interactions with ECM components and with adjacent cells, as well as accompanying biochemical processes supportive of active cell movement [1]. Critical factors in tumor cells invasion, include the detachment of invading cells, the synthesis and

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deposition of ECM components by tumor cells and mesenchymal cells, the release of ECM-degrading activities for remodeling interstitial space, and the expression of adhesion molecules on glioma cell surfaces that specifically recognize and adhere to ECM components [1]. ECM provides the microenvironment for the cells and serves as a tissue scaffold, guiding cell migration during embryonic development and wound repair. Changes in the ECM components play a crucial role in peritumoral invasion forming structural elements for cellular attachment and migration, although specific interactions and exact mechanisms are unknown. Glioma cells express and release ECM-degrading enzymes for ECM remodeling and infiltration. The function of these proteases is controlled by specific inhibitors and an imbalance in their expression levels facilitates invasion. Malignant gliomas are also characterized by extensive microvascular proliferation. Neo-vascularization in brain tumors correlates directly with their biological aggressiveness, degree of malignancy and clinical recurrence and inversely with the post-operative survival of patients affected by gliomas. Glioma vasculature is structurally and functionally abnormal and it correlates and leads to vasogenic edema, increased interstitial pressure, and heterogeneous delivery of oxygen and drugs [7]. The new blood vessel growth is stimulated by the secretion of growth pro-angiogenic factors. These factors bind to receptors present on endothelial cells, so as to activate them. Glioma cells first accumulate around the existing cerebral blood vessels and lift off the astrocytic foot processes, which leads to the disruption of the normal contact between endothelial cells and the basement membrane [8]. The deposition of pro-angiogenic matrix is essential for newly sprouting vessel. This involves breakdown of the vascular basement membrane and extracellular matrix through the action of cathepsin B, matrix metalloproteases and other enzymes as well as the expression of matrix proteins such as fibronectin, laminin, tenascin-C and vitronectin [9]. After breakdown of the basement membrane, endothelial cells proliferate and migrate toward the tumor cells expressing pro-angiogenic compounds.

Surgical treatment is invasive but represents the first approach for the vast majority of brain tumors due to difficulties arising in early stage detection. Principal objective of the surgery is the reduction of the tumor size and of the intracranial pressure. Aggressive treatments have extended the median survival but it is often associated with significant impairment in the quality of life [10]. Radiation therapy and chemotherapy are non-invasive options often used as adjuvant therapy, but may also be effective for curing early-stage tumors. Adjuvant radiotherapy gives limited benefits and causes debilitation side effects which reduce its efficacy [11]. Patients treated with radiotherapy are at high risk of developing some complications such as post-radiation leukoencephalopathy, characterized by dementia, gait disturbance, incontinence, and a deficit in attention and executive functions [11-13]. The effectiveness of systemic chemotherapy is limited by toxic effects on healthy cells, generally resulting in morbidity or mortality of the patient. Moreover, the presence of the BBB limits the passage of a wide variety of anticancer agents [14,15].

Recent advances in the understanding of the deregulated molecular pathways of gliomas have brought about targeted therapies that have the ability to increase therapeutic efficacy in tumors while decreasing toxicity. Multi-targeted kinase inhibitors, novel monoclonal antibodies, and new vaccines have been developed. Promising therapeutic approach is also represented by RNA and DNA oligonucleotides, including antisense, microRNAs, small interfering RNAs, and nucleic acid aptamers. The concept of antisense-mediated gene inhibition is now emerging as a potentially powerful alternative or adjunct to conventional cancer chemotherapy. Clinical applications

for AONs have been envisioned in many fields including oncology, vascular and genetic diseases, cardiovascular disease, hematological disease, inflammatory disorders, and in the treatment of the human immunodeficiency virus and other viral infections.

In this review clinical and experimental studies about the use of AONs in cerebral gliomas treatment are reported.

Antisense Therapy

In eukaryotic organisms, pre-mRNA is transcribed in the nucleus, introns are spliced out and then the mature mRNA is exported from the nucleus to cytoplasm. The small subunit of the ribosome usually starts by binding to one end of the mRNA and is joined there by other eukaryotic initiation factors, forming the initiation complex. This multi-enzymatic complex scans along the mRNA strand until it reaches a start codon, and then the large subunit of ribosome attaches to the small subunit so that the translation of a protein begins. This process, by which the information of a gene is converted into protein, is referred to as "gene expression". Gene expression can be blocked using three principal strategies [16,17]: a) inhibitor approaches based on chemical compounds and monoclonal antibodies, b) anti-gene approaches, and c) siRNA approach. The anti-gene approaches can be subdivided into others three groups: the antisense molecules [18] targeted to the complementary sequence in mRNA, (antisense RNA, AONs and ribozymes), the triple helix-forming oligomers [19] targeted to the double stranded DNA gene, and the sense oligonucleotide designed to act as decoys to trap regulatory proteins [20].

RNAi or post-transcriptional gene silencing is a conserved biological response that mediates resistance to both endogenous parasitic and exogenous pathogenic nucleic acids. RNAi is also involved in transcriptional regulation, acting as a process within living cells that moderates the activity of their genes. It plays a fundamental role in diverse eukaryotic functions including viral defence, chromatin remodeling, genome rearrangement, developmental timing, brain morphogenesis, and stem cell maintenance [21]. In this mechanism, a short sequence of dsRNA specifically downregulates the eukaryotic expression of an associated gene [22]. The mediators of the sequence-specific mRNA degradation process are 21-25 nucleotide interfering RNAs generated from long dsRNAs by the DICER ribonuclease cleavage. The siRNAs are double stranded molecules, consisting of a guide strand that is perfectly complementary to a target mRNA and a passenger strand [23]. The siRNA is then incorporated into one or more of the Argonaute proteins in RISC where the RNA serves as a sequence specific guide for complementary base pairing with the target and guides RISC for sequence specific target degradation or translational inhibition [23]. The limit in therapeutic use of siRNA to modify gene expression is represented from the transient effect. These antisense molecules, for the lack of the RNA-dependent RNA polymerase, should be require the continuous transfection into target cells by plasmid vectors. In the extracellular compartment, siRNAs are highly susceptible to degradation by enzymes found in serum and tissues. To be effective siRNAs must also reach their target cells in the specific tissues that express the aberrant gene. The large size and negative charge of naked siRNAs thwarts their diffusion across the plasma membrane and prevents intracellular accumulation. Meanwhile, siRNA delivery strategies that take advantage of endocytosis also must provide for endosomal escape. In the cytoplasm, siRNAs remain vulnerable to degradation by intracellular RNAses and still need to be recognized by and incorporated into RISC with high efficiency. A peculiar distinctive characteristic between RNAi and the other antisense approaches is represented by the extreme selectivity.

In this way siRNA may inhibit selectively the expression of oncogene containing a single point mutation, without suppresses the expression of the wild-type transcript [24].

RNA can act as an enzyme and is capable of catalyzing RNA splicing and cleavage, as well as several other chemical reactions. These novel activities of RNA now permit the development of enzymatic RNA molecules as therapeutic agents that can suppress the expression of altered proteins by catalyzing the *trans*-cleavage of the corresponding mRNAs [25]. RNA targets for ribozyme-based therapeutics may encode oncoproteins, growth factors, proinflammatory cytokines and their corresponding cell-surface receptors, and signal transduction molecules; viral and microbial mRNAs or genomic RNAs are also readily cleaved by this approach. Some ribozymes have a self-cleavage catalytic action while other ones are true catalysts and can carry out RNA slicing by transesterification (spliceosome) and peptidyl transfer (in ribosomes) [26]. These molecules, transcribed from DNA sequences different, should play a crucial role in the epigenetic mechanisms of gene expression and in cell function.

Antisense oligonucleotides

AONs show the important ability to identify and determine the role of a specific gene in a physiological process. The AONs own, also, the capacity to detect genetic mutations and telomere size, the potential uses as nucleic acid biosensors and more several other diagnostic applications [27,28]. Others AONs applications are in chemistry and technology, e.g. as electrochemical biosensors, and in optical data storage [29,30]. In medical applications these antisense molecules may be introduced into a cell to silence one of many genic functions physically obstructing the translation machinery [5].

AONs are relatively small, single-stranded deoxyribonucleotide, 13-25 nucleotides long, that are complementary to the target mRNA [5,31,32]. Their binding to this mRNA by Watson-Crick base-pairing stops translation and thereby reduces synthesis of the encoded protein. The biological activity of AONs can be expressed through multiple mechanisms, including inhibition of the interaction with proteins or other nucleic acids, consequently inhibiting or preventing RNA transport, splicing, and translation; disruption of RNA structure; covalent modification of target nucleic acid; induction of RNase L; and induction of RNase H [33]. However, the most important mechanism appears to be the utilization of endogenous RNase H enzymes [18,34]. This enzyme specifically cleaves the RNA strand of RNA-DNA duplexes. This releases the AON intact, which can then bind to a new mRNA strand. This process produces a targeted destruction of mRNA and a correction of genetic aberrations. Most of the antisense drugs currently in clinical trials utilize the RNase H mechanism [34]. Regulation of RNA processing is another efficient mechanism in which oligonucleotides can be utilized to regulate gene expression. New studies have demonstrated that AONs can be used to regulate RNA splicing in both cell based assays and in rodent tissues [35,36]. Other AONs mechanisms include translational arrest by steric hindrance of ribosomal activity, interference with mRNA maturation by inhibiting splicing and destabilization of pre-mRNA in the nucleus.

Oligonucleotides are polyanionic macromolecules and multiple obstacles in reaching their intracellular site of action are evident. Due to the high molecular weight and polyanionic characteristics, AONs are subjected to relatively rapid clearance from the blood circulation and show a reduced bioavailability. Low cellular membrane permeability and lack of cell-type specific uptake are additional issues that need to be addressed to advance these molecules into the clinic. To

overcome these limits chemical modifications such as the introduction of phosphorothioate linkages and 2'-O-methylation of the ribose moieties have been developed [37,38]. The chemical modifications can significantly improve the therapeutics properties of AONs and may be unavoidable for their clinical exploration. Based on variations of these modifications three AONs generations can be identified. AONs first generation contains backbone modifications such as replacement of the oxygen atoms of the phosphate linkage by sulphur (phosphorothioates), methyl group (methylphosphonates) or amines (phosphoroamidates). The phosphorothioates have been widely used for gene silencing because of their sufficient resistance to nucleases and ability to induce RNase H functions [18,34]. The second generation of AONs was characterized by substitutions of position 2' of ribose with an alkoxy group. 2'-O-Methyl and 2'MOE (2'-O-methoxyethyl) derivatives can be further combined with a phosphorothioate linkage. 2'MOE AONs show an improved resistance against nuclease-mediated metabolism as well as tissue half-life *in vivo*, which produces a longer duration of action [18,34]. The third generation of AONs contains structural elements, such as zwitterionic oligonucleotides, LNA, morpholino, PNA and hexitol nucleic acids. These modifications enhanced AONs in terms of nuclease resistance, specific binding and cellular uptake with agents such as PNA and morpholino.

Systemic AONs treatment is well tolerated and side-effects are dose-dependent. Dose-limiting toxicities include thrombocytopenia, hypotension, fever and asthenia [5]. The most common acute toxicities associated with AONs administration *in vivo* are activation of the transient complement cascade and inhibition of the clotting cascade. Both these toxic effects are dependent on AONs backbone chemistry. The toxicity is largely produced by the non-specific binding properties of phosphorothioate-AONs to proteins at high plasma concentrations. The complex phosphorothioate-AON binds to multiple coagulation factors, such as VIIIa, IXa X and II, leading to a transient self-limited prolongation of activated partial thromboplastin times [39,40]. Splenomegaly, lymphoid hyperplasia and diffused multi-organ mixed mononuclear cell infiltrates are often evidenced [41]. This is due to an unmethylated CpG motif in the AON sequence that can be recognized by toll-like receptor-9 in immune cells, resulting in the release of cytokines (IL-6, IL-12 and interferon-g), B cell proliferation, antibody production and activation of T lymphocyte and natural killer cells [42]. Increase of the liver enzymes aspartate aminotransferase, alanine aminotransferase, and a prolonged partial thromboplastin time has also been reported [42].

Oligonucleotides delivery to the brain

The brain is one of the least accessible organs and the BBB limits the delivery of therapeutic agents. BBB function is to maintain a constant internal environment inside the brain by strictly regulating the composition of the cerebral extra-cellular fluid and to protect the brain against potentially toxic substances. In fact, except leakage in areas of BBB dysfunction, peptides, recombinant proteins, monoclonal antibodies, RNA interference-based drugs, generally do not cross the barrier [5,15]. Innovative drug delivery systems may make it possible to use certain chemical entities or biologic that were previously impractical because of toxicities or because they were impossible to administer. Several recent papers describe the possibility to apply the brain drug-targeting technology for the diagnosis or therapy of many brain disorders [43,44]. Peptidomimetic monoclonal antibodies that bind endogenous transport system within the BBB, such as the insulin receptor, the Tfr, or the leptin receptor, have been used for targeting neuropeptides, siRNAs, or antisense agents through the BBB *in vivo*. Nanoparticles

delivery systems in cancer therapy provide better penetration of therapeutic agents with a reduced risk in comparison to classical treatment. Encapsulated molecules can be released from nanocarriers in a controlled manner over time to maintain a drug concentration within a therapeutic window, or the release can be triggered by some stimulus unique in the delivery site [45]. The surface of the nanocarrier can be engineered to increase the blood circulation half-life and influence the bio-distribution, while attachment of targeting ligands to the surface can result in enhanced uptake by target tissues [46]. Several types of nanoparticles such as polymers, dendrimers, liposomes, and micelles have been synthesized or engineered as carriers for brain-specific drug delivery [47-49]. One of the most investigated approaches uses liposomes as sub-micron delivery vehicles. They are particularly useful as gene therapy devices due to their ability to pass through lipid bi-layers and cell membranes. Glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) are among the most widely used neutral lipids. Simply mixing siRNA with DOPC results in more than 65 percent encapsulation, and these complexes have been shown to bring about siRNA-mediated silencing in cancer cells *in vivo* [50]. Immunoliposomes have been generated carrying small hairpin RNA expression plasmids for RNA interference effect [32]. The immunoliposomes are then engineered with PEG, which stabilizes their structure in circulation. Recently, lipid-based delivery systems have demonstrated that some synthetic lipid-like materials (termed "lipidoids") form complexes with siRNA or miRNA that facilitate intracellular delivery of the oligonucleotides [51].

Molecular Targets

The number of potential targets for AONs treatment of glioma cells is extremely large, including genes coding for growth factors and their receptors, cellular proteases, kinases, second messengers, proto-oncogenes, and factors and proteins important in cell cycle control and apoptosis. The classical examples of use of antisense oncogenes are that of *c-myb*, or *bcl-2*. In neoplastic progression, several growth factor receptors, such as EGFR, VEGFR, TGF-beta, IGF-1R are over-expressed, amplified and/or mutated in gliomas.

Oncogenes

The capacity of AONs to detect specific gene sequences and to down-regulate gene expression make them optimal agents for use in targeting oncogenes expressed in brain tumors.

The *c-myb* proto-oncogene encodes for the nuclear protein Myb, which acts as a sequence-specific DNA transcription factor. The inhibition of proliferation of T98, U87, and U373 glioma cells after treatment with phosphorothioate AONs to either the 5'-cap initiator region or the transactivation sequence of *c-myb* was obtained [52]. The mechanism is probably mediated through cell surface growth factor receptor expression. A most recent demonstrated the therapeutic effect of oncogene *c-myb* AON on C6 glioma in nude mice. The results showed that the expression of *c-myb* and *bcl-2* proteins was significantly decreased in the AON group [53].

The *c-sis* oncogene encodes for the B-polypeptide chain of PDGF. In the A172 GBM cell line, AONs complementary to *c-sis* mRNA inhibited cell proliferation in a time- and dose-dependent fashion [54]. By flow cytometric analysis, AONs were shown to block the *de novo* synthesis of intracellular *c-sis* protein in A172 cells [54].

The *c-myc* proto-oncogene encodes nuclear transcription factors proteins that play important roles in cellular proliferation and differentiation. The *c-myc* has been observed to promote cellular

proliferation as well as programmed cell death, or apoptosis. Over-expression of the *c-myc* has been also demonstrated in gliomas [55]. The *c-myc* has been observed to be expressed at the mRNA level in human gliomas, and in human glioma and GBM cell lines [56]. In an experimental study, sequence-specific antisense inhibition of *c-myc* protein expression reduced cellular proliferation in malignant rat glioma cells [57]. A variety of mechanisms to explain this phenomenon are possible, such as augmented production of *c-myc* mRNA, or activation of pathways for cellular proliferation independent of *c-myc*.

The *c-met*, a receptor tyrosine kinase, and its ligand, HGF, are critical in cellular proliferation, motility, and invasion. The complex HGF/*c-met* played an important role in the gliomas formation and progression, and can promote tumor proliferation and intratumoral microvascular formation [58]. It was demonstrated *in vitro* and *in vivo* that AONs against *c-met* (FAM-labeled *c-met* nonsense AONs-LIPOFECTAMINE PLUS™) markedly suppressed the expression of *c-met* mRNA in human glioma cells and cell growth and enhanced significantly the cytotoxic effect of radiation on human U251 glioma cells in culture [59]. Recently has also been demonstrated that *c-met*-AONs increase the sensitivity of human glioma cells to paclitaxel. A combination of paclitaxel with antisense *c-met*-AONs inhibited cell growth, induced apoptosis and induced *c-met* protein expression in U251 and SHG44 human glioma cells more significantly than either paclitaxel or the AONs on their own [60].

Apoptotic Pathway

Apoptosis, or programmed cell death, is a highly organized physiologic event that plays an essential role in controlling cell number in many normal processes, ranging from fetal development to adult tissue homeostasis [61]. One of the features of cancer cells is their ability to evade programmed cell death. Apoptosis can occur by up-regulation of antiapoptotic proteins, by down-regulation or loss of proapoptotic proteins or by defective functioning of proapoptotic proteins [62]. Apoptosis can be initiated by stress signals from within the cell or by external environmental. This death signal then involves widespread proteolysis by caspases, nucleosomal fragmentation by endonucleases, and cell surface tagging for phagocyte engulfment [63]. Extrinsic apoptosis is regulated by members of the TNF receptor protein family. Intrinsic apoptosis is regulated at the mitochondrial membrane by members of the *Bcl-2* protein family. Extrinsic apoptosis activates caspases 8 and 10 in the death-inducing signaling complex (DISC), while intrinsic apoptosis activates caspase 9 within the apoptosome. These initiator caspases go on to activate the effector caspases 3 and 7 that amplify the proteolytic caspase cascade, committing the cells to die. However, this cascade can be blocked by IAPs, which bind active caspases and prevent further proteolysis.

Bcl-2 family proteins are the regulators of apoptosis and are overexpressed in many cancers [64]. *Bcl-2* gene is a proto-oncogenes located at the breakpoints of t(14;18) chromosomal translocations in low-grade B-cell non-Hodgkin's lymphomas. Normally, in response to DNA damage and to cellular damage, *bcl-2* induces the release of cytochrome c from mitochondrial matrix to cytosol, where it activates caspase-9 and caspase-3. *Bcl-2* over-expression has been observed in several glioma cell lines and in glioma surgical specimens. It has been also demonstrated that *bcl-2* immunohistochemical positivity is inversely correlated with survival and that *bcl-2* protein promotes migration and invasiveness of human glioma cells [65]. More, experimental observations support the hypothesis that *bcl-2* and *bcl-xL* are important in preventing cell death in GBM cells. A decrease in cell growth and an increase in apoptotic death, by using AONs against

the first six codons of the human *bcl-2* gene transfected into malignant glioma cells (Jon52 and Roc GBM cell lines), has been demonstrated [66]. In GBM cells lines U87 and NS008 with *bcl-2/bcl-xL* bispecific AON, down-regulation of *bcl-2* and *bcl-xL* resulted in spontaneous cell death. The mechanism of cell death was partially caspase-dependent [67]. An experimental study has evidenced the therapeutic effect of oncogene *bcl-2* AON on C6 glioma in nude mice. The results showed the expression of *bcl-2* proteins was significantly decreased in the AON group [53]. More, it has been demonstrated that the antisense modulation of *bcl-2* expression could increase the effectiveness of conventional chemotherapeutic agent. Guensberg et al. showed that resistance to chemotherapy in GBM is linked to the expression of *bcl-2* family members, including *bcl-xL* [68]. In this experimental study, the authors demonstrated a valid correlation between reduction of *bcl-xL* protein expression, induction of intrinsic apoptotic pathway and enhancement of cytotoxic responses to paclitaxel treatment, in M059K GBM cell lines treated with anti-*bcl-xL* AONs (ISIS 16009, ISIS 16967) [68].

Various cancers, including GBM, over-express members of the IAP family. Nowadays, seven members have been identified in mammalian cells: X-linked IAP (XIAP), cellular IAP1 and -2 (cIAP1, cIAP2), neuronal apoptosis inhibitory protein (NAIP), survivin, BIR repeat containing ubiquitin-conjugating enzyme6 and melanoma/kidney IAP (ML-IAP/KIAP) [69]. The principle mechanism underlying the antiapoptotic activity is represented by direct caspase inhibition. It has been reported that the infection of malignant glioma cells with adenoviruses encoding antisense RNA to XIAP depletes endogenous XIAP levels and promotes global caspase activation and apoptosis. More, AON-XIAP induces cell death in intracranial glioma xenografts, and prolongs survival in nude mice [69]. Apollon, a new human IAP, is a human homolog of the recently reported BRUCE isolated from mouse cells. Glioma cell line expressing a large amount of Apollon showed resistance to chemotherapeutic drugs. Western blot analysis revealed that Apollon was expressed in four of six human brain cancer cells. In this study, treating the cells with AON reduced the Apollon protein expression and sensitized the cells to chemotherapy-induced apoptosis [70].

Targeting death receptors to trigger apoptosis in tumor cells is an interesting option for cancer therapy. Stimulation of death receptors of the tumor necrosis factor receptor superfamily such as CD95 (APO-1/Fas) or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors results in caspase-8 activation, which can directly translate into cleavage of downstream effectors caspases. The mitochondrial pathway is initiated by the release of apoptogenic factors such as cytochrome c, apoptosis-inducing factor (AIF), Smac (second mitochondria-derived activator of caspase)/DIABLO, or endonuclease G from the mitochondrial intermembrane space. Malignant glioma cells express high levels of TRAIL DRs and appear to be susceptible to TRAIL-induced cell death. It has been demonstrated that malignant glioma cells primarily express DR5, suggesting that TRAIL-induced apoptosis may occur primarily through DR5 activation [71].

The urokinase plasminogen activation system plays an important role in the activation of matrix-degrading enzymes that enhance the invasion of cancer cells. This complex comprises the ligand urokinase plasminogen activator and the receptor urokinase plasminogen activator receptor (uPAR). Reduced expression of this receptor may diminish migration, mitogenicity, or induction of neovascularization. In an experimental study SNB19 GBM cells has been treated with an uPAR AON. Probably, silencing of uPAR expression, by the

introduction of an AON, cause blocks mitogenic signals from feeding into the cell cycle, rendering SNB19 cells susceptible to TRAIL [72].

Survivin is a member of the IAP family that is expressed at high levels in most human cancers including gliomas. Survivin may contribute to resistance of tumors by facilitating evasion from apoptosis and aberrant mitotic progression [61]. These features make survivin an attractive target in the mechanism of cancer resistance. In a valid study, to inhibit survivin expression, phosphothiorate AONs against survivin expression was adopted. In GBM cells (U373MG and A172) down-regulation of survivin using survivin AON sensitized cells for TRAIL-induced apoptosis was demonstrated [73].

PTEN/PI3K/Akt Pathway

The PTEN/MMAC, which consists of nine exons, is located on chromosome 10q23.3 and encodes a 403-amino acid cytoplasmic protein that contains two domains in the N-terminus, a region with tyrosine phosphatase activity and a region that interacts with the cellular cytoskeleton [74]. PTEN or MMAC 1, is a tumor-suppressor gene regulating cell growth, apoptosis, interaction with extracellular matrix and inhibiting cell migration, as well as spreading and focal adhesion [75]. PTEN mutations and loss of function are frequent in malignant gliomas and are responsible for the abnormally high levels of activity in the PI3K/Akt signaling pathway that have been demonstrated in these neoplasms [76]. PTEN own the phosphatidylinositol phosphatase activity, specifically removing phosphates from the 3' position of the inositol ring [77]. This finding implicated PTEN as a regulator of PI3K-mediated cell signaling pathways. The PI3K family of kinases catalyzes the transfer of the γ -phosphate of ATP to the D3-position of the head group of phosphatidylinositols (PtdIns), a form of membrane lipid. After its production, PtdIns recruits the serine/threonine kinase Akt. Several researchers have discovered the substrates of Akt that are involved in the pro-cell survival effects, which thus far include glycogen synthase kinase-3, mTOR, MDM2, p21, HIF-1, IKK, Bad, and caspase 9 [78]. Akt must be activated for GBMs to form genetically modified neural progenitors and normal human astrocytes, suggesting that the activation of Akt plays an important role in glioma formation and progression [79]. Decreased expression of PTEN and over-expression of the Akt proto-oncogene, which is located downstream of PI3K, have been demonstrated in human GBM [80].

In a recent study, a combined PTEN and antisense hTERT gene therapy for experimental glioma in vitro and in vivo has been evaluated. Infection with antisense-hTERT and wild-type-PTEN adenoviruses significantly inhibited human U251 glioma cell proliferation in vitro and glioma growth in a xenograft mouse model. The efficacy of therapy was higher in the tumor xenografts infected with both PTEN and antisense hTERT [81].

ILK, a serine-threonine protein kinase, is a key component of cell-ECM adhesion and has been shown to anchor to integrins by interacting at its C-terminal domain to the cytoplasmic domain of $\beta 1$ and $\beta 3$ integrin subunits [82]. ILK is critical for the PTEN-sensitive regulation of PKB/Akt-dependent cell cycle progression and cell survival. To confirm these data, the transfection of ILK antisense into U87MG xenograft inhibits serum-independent PKB/Akt-Serine-473 (Ser-473) phosphorylation as well as PKB/Akt kinase activity, and leads to apoptosis or apoptosis sensitivity [83]. Edwards et al. targeted the phosphatidylinositol 3-kinase/protein kinase B (PKB)/Akt and the Ras/MAPK pathway. The GBM cell lines U87MG, SF-188, and U251MG were transfected with an AON targeting ILK (AON-ILK).

GBM cells transfected with AON-ILK exhibited reduced levels of ILK and phosphorylated PKB/Akt on Ser473 [84].

In an experimental study, AON Akt2 (AS-Akt2) were transfected into rat C6 glioma cells with elevated endogenous Akt2 expression. In glioma transfected cells down-regulation of proliferation and growth rate, induction of apoptosis was evidenced. The inhibition of Akt2 expression was also demonstrated [85,86]. In an analogue research, AON Akt2 was transfected into glioma cell line TJ905 and inhibition of Akt2 was observed [87].

Growth Factors Pathways

Overexpression of EGFR has been associated with numerous malignancies and has become an interesting target in cancer research [88]. In malignant gliomas, EGFR signaling is increased via overexpression or mutation in 40%-50% of all tumors. In a recent experimental study, the stereotactic injection of AON-EGFR-FA-PAMAM complexes into rat C6 intracranial gliomas caused a greater suppression of tumor growth and longer survival time of tumor-bearing rats compared with PAMAM and oligofectamine-mediated AON-EGFR therapy [89]. In this study, the authors coupled the folic acid (FA) to the surface amino groups of G5-PAMAM dendrimer (G5D) through a 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide bond. Successively, the AON corresponding to rat EGFR were then complexed with FA-PAMAM [89]. In another research, the treatment of U87MG cells with AON-EGFR and with wild-type PTEN inhibited the cellular growth by 91.7% [90]. EGFRvIII is a mutated isoform characterized by a truncated extracellular domain. This mutant isoform has been detected in several human cancers including NSCLC, breast carcinomas, ovarian carcinomas and gliomas. Recent studies in human GBM cells and in human xenografts models suggest that EGFRvIII promotes tumor growth and progression via constitutive activation of the PI3K/Akt pathway; it also induces up-regulation of cell proliferation via the MAPK/ERK1/2 signal transduction pathway. The use of AONs into glioma murine models, through liposomes tagged with monoclonal antibodies, facilitates tumor targeting and induces reduction of HER1/EGFR expression increasing survival [91]. In another study, in U251 glioma cells treated with AON-EGFR and siRNA, a significantly inhibition of cell growth was evidenced [92]. More, GBM U87MG cells, which showed an EGFR over-expression, were transfected with AON-EGFR. U87MG cells transfected presented smaller cell bodies and longer processes, and expressed higher level of GFAP compared with the control cells. Telomerase activity was, also, significantly decreased in the AON-EGFR treated cells [93,94]. The effect of combination therapy with GKR and AON-EGFR was, also, evaluated. In C6 glioma models and in orthotopic xenografts, the combined treatment with AON-EGFR and GKR, caused an important growth rate and a significantly suppression of cell proliferation. A cell apoptosis *in vitro* induction was also obtained [95].

The VEGF pathway show a primary function in the neovascularization process and it represent the principal target of most antiangiogenic therapies. In tumorigenic mice over-expression of AON-VEGF (C6-VEGF(-/-) mice) significantly suppressed tumor growth, decreased angiogenesis and reduced tumoral edema [96]. In C6 cells with expression vectors containing sense (C6/VEGF+) or antisense (C6/VEGF-) VEGF complementary DNA, VEGF expression, water content, and morphological characteristics were investigated. The authors evidenced that VEGF can aggravate edema in tumor tissues and plays critical roles in the stickiness of tumor cells to vessel wall and in the integrity of the vessels basal lamina [97]. In an *in vivo* murine model, with U-87 MG glioma cells transfected with AON-VEGF cDNA

coupled to the recombinant adenoviral vector Ad5CMV- α VEGF, a reduction of VEGF mRNA endogenous level was demonstrated [98].

TGF- β 2 plays a key mechanism of carcinogenesis, in particular immunosuppression and metastasis, and is frequently overexpressed in malignant tumors. The TGF- β 2 is overexpressed in more than 90% of gliomas, and its levels are closely related to tumor progression [99]. In patients with malignant glioma, intratumoral treatment with trabedersen is currently evaluated in a pivotal, randomized and active-controlled phase III study [100]. Trabedersen (AP-12009) is a synthetic AON designed to inhibit the production of TGF- β 2 and enhanced the immune cell mediated cytotoxic antitumor response [100-103]. Preclinical studies demonstrated that trabedersen reduced the secretion of TGF- β 2 in cultured tumor cells and showed antitumor activity *ex vivo*. Improved survival, compared with patients receiving standard chemotherapy, in patients with brain tumors who were administered trabedersen was observed [104]. TGF- β 2 binds to TGF- β receptors and promotes a signaling cascade *via* cytoplasmic signaling mediators (SMAD) into the nucleus, inducing regulation of target gene expression [105]. A randomized phase III study relates trabedersen 10 mM *versus* conventional alkylating chemotherapy in patients with recurrent or refractory anaplastic astrocytoma after standard radio- and chemotherapy [106,107]. The results demonstrated that the 2-year survival rate for 10 mM trabedersen was 39% *vs.* 22% for standard chemotherapy in the enrolled patients.

IGF-I is involved in neural development, neurogenesis, glial differentiation and glucose metabolism, acting locally with autocrine/paracrine fashion [108]. Its overproduction is considered to be a participating factor in cancer development in the brain [108]. Rats submitted to injections of C6 glioma cells and treated with AON-IGF-IR did not develop tumors, and were protected from a subsequent challenge with wild-type C6 glioma cells for at least 3 months [109]. The authors observed that inhibition of IGF-IR in the experimental glioma model results in apoptosis of tumor cells, inhibition of tumor genesis and immune anti-tumor response.

Conclusion

Malignant gliomas remain a poorly understood form of cancer associated with high rates of morbidity and mortality. Despite all the advances in understanding of pathomechanism, diagnosis by imaging and availability of powerful therapeutic tools, the life expectancy of patients with gliomas and especially GBM has been prolonged only slightly. None of the currently available surgical tools, including operative microscopes, and image guided surgery enable detection and removal of all of the tumor tissue. Current conventional treatments protocols include maximally safe surgical resection followed by fractionated radiation therapy of the tumor and surrounding brain parenchyma and systemic chemotherapy. However, the intensive proliferation activity, the formation of abnormal tumor vasculature, and the glioma cell invasion along white matter fibers are responsible for the high recurrence rate and for the resistance of gliomas to treatment [110]. More, radiation therapy is limited to a largely palliative role, and chemotherapy has provided only a modest benefit in clinical outcome. There are several factors underlying the disappointing results in brain cancer therapeutics including limited tumor cell drug uptake, intracellular drug metabolism, inherent tumor sensitivity to chemotherapy, and cellular mechanisms of resistance. The commonly used drugs are nonspecific and unable to modify the transformed phenotype of malignant brain tumor cells. The transformed phenotype of malignant brain tumor cells is highly complex and involves amplification or over-expression of oncogenes, as well as loss or

lock of expression of tumor suppressor genes [1,3]. Glioma gene expression during gliomagenesis may help to better understand the role of important molecules involved in tumor-safe brain parenchyma relationships. These molecules, such as ECM proteases, cell adhesion molecules, and their related signaling pathways, show an important role in glioma cell migration and invasion and represent ideal targets in gliomas treatment. The complexity of the signal transduction pathways limits the potential efficacy of targeting a single receptor or molecule. Besides, we think it could be very important to detect more molecular targets of the same pathway due to multiple phenotypes inside glioma. A combination therapy with different AONs or, also, the use of AONs in conjunction with conventional chemotherapeutic agents may have a therapeutic value. The recent discovery of many RNA molecules non-coding for protein, transcribed from DNA sequences different than II class genes (gene coding for protein), has modified strongly the classic idea of human genome, genome-phenotype correlation, and gene expression regulation [21]. The knowledge of the gliomas genetic bases and of their invasive behavior may suggest new molecular targets to overcome the mechanisms of multi-drug resistance of the actual therapeutic approaches.

Antisense therapy approach has been applied in various kinds of tumors and shows a very high specificity and efficacy against cancer cells. The goal of an antisense molecules-based approach is to selectively suppress the expression of a protein by exploiting the genetic sequence in which it is encoded. The identification and validation of antisense inhibitors is the fastest way to identify inhibitors of gene expression. Many AONs currently in phase II and phase III clinical trials have shown sharp reduction in target gene expression and promising activity against a variety of human malignancies. Several experimental *in vitro* and *in vivo* studies in cell lines cultures and animal models showed inhibition of genes involved in cell proliferation, apoptosis and angiogenesis [5]. The development of tumor-specific, systemic delivery systems, such as the folate-liposome complex and/or ligand-liposome complex can also increase the clinical potential of AONs agents. In this field, nanoparticles-based delivery systems could increase the overcoming of BBB by the drug with a targeted-cell specificity modality. This approach permits the use of a lower dose of drug, a selective drug delivery to target tumor cells, both into the central core of tumor and into the distal foci of tumor cells within areas often characterized from integrity of BBB [61]. Generally, a gene knockdown agent should achieve high sequence specificity and should lack off-target effects. However, improvements in AONs efficacy via chemical modifications are ongoing. The most important modification in AONs chemical structure were the introduction of phosphorothioate internucleotide linkages, and the addition of 2'-O-methyl-modified nucleotides at the 3' and 5' ends, which protected the AONs from degradation by nucleases. However, hybridization-independent toxicity profile varies with different sequences. The effects include increased coagulation time, pro-inflammatory effects and activation of the complement pathway. In addition, phosphorothioate AONs that contain certain sequences induce a strong immunostimulatory response through their interactions with toll-like receptors or they bind directly to proteins, leading to unexpected spurious effects. Morpholinos are virtually free of off-target effects, probably because they cannot interact electrostatically with proteins. Morpholinos also achieve exquisite sequence specificity and this constitutes sufficient sequence information to uniquely target a selected gene transcript [111].

Future and potential interesting molecular target are represented

by the inhibitor of apoptosis family (IAP, c-IAP1, livin), by metalloproteinases, and by clusterin. The heat shock proteins expression (HSP) in some cancer types is correlates with poor prognosis. A potential target is, also, the IL-8 that acts within HIF-1 α pathway, crucial step in the angiogenic process [5,112,113]. An optimal realization of a system that overcomes the noted problems in brain tumors treatment requires the identification of new and specific neoplastic markers, the development of technology for the biomarker-targeted delivery of therapeutic agents, and the simultaneous capability of avoiding biological and biophysical barriers.

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