Mini-review

Targeting apoptosis pathways in glioblastoma

Günter Eisele *, Michael Weller

Department of Neurology, University Hospital Zurich, Frauenklinikstrasse 26, CH-8091 Zurich, Switzerland

A R T I C L E   I N F O

A B S T R A C T

The treatment of glioblastoma remains a major challenge for clinicians since these highly aggressive brain tumors are relatively resistant towards radio- and chemotherapy. The pathways that control apoptosis are altered in glioblastoma cells leading to resistance towards apoptotic stimuli in general. In this review we describe the alterations affecting the p53 pathway, the BCL-2 protein family, the inhibitor of apoptosis proteins and several growth factor pathways involved in the regulation of programmed cell death and define possible targets for new therapies within these apoptotic pathways in glioblastomas. Moreover, we review strategies to target death receptor pathways, most notably to render the glioblastoma cells more susceptible towards this approach without enhancing toxicity in general. Most of the strategies targeting apoptosis in glioblastomas presented here are in a pre-clinical stage of development, however, they all share the ultimative goal to improve the outcome for glioblastoma patients.

1. Introduction

Glioblastomas are highly aggressive brain tumors thought to be of glial, that is astrocytic or oligodendrocytic origin. Typical histological features comprise high cellularity, nuclear atypia, mitosis, atypical vasculature and pseudopalisading necrosis. Despite recent improvements of care in the fields of surgical resection, radiotherapy and alkylating chemotherapy, the median survival is still less than 6 months in population-based analyses [1]. The dismal prognosis of glioblastoma has several reasons. In addition to their infiltrating growth pattern, glioblastomas are characterized by an immunosuppressive phenotype. Moreover, glioblastoma cells are virtually resistant to different apoptotic stimuli. Modifications in the apoptotic pathways may not only contribute to gliomagenesis, but also to the resistance towards classical genotoxic approaches of therapy [2].

In the clinic, regressions of glioblastomas are rarely achieved as judged from contrast-enhancing lesions on neuroimaging. At least with radio- or chemotherapy, it is more likely, if at all, to achieve stable disease in glioblastoma patients. Therefore, the therapeutic interventions slow down the growth of the tumor cells rather than induce death including apoptotic cell death. Garcia-Barros and colleagues introduced the concept of regulation of tumor response towards radiotherapy by endothelial cell apoptosis [3]. According to this theory, the response of glioblastomas to radiotherapy might in part be mediated by an anti-angiogenic effect followed by malnutrition of the tumor which in turn prevents further growth. However, this has never been proven experimentally in glioblastoma. Nevertheless, it is unlikely that endothelial cells within the tumor survive the cumulative irradiation doses up to 60 Gy given in glioblastoma patients. In a mouse model of glioblastoma the neo-vasculature following radiotherapy and thus tumor recurrence were critically dependent on the recruitment of bone marrow-derived cells to the tumor, indicating impairment of neo-vasculogenesis from residing cells in the tumor following irradiation [4].

The dominating mode of spontaneous cell death found in glioblastomas in histopathology is necrosis, which is

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* Corresponding author. Tel.: +41 44 2555500; fax: +41 44 2554507.
E-mail address: guenter.eisele@usz.ch (G. Eisele).

one of the hallmarks for the neuropathological diagnosis of glioblastoma. Spontaneous apoptosis is also observed in glioblastomas and upon quantification, apoptosis is enhanced in glioblastoma compared with low-grade astrocytoma and anaplastic astrocytoma. However, whether this observation has any prognostic impact has remained controversial [5–8]. Interestingly, other brain tumor entities like oligodendroglial tumors are sometimes at least transiently almost completely eradicated following radio- or chemotherapy [9–12]. The apparent absence of an inflammatory reaction by magnetic resonance imaging suggests apoptotic cell death in these patients, but this has not been confirmed histologically because of limited access to regressing tumor tissue. The molecular differences between oligodendroglial tumors and glioblastomas leading to this differential sensitivity towards therapy-induced apoptosis remain to be determined. Of the three well-defined molecular markers linked to favourable outcome in gliomas, the 1p/19q codeletion, O^6-methylguanine-DNA methyltransferase (MGMT) promoter hypermethylation and mutations in the gene coding for isocitrate dehydrogenase (IDH)-1 [12], only the 1p/19q codeletion is much more prevalent in oligodendroglial tumors. In turn, this also means that tumors derived from glial cells can be per se susceptible towards therapy-induced apoptosis.

A lot of effort has been made in the last decades to dissect the alterations in the molecular pathways of apoptosis in glioblastoma cells and subsequently to render these cells more susceptible towards therapy-induced apoptosis. However, interventions are complicated by resistance mechanisms towards therapeutic agents building up a threshold for therapeutic efficacy. The alkylating cytostatic drug temozolomide constitutes, in combination with radiotherapy, the current standard of care for glioblastoma [13]. However, the action of temozolomide may be counteracted in the tumors by expression of the DNA repair enzyme MGMT which repairs the temozolomide-induced DNA lesion [14]. The metabolic state of the glioblastoma cells might further influence the sensitivity towards pro-apoptotic stimuli. Within the Cancer Genome Atlas Network, mutations in the genes for IDH 1 and to a lesser extent for IDH 2 were identified as common in secondary glioblastomas [15]. Altogether, these mutations are much more common in WHO grade II and III gliomas than in glioblastomas [16]. The mutation in the IDH 1 gene seems to be a positive prognostic factor for progression-free and overall survival in glioblastoma [17]. IDH 1 catalyzes the decarboxylation of isocitrate to α-ketoglutarate and the point mutation usually observed in glioblastomas inactivates this function. The cellular level of α-ketoglutarate is consecutively lowered, which in turn may lead to enhanced levels of hypoxia-inducible factor subunit HIF-1α [18]. Moreover, mutated IDH 1 may acquire a new biological activity with the generation of 2-hydroxyglutarate which has been proposed to act as an oncometabolite and to contribute to malignant progression [19]. So far, it has not been clarified whether the nevertheless more favourable outcome of glioblastomas with IDH 1 mutations is linked to a different natural course of the disease or to a better response to therapy.

2. Targeting apoptotic pathways

In glioblastoma cells, several key regulatory elements of cell homeostasis and apoptosis are altered at the levels of loss of heterozygosity (LOH), inactivating mutations, methylation, or altered expression, including the p53 protein, the BCL-2 protein family, the inhibitor of apoptosis proteins (IAPs) or receptor tyrosine kinases like the epidermal growth factor receptor (EGFR) and their down-stream signalling cascade. These alterations are in principle attractive targets for therapeutic interventions.

2.1. p53

The TP53 gene product, p53, plays an essential role in cellular responses to DNA damage and regulation of cell cycle and apoptosis [20,21]. Following DNA damage, p53 is activated and induces the transcription of response genes like p21$^{Waf1/Cip1}$, a regulator of cell cycle progression, or BAX, a mediator of mitochondrial apoptosis. p53 is regulated by murine double minute (MDM) 2 and MDM4 (also called MDMX) which inhibit p53 stability or activity. MDM2, in turn, is inhibited by p14^{ARF} (Fig. 1). In addition to its transcriptional activity, p53 may also promote apoptosis through transcription-independent mechanisms and direct interactions with members of the BCL-2 family of proteins in the cytosol or mitochondria [22], a mechanism which still has to be investigated in the glioma context. In glioblastomas, TP53 mutations are common and more frequent in secondary (65%) than in primary (28%) glioblastomas, according to a population-based analysis [23]. In a comprehensive genomic characterization of human glioblastoma genes and core pathways within the Cancer Genome Atlas Research Network, p53 signalling was altered in 87% of all patients in at least one component of the pathway [15].
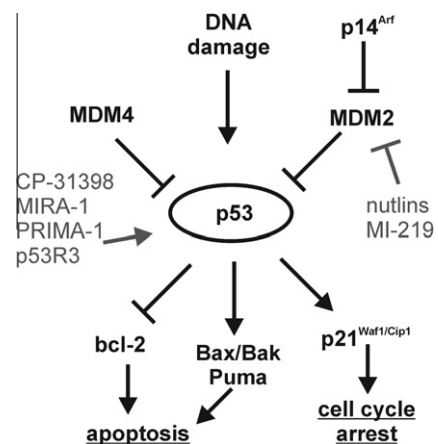

Fig. 1. Targeting the p53 pathway of apoptosis in glioblastomas. Following DNA damage, p53 is activated and this leads to apoptosis or cell cycle arrest. p53 functions as a transcription factor for genes relevant for the regulation of the cell cycle (e.g. p21) or apoptosis (e.g. BAX, BAK, PUMA, BCL-2). Moreover, p53 may act directly in the cytosol or mitochondrium via protein–protein interactions in a transcription-independent manner. Therapeutic interventions aim at restoring and enhancing p53 function or at inhibiting MDM2-p53 interactions.

Given the relative importance of the p53 pathway for the regulation of apoptosis in human glioblastomas, several efforts have been made to develop therapeutic strategies. In a phase I clinical trial of p53 gene therapy, an adenovirus vector (Ad-p53, INGN 201) was injected intratumorally via an implanted catheter and, following resection, in the post-resection cavity [24]. While minimal toxicity was found in the 12 patients treated this way, exogenous p53 was only found in tumor cells within on average 5 mm of the resection site, indicating a major delivery problem of this approach, a problem which might be circumvented by the use of small molecules. CP-31398 is such a small molecule [25], which stabilizes p53 and leads to p53-dependent but also -independent glioma cell death [26]. The exact mode of action of this compound has never been clarified and no further research was apparently pursued because of unspecific toxicity of the compound. Other compounds like 1-(propoxymathyl)-maleimide (MIRA-1), proline-rich membrane anchor p53 reactivation and induction of massive apoptosis (PRIMA-1) or p53R3 restore or enforce DNA binding of mutant or wild-type p53, restoring its pro-apoptotic function [27–29]. Moreover, p53R3 enhanced the cell surface expression of receptors for TNF-related apoptosis-inducing ligand (TRAIL) and thus sensitized glioma cells to TRAIL-induced apoptosis. A different approach targets MDM2, which counteracts p53, to induce cell cycle arrest and apoptosis. MDM2 binds and forms a complex with p53 and thus inactivates its function. Several small molecules have been developed to disrupt the MDM2-p53 interactions, like the *cis*-imidazoline analogs *nutlins* [30] or the spiro-oxindole

inhibitor MI-219 [31], however, only few data exist in the glioblastoma context. In the C6 rat glioma model, nutlin-3 enhanced activation of p53 [32], acting together with p19^{ARF} gene transfer. p19^{ARF} might sequester MDM 4 [33] and sensitize cells to treatment with nutlin-3 [34]. However, this mechanism as an explanation for the combined action of p19^{ARF} and nutlin-3 was not confirmed [32]. In another approach, peptides termed (D)PMI-alpha and -gamma were packaged in liposomes decorated with an integrin-targeting cyclic-RGD peptide in order to enable cell penetration. These peptides disrupted the MDM2-p53 interaction in human glioblastoma cells *in vitro* and *in vivo*, resulting in growth inhibition of the tumor cells [35].

2.2. BCL-2 family and inhibitor of apoptosis proteins

The mitochondrial pathway of apoptosis is tightly regulated by proteins of the BCL-2 family. They share protein–protein interaction motifs, the BCL-2 homology (BH) domains. These proteins have either anti- (e.g. BCL-X$_L$, BCL-2) or pro-apoptotic (e.g. BAX, BAK, BID, BAD) activities. Correlation studies of expression of proteins of the BCL-2 family with WHO grade of malignancy of gliomas and patient outcome have yielded conflicting results (Table 1). Paradoxically, the levels of anti-apoptotic proteins might be even higher in low-grade gliomas compared to glioblastomas [36] and higher levels of BCL-2 correlated with longer survival of the patients [37]. In this context, it might be more important to assess the BCL-2 family as a whole system instead of single proteins to get an estimate on the

Table 1
Expression of BCL-2 in astrocytic tumors. Correlation with WHO grade of malignancy and survival.

Ref.	Number of astrocytic tumors of WHO grade			Method	Expression of BCL-2 correlation with	
	I/II	III	IV		Increasing WHO grade	Survival
[111]	10	7	20	IHC	BCL-2 decrease	No
[112]	16	19	46	IHC	BCL-2 decrease	n.d.
[113]	16	10	11	WB; IHC	% Positive cases increase % positive cells decrease	No
[36]	24	6	19	IHC	BCL-2 decrease	n.d.
[114]	15	7	7	IHC	BCL-2 decrease	n.d.
[115]	28	17	31	IHC	BCL-2 increase[a]	n.d.
[116]	–	29	57	IHC	BCL-2 index decrease	Yes[b]
[117]	8	10	10	RT-PCR	BCL-2 decrease	n.d.
[118]	17	13	26	IHC	No correlation	n.d.
[119]	18	20	21	IHC	No correlation	n.d.
[120]	2	12	23[c]	IHC	No correlation	Yes[d]
[121]	21	9	29	IHC	No correlation	No
[37]	–	99	88	IHC	No correlation	Yes[e]
[122]	8	15	20	IHC	No correlation	No
[123]	6	–	6	IHC, WB, LCM	IHC: BCL-2 decrease WB: BCL-2 increase	No[f]
[124]	–	–	20	IHC	n.d.	No[g]
[125]	–	–	37	IHC	n.d.	No[h]

IHC: immunohistochemistry; RT-PCR: reverse transcriptase polymerase chain reaction; WB: immunoblot; LCM: laser confocal microscopy.
[a] Authors discuss sampling differences to explain differing results.
[b] In multivariate analysis positive staining for BCL-2 correlates with poor survival only in grade III anaplastic astrocytoma, not in grade IV.
[c] Only adult patients.
[d] Six patients with grade III/IV tumors without BCL-2 expression had shorter mean survival.
[e] Higher BCL-2 expression correlated with longer overall, but not progression-free survival.
[f] For a BAX/BCL-2 ratio compared in grade II and IV gliomas.
[g] Trend for earlier progression in BCL-2-high-expressing tumors.
[h] No significant correlation of BCL-2 low/high expression and progression-free survival.

balance of pro- and anti-apoptotic factors present in a single patient, which might influence the response towards radio- and chemotherapy.

One approach to target anti-apoptotic BCL-2 or BCL-X$_L$ are antisense strategies which led to cell death and enhanced chemosensitivity of glioblastoma cell lines in pre-clinical studies [38,39]. However, these strategies face problems with the delivery of the antisense molecules into the tumor cells in vivo. Another pre-clinical approach used an adenoviral gene transfer of natural born killer (NBK), a pro-apoptotic BH-3 only member of the BCL-2 family which heterodimerizes with and inactivates BCL-2 and BCL-X$_L$ [40]. However, this approach is limited by cytotoxicity towards non-transformed rat glial cells and neurons.

Another pre-clinical approach tested in glioblastoma targeting the BCL-2 proteins uses gossypol, a polyphenolic compound derived from the cotton plant. (−)-gossypol binds several members of the anti-apoptotic BCL-2 protein family but BAX or BAK do not seem to be necessary for the cytotoxic action [41]. In glioblastoma cells (−)-gossypol triggered autophagic cell death and led to cytochrome c release. However, cell death occurred without effector CAS-PASE activation and lentiviral knock-down of BECLIN1 and ATG5 in U87MG, U343, and MZ-54 glioblastoma cells diminished the extent of cell death induced by (−)-gossypol and combined treatment of (−)-gossypol and temozolomide. This might indicate that autophagy contributed to this type of cell death [42]. In a phase I study (NCT00390403), gossypol was investigated when given together with temozolomide with or without radiation therapy in treating patients with newly diagnosed glioblastoma. The results await publication. BH3 mimetics should antagonize BCL-2 and kill cells specifically by acting through BAX and BAK. However, many so called BH3 mimetics act independently of BAX and BAK and therefore their predominant activity may involve pathways other than those regulated by BCL-2 [43]. Solution competition assays revealed lower binding affinities for a panel of BH3 mimetics (HA14-1, BH3I-1, Antimycin A, Gossypol; IC_{50} in µM range) compared to BIM (IC_{50} 4.5 nM) [43].

In contrast to other BH3 mimetics, the small-molecule inhibitor ABT-737 specifically targets BCL-2, BCL-X$_L$ and BCL-w with high affinity [43], which might reduce unspecific toxicity. In glioblastoma cells, ABT-737 released BAX from its binding partner BCL-2 and thus induced apoptotic cell death in vitro and in vivo [44]. The orally active analog ABT-263 is currently investigated in phase I/II studies in solid and haematological malignancies alone or in combination with other anti-cancer agents (NCT01009073, NCT00406809, NCT00868413, NCT00891605, NCT-00888108, NCT00887757, NCT00788684, NCT00982566, NCT01053520, NCT00878449, NCT00481091, NCT-01087151). Taken the heterogeneity of the BCL-2 protein family into account, it might be indispensable to modulate the levels of several proteins at the same time to shift the balance towards a pro-apoptotic phenotype.

A novel approach comprises the use of micro RNAs (miRNA) to modulate the expression levels of BCL-2 family proteins. miRNA are small, non coding RNAs of 20-22 nucleotides that bind to complementary sequences of mRNA, leading to either degradation or inhibition of translation of the mRNA. Therefore, miRNAs are considered master regulators of the expression levels of several genes and might determine a certain cell phenotype. microRNA-21 (miR-21) is overexpressed in glioblastomas and miRNA-21 silencing using antisense oligonucleotides reduced cell viability paralleled by elevated levels of CASPASES in vitro [45]. The transfection of miRNA-153 led to reduced levels of BCL-2 and overexpression of miRNA-21 might contribute to resistance towards treatment with the alkylating chemotherapeutic agent temozolomide via a decreased BAX/BCL-2 ratio. [46,47].

The inhibitor of apoptosis proteins (IAP) are a late cellular checkpoint to inhibit apoptosis by inhibiting effector

Table 2
Expression of the IAP SURVIVIN in astrocytic tumors. Correlation with WHO grade of malignancy and survival.

Ref.	Number of astrocytomas of WHO grade			Method	Expression of SURVIVIN/BIRC5
	I/II	III	IV		
[126]	4	3	12	IHC	↑ With increasing grade of malignancy[a]
[127]	–	–	39	IHC	80% of tumors positive
[128]	12	13	56	WB	↑ With increasing grade of malignancy; correlates with poor survival
[122]	8	15	20	RT-PCR	↑ With increasing grade of malignancy; correlates with poor survival
[129]	9	12	8	IHC	↑ With increasing grade of malignancy[b]
[130]	33	11	12	IHC	↑ with increasing grade of malignancy[c]
[131]	–	–	104	IHC	100% of tumors positive no correlation with survival
[132]	19	16	43	IHC; RT-PCR; WB	↑ With increasing grade of malignancy[d]
[133]	–	19	32	IHC	100% of tumors positive correlates with poor survival[e]
[117]	8	10	10	RT-PCR	↑ With increasing grade of malignancy
[134]	18	34	47	IHC	↑ With increasing grade of malignancy; correlates with poor survival[f]
[135]	–	–	66	IHC	Correlates with poor survival[g]

BIRC5 (baculoviral IAP repeat-containing protein 5); IHC: immunohistochemistry; RT-PCR: reverse transcriptase polymerase chain reaction; WB: immunoblot.
[a] Staining intensitiy (only a trend).
[b] Mean percentage of positive cells.
[c] Immunoreactivity score.
[d] Only for nuclear BIRC5 in IHC and not for cytoplasmic BIRC5.
[e] Combined nuclear and cytoplasmic staining vs. nuclear or cytoplasmic alone.
[f] "High" vs. "low" expression.
[g] Only the staining score (cell positivity and staining intensity) for nuclear staining, not for cytoplasmic staining.

CASPASES. Moreover, IAP also modulate inflammatory signalling and immunity, mitogenic kinase signalling, proliferation and mitosis, as well as cell invasion and metastasis in several types of cancer [48]. The IAP family consists of several proteins, like X-linked IAP (XIAP), *BIR* repeat-containing ubiquitin-conjugating enzyme (BRUCE) or the atypical IAP SURVIVIN. Table 2 summarizes investigations on the expression of SURVIVIN in astrocytic tumors, including glioblastoma. The expression of IAP family members is thought to contribute to the resistance of glioblastoma cells to apoptosis induced by radio- and chemotherapy [49]. One approach to target IAP includes the second mitochondria derived activator (SMAC), a mitochondrial protein promoting CASPASE activation in the cytochrome *c*/CASPASE-9/APAF-1 pathway by inhibiting IAP [50]. The gene transfer of SMAC or exposure to cell-permeable SMAC peptides sensitized glioma cells to apoptosis *in vitro* and *in vivo*, induced by death receptor ligation by TRAIL or cytotoxic drugs [51] and full-length SMAC sensitized glioma cells to gamma irradiation-induced apoptosis *in vitro* [52]. This approach is limited by the delivery of SMAC peptide into the tumor but the development of small molecule compounds may circumvent this problem. One study using such a SMAC mimetic in glioma cells *in vitro* was published with similar results as compared to the peptides [53]. The small molecule used in this study functioned similarly to SMAC N-terminal peptides at 10^5–10^6-fold lower concentrations and the compound penetrated cell membranes and bound XIAP with an affinity equal to that of SMAC protein.

Altogether, further *in vivo* studies are warranted and currently, the SMAC mimetic AT-406 is investigated in a phase I clinical trial in solid malignancies and lymphoma (NCT01078649) and according to a patent review [54], 4 small molecule pan-IAP antagonists have been approved to enter human clinical trials, however, dealing with other cancers than glioblastoma.

2.3. Growth factor pathways

Several growth factor pathways have been implicated in transmitting survival signals in glioblastoma cells and thus can be promising targets to facilitate apoptosis. Alterations in these pathways in glioblastomas are well recognized and comprise cell surface receptors as well as down-stream molecules, including the epidermal growth factor receptor (EGFR), the platelet-derived growth factor receptor (PDGFR) and the major down-stream signalling pathway via phosphoinositide-3 kinase (PI3K)/AKT and mammalian target of rapamycin (mTOR), including the tumor suppressor molecule phosphatase and tensin homolog deleted on chromosome 10 (PTEN) which inhibits the PI3K/AKT pathway [55].

EGFR amplification or overexpression occur in approximately 40% of primary, but rarely in secondary glioblastomas [56,57]. Moreover, glioblastomas frequently express constitutively active mutant variants of the EGFR, most notably the deletion variant EGFRvIII. However, phase I/II clinical trials employing erlotinib, an orally active inhibitor of the EGFR tyrosine kinase, failed to exhibit a significant benefit in glioblastoma patients [58–61]. A major goal comprises here the identification and further validation

of molecular profiles of glioblastomas which might help to predict the response to EGFR inhibitors like the co expression of PTEN and EGFRvIII [62]. The PDGFR isoform α is also overexpressed in astrocytomas [57]. However, the tyrosine kinase inhibitor imatinib, a small-molecule inhibitor of PDGFR, BCR-ABL and c-KIT, exhibited only modest antitumor activity, alone or in combination with hydroxyurea [63,64]. Sorafenib, an inhibitor of PDGFR, RAF kinase, c-KIT and vascular endothelial growth factor receptor (VEGFR) 2 inhibits proliferation of glioblastoma cells *in vitro* in combination with the proteasome inhibitor bortezomib [65] and rottlerin, which inhibits protein kinase C [66]. However, the addition of the multikinase inhibitor sorafenib to standard therapy in the first-line treatment of glioblastoma did not improve efficacy of treatment [67]. Overall, agents blocking PI3K/AKT signalling at the level of the receptor tyrosine kinases appear to have little clinical activity. Efficacy is probably limited due to bypass signalling making common down-stream molecules like mTOR an attractive target. In a phase II trial in recurrent glioblastoma, the mTOR inhibitor temsirolimus showed only limited clinical benefit with a progression-free survival at 6 months of only 7.8% of the patients [68]. However, in PTEN-deficient glioblastoma cells, rapamycin, an inhibitor of mTOR enhances cell death and reduces cell proliferation when combined with erlotinib [69]. In a phase II trial in recurrent glioblastomas, the combination of erlotinib and sirolimus, another mTOR inhibitor, again had only negligible activity [58]. However, in this study, patients were heavily pretreated with 53% of the patients having received 3 or more chemotherapies. There are currently more trials running, investigating combination therapies including erlotinib and sirolimus (NCT 00509431, NCT00672243) or sorafenib and temsirolimus (NCT00329719) or vandetanib, an EGFR and VEGFR2 inhibitor and sirolimus (NCT00821080). Further studies investigating efficacious combinatory therapies targeting growth factor pathways are warranted.

3. Targeting death receptors

The death receptor pathway of apoptosis is activated via receptors on the cell surface, belonging to the family of tumor necrosis factor receptors (TNFR). The receptors are activated via ligand binding and trimerization, followed by the assembly of a death inducing signalling complex (DISC) and subsequent activation of a killing cascade. The most important death receptor–ligand systems are TNFR-α with TNF-α, CD95/FAS/APO-1 and CD95 ligand (CD95L/APO-1L/FASL) and TNF-related apoptosis-inducing ligand receptor (TRAIL-R/APO-2) and TRAIL/APO-2L with CD95L and TRAIL having the most promising therapeutic impact for glioblastoma. Tables 3–5 summarize investigations on the expression of CD95/CD95L, TRAIL/TRAIL-R and CASPASES in astrocytomas.

3.1. CD95L

The concept of inducing apoptosis in cells with antibodies targeting cell surface receptors was introduced in 1989

Table 3
Expression of CD95 and CD95L in astrocytic tumors. Correlation with WHO grade of malignancy and survival.

Ref.	Number of astrocytic tumors of WHO grade			Method	Expression of CD95/CD95L
	I/II	III	IV		
[136]	13	12	9	RT-PCR	Expression of CD95 correlates with grade of malignancy[a]
[137]	3	–	11	RT-PCR; IHC	IHC: CD95 in 1/3 grade II and 7/9 grade IV; CD95L in 1/3 grade II and 9/9 grade IV. RT-PCR: CD95 and CD95L in all tumors expressed
[138]	4	2	6	WB; IHC	CD95L detected in all grade I/II and in 6/7 grade III/IV tumors
[115]	28	17	31	IHC	Expression of CD95 and CD95L correlates with grade of malignancy
[139]	3	3	14	WB; IHC	Expression of CD95 and CD95L correlates not with grade of malignancy
[140]	–	18	33	IHC	Expression of CD95 higher in grade IV than grade III; no correlation with survival
[141]	25	–	50[b]	IHC	Expression of CD95 and CD95L upregulated in grade IV

IHC: immunohistochemistry; RT-PCR: reverse transcriptase polymerase chain reaction; WB: immunoblot.
[a] Percent positive tumors per WHO grade.
[b] 25 primary glioblastomas, 25 paired initial and recurrent glioblastomas.

Table 4
Studies on the expression levels of TRAIL and TRAIL receptors in astrocytic tumors. Correlation with WHO grade of malignancy and survival.

Reference	Number of astrocytic tumors of WHO grade			Method	Expression of TRAIL/-R
	I/II	III	IV		
[142]	7	5	11	ICH	100% of tumors positive for TRAIL
[90]	4	–	5	RT-PCR	TRAIL and TRAIL-R1-3 expressed in all WHO grade IV. TRAIL expressed in 1, TRAIL-R3 in 3 and TRAIL-R 1-2 in all WHO grade II. TRAIL-R4 is not expressed
[91]	–	–	62	IHC; RT-PCR	TRAIL-R2 expression is higher than TRAIL-R1 expression correlates with survival[a] and inversely with WHO grade of malignancy. TRAIL-R2 expression correlates with survival but not with WHO grade of malignancy. TRAIL is expressed preferentially in the perinecrotic zone

IHC: immunohistochemistry; RT-PCR: reverse transcriptase polymerase chain reaction.
[a] Only true for a subgroup analysis (patients having received radiotherapy) and % TRAIL-R1 expression or the antigenic load (product of % positive cells and semi-quantitative staining score).

Table 5
Studies on the expression levels of several CASPASES in astrocytic tumors. Correlation with WHO grade of malignancy and survival.

Reference	Number of astrocytic tumors of WHO grade			Method	Expression of CASPASES
	I/II	III	IV		
[143]	n.d.	n.d.	n.d.	IHC	CASPASES 3 and 6 in >50% and CASPASES 8 and 9 in >10% of tumor cells
[144]	3	5	11	WB	CASPASE 8: expression low or absent,[a] no correlation with grade of malignancy
[145]	7	9	11	WB	CASPASE 3: highest expression in WHO grade III
[132]	19	16	43	IHC; WB	CASPASES 3, 6, 7, 8, 9, 10 and 14 expressed,[b] no correlation with grade of malignancy[c]
[146]	14	22	21	IHC	Cleaved CASPASE 3: highest expression in WHO grade I, correlation with survival[d]
[147]	–	–	30	IHC	CASPASE 3: expression in 93% of tumors, 7–60% of tumor cells

IHC: immunohistochemistry; WB: immunoblot.
[a] In relation to protein level in glioma cell line U373.
[b] CASPASES 3 and 9 expression higher compared to other CASPASES in IHC.
[c] Tendency of higher amounts in high grade tumors for CASPASES 3, 8 and 9 in WB.
[d] Among WHO grades II, III and IV and within grade III. n.d. not defined.

with the identification of the receptors APO-1 [70] and FAS [71], which were found to be identical in 1992 [72]. This receptor is now called CD95 according to the *cluster of differentiation* nomenclature and the first ligand for CD95 was described in 1993 [73]. Soon, these findings were used to target also glioma cells with agonistic antibodies to CD95 [74] or with CD95L [75] *in vitro*. Some efforts were made to improve activity of CD95L. Since hexameric forms of CD95L were highly competent to signal apoptosis via formation of a DISC, Holler and colleagues developed a hexameric formulation of CD95L, the *MegaFAS Ligand*, now called APO010 [76]. Compared with a cross-linked soluble

CD95L or a CD95-agonistic antibody, APO010 exhibited superior activity in glioma cell lines expressing CD95 and triggered CASPASE-dependent cell death [77].

Unfortunately, the concept of targeting CD95 has experienced some limitations and drawbacks. Despite some evidence for the feasibility of a local application of CD95L [77,78], *in vivo* applications are critical because of hepatotoxicity upon systemic delivery of an anti-CD95 antibody observed in mice [79] and the fear of potential neurotoxicity since neurons express CD95 [80,81]. However, upon maturation motor neurons become resistant towards CD95L [82] and in rat cerebellar granule neurons the protein lifeguard, which interacts directly with CD95 contributed to this resistance [83]. A more recent constriction for the use of anti-CD95 strategies comes from studies indicating a tumor-promoting role of CD95 signalling via enhancing invasiveness in a mouse model of glioma [84] and tumor growth in mouse models of liver and ovarian cancer [85]. Thus, the CD95/CD95L system remains to be a complex target in terms of cancer therapy, including glioblastoma.

3.2. TRAIL

Given the toxicity associated with CD95 agonism, TRAIL became the most promising death ligand targeting various types of cancer [86]. TRAIL binds to at least five receptors. The death receptors (DR)4/TRAIL-R1 and DR5/TRAIL-R2 transmit an apoptotic signal whereas the decoy receptors DcR1/TRAIL-R3 and DcR2/TRAIL-R4 do not. Further, OSTEOPROTEGERIN is a soluble receptor for TRAIL with uncertain significance [87,88]. TRAIL-R are expressed in human glioma cell lines and in primary human glioma samples [89,90]. One study demonstrated a preponderance of the expression of TRAIL-R2 compared to TRAIL-R1 in primary glioblastoma samples and expression of TRAIL-R2 correlated with survival [91]. Another study confirmed the correlation of TRAIL-R2 expression with better survival in gliomas and found a higher expression level of TRAIL-R2 in WHO grade II compared to grade III gliomas [92]. However, also in normal human brain tissue, expression of TRAIL-R 1-4 was demonstrated on neurons, astrocytes and/or oligodendrocytes [93] and TRAIL can induce human brain cell death [94]. TRAIL-R might be targeted by soluble TRAIL preparations or specific antibodies. APO2L.0, a natural human TRAIL containing amino acids 114-281 is presumed to be well-tolerated while retaining tumor activity [95]. Further recombinant TRAIL preparations aim e.g. at a relative receptor selectivity [96] or use trimeric soluble TRAIL [97] to potentially reduce toxicity while enhancing activity. A recent study investigated full anti-human TRAIL-R monoclonal antibodies selective for TRAIL-R1 or -R2 and only anti-TRAIL-R2 antibodies induced cytotoxicity in human glioma cell lines suggesting that TRAIL-R2 is the main TRAIL receptor responsible for mediating apoptotic signals in human glioma cells [98].

However, a major drawback of the use of TRAIL to target glioblastoma is intrinsic resistance of the tumor cells. Possible factors responsible for resistance comprise low expression levels of mediators of TRAIL signalling, e.g. CASPASE 8, high expression of decoy receptors TRAIL-R3 and -

R4 or high expression of proteins inhibiting TRAIL signalling, e.g. cellular FLICE-inhibitory protein. Moreover, primary glioblastoma cell cultures were resistant to APO2L.0 and showed low expression levels for TRAIL-R2. In these cells, transient overexpression of TRAIL-R2 conferred sensitivity to APO2L.0 challenging the view that specific cell lines harbour specific mechanisms of resistance to TRAIL [99].

To overcome intrinsic resistance, several approaches aim at enhancing the sensitivity of glioblastoma cells towards TRAIL. Pre-clinical studies demonstrated a synergistic, that is a more than additive effect of TRAIL and radio-/ or chemotherapy, e.g. with temozolomide, cisplatin, or nimustine [100–105]. The molecular mechanisms involved in these synergistic effects are numerous, including enhanced release of cytochrome *c* or enhanced protein levels of BID/BAX and therefore enforcement of the mitochondrial loop of induction of apoptosis or enforcing the activation of effector CASPASES. The role of an upregulation of TRAIL-R in such synergistic settings has been discussed controversially [100,102]. Moreover, numerous pre-clinical studies investigated the role of several agents to act together with TRAIL and a complete listing of these substances is beyond the scope of this review but some recent developments are highlighted in the following. The combined use of SMAC agonists and TRAIL has been discussed above [51]. Inhibitors of the proteasome are known to enhance sensitivity for TRAIL as well. A novel small-molecule proteasome inhibitor SC68896 not only induced apoptosis by itself, but upregulated TRAIL-R1 and -R2 expression on the cell surface which might contribute to the enhanced sensitivity of the treated cells towards TRAIL. Moreover, SC68896 is the first proteasome inhibitor showing anti-glioma activity also *in vivo* [106]. The PI3K inhibitor Ly294002 made glioma cells, including primary human glioma cells, more susceptible towards TRAIL- or chemotherapy-induced apoptosis by acting together in triggering mitochondrial membrane permeabilization, activation of CASPASES and subsequent apoptosis [107]. In another approach the synthetic chemical inhibitor molecules BH3I-2, which can bind both, BCL-2 and BCL-X$_L$ and HA 14-1 which is supposed to bind BCL-2 were used to target members of the BCL-2 protein family in order to enhance TRAIL sensitivity [108]. miRNA-21 is another valuable target in the treatment of glioblastoma and knock-down of miRNA-21 combined with a secretable form of TRAIL led to synergistic cytotoxicity [109]. c-FLIP is an anti-apoptotic protein believed to be involved in cellular resistance towards TRAIL. The anti-diabetic drug troglitazone, a PPAR-gamma agonist, reduces protein levels of cFLIP and thus sensitizes glioma cells to TRAIL [110]. Since the mRNA levels of cFLIP remained unaltered upon treatment with troglitazone, a posttranslational mechanism has to be postulated. Additionally, troglitazone downregulated SURVIVIN on mRNA and protein level and upregulated TRAIL-R2.

In contrast to the numerous pre-clinical studies, to date no clinical trial has investigated TRAIL as a possible therapeutic agent in human glioblastomas. At least, clinical trials are testing TRAIL in several solid tumors and in non-Hodgkin lymphoma and the results from these trials

may help to design a clinical protocol for glioblastoma patients.

4. Conclusion

The under physiological conditions tightly regulated program of apoptotic cell death is compromised in glioblastoma, leading to a survival advantage of the tumor cells. Nevertheless, key players of the apoptotic cascades are present in glioblastoma cells and therefore prone to therapeutic interventions. A lot of effort has been made to identify possible targets in pre-clinical investigations. However, to date, data from clinical trials, mostly targeting growth factor pathways, have shown only little activity. A major challenge remains the translation of pre-clinical findings in clinical trials and to avoid toxic side-effects to healthy tissues while retaining efficacy with therapies targeting apoptotic pathways in glioblastoma.

Conflict of interest

The authors declare no conflict of interest.

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