Inhibition of CD95/CD95L (FAS/FASLG) Signaling with APG101 Prevents Invasion and Enhances Radiation Therapy for Glioblastoma

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Abstract

CD95 (Fas/APO-1), a death receptor family member, activity has been linked to tumorigenicity in multiple cancers, including glioblastoma multiforme (GBM). A phase II clinical trial on relapsed glioblastoma patients demonstrated that targeted inhibition of CD95 signaling via the CD95 ligand (CD95L) binding and neutralizing Fc-fusion protein APG101 (asunercept) prolonged patient survival. Although CD95 signaling may be relevant for multiple aspects of tumor growth, the mechanism of action of APG101 in glioblastoma is not clear. APG101 action was examined by in vitro proliferation, apoptosis, and invasion assays with human and murine glioma and human microglial cells, as well as in vivo therapy studies with orthotopic gliomas and clinical data. APG101 inhibits CD95L-mediated invasion of glioma cells. APG101 treatment was effective in glioma-bearing mice, independently of the presence or absence of CD4 and CD8 T lymphocytes, which should be sensitive to CD95L. Combined with radiotherapy, APG101 demonstrated a reduction of tumor growth, fewer tumor satellites, reduced activity of matrix metalloproteinases (MMP) as well as prolonged survival of tumor-bearing mice compared with radiotherapy alone. Inhibiting rather than inducing CD95 activity is a break-of-paradigm therapeutic approach for malignant gliomas. Evidence, both in vitro and in vivo, is provided that CD95L-binding fusion protein treatment enhanced the efficacy of radiotherapy and reduced unwanted proinfiltrative effects by reducing metalloproteinase activity by directly affecting the tumor cells.

Implications: APG101 (asunercept) successfully used in a controlled phase II glioblastoma trial (NCT01071837) acts anti-invasively by inhibiting matrix metalloproteinase signaling, resulting in additive effects together with radiotherapy and helping to further develop a treatment for this devastating disease. Mol Cancer Res; 1–10. ©2018 AACR.

Introduction

Glioblastoma, the most frequent primary brain tumor, still has a dismal prognosis with a mean overall survival of approximately 14 months in study cohorts. The standard treatment comprises maximal safe resection and a combined radiochemotherapy with the concomitant administration of the alkylating drug temozolomide followed by six maintenance cycles of temozolomide over 6 months. The prognosis is, in particular, bad for those patients having tumors with a unmethylated O6-methylguanine DNA methyltransferase (MGMT) promoter status responsible for resistance against chemotherapy with temozolomide (1).

New treatment concepts for patients with glioblastoma investigated over the years include antiangiogenesis (2, 3), targeted treatments with the aim to decipher subgroups (4), and immune activation (5).

CD95 (APO-1/Fas) is a prototypic death receptor that regulates tissue homeostasis mainly in the immune system by induction of apoptosis. Binding of CD95-ligand (CD95L) to CD95 induces receptor trimerization, followed by intracellular
formation of the death-inducing signaling complex (DISC; ref. 6), ultimately triggering the extrinsic apoptosis pathway. Inducing apoptosis in cancer cells by pharmacologic intervention with the CD95/CD95L system has long been in the focus of anticancer research (7). However, there is compelling evidence that CD95 activation can also promote tumor growth: in many types of cancers, tumor growth and cell proliferation depend on a constitutive activation of CD95 (8). Moreover, CD95 activation increases the pool of breast cancer stem cells (9) and promotes the metastatic spread of pancreatic adenocarcinoma (10). Likewise, induction of survival and migration by the CD95/CD95L system has also been described for glioblastoma (11, 12) where activation of CD95 by CD95L stimulates AKT kinase and inhibition of glycogen synthase kinase (GSK)3β (11). A first clinical phase II trial in relapsed glioblastoma patients demonstrated that inhibition of CD95 signaling via the CD95L-binding protein APG101 in combination with radiotherapy prolongs patient survival, especially in patients with presence of CD95L in the tumor tissue (13). APG101 is a CD95L-binding Fc-fusion protein consisting of the extracellular domain of human CD95 and the Fc region of human IgG1. The molecule was designed to interfere with CD95-dependent signaling by direct binding to CD95L, thereby preventing CD95L/CD95 interaction and subsequent intracellular signaling (14). Ex vivo preinhibition of CD95 signaling on glioma cells results in reduced tumor engraftment rates in vivo following implantation of the preconditioned cells (11). Yet, the therapeutic mechanism behind the systemically applied drug APG101 may be more sophisticated: Apart from a direct effect on the tumor cells, it is conceivable that other components of the tumor microenvironment may respond to an interference with the CD95 pathway and may be involved in the response toward APG101. Tumor cells of various cancers actively induce the expression of CD95L on tumor-associated endothelial cells. The corrupted endothelial cells then form a unique barrier that selectively prevents an infiltration of cytotoxic T cells but allows an entry of T regulatory cells (15). Similarly, CD95L-mediated killing of tumor-infiltrating cytotoxic T cells as part of a “tumor counterattack model” (16) has been suggested for gliomas as well, but is still controversially discussed. It remains to be determined whether this model is really relevant for the efficacy of a systemic CD95L inhibition. Finally, radiotherapy may contribute to an invasive phenotype discussed. It remains to be determined whether this model is finally radiotherapy may contribute to an invasive phenotype discussed. It remains to be determined whether this model is

Materials and Methods

Cell culture and radiotherapy

The murine glioma cell line SMA-560 was provided by D.D. Bigner (Duke University School of Medicine, Durham, NC). The human glioma cell line NCH-89 and the murine glioma cell line NMA-34 were provided by A. Martin-Villalba (German Cancer Research Center, Heidelberg, Germany). All cells were maintained in DMEM containing 10% FCS and penicillin (100 IU/mL)/streptomycin (100 µg/mL). SMA-560 cells were tested for mycoplasma and cell contamination via Multiplex PCR-based amplification of contaminants. All cell lines were not passaged higher than 25 times. The primary glioblastoma-initiating cell (GIC) line S24 was established

Collagen invasion assay

The in vitro invasive properties of glioma cells were assessed in an xCELLigence Real-Time Cell-Analyzer DP system (RTCA; Roche Diagnostics). Following different pretreatments [20 µg/mL APG101 (batch 071215-02) for 1 hour; irradiation at 8 Gy; 5 ng/mL CD95L for 1 hour], glioma cells were harvested and added in quadruplicates at a total of 4 × 10⁴ cells to the chambers of the RTCA. Invasion was monitored in real-time for 24 hours, and growth curves were analyzed using the RTCA software version 1.2. The dimensionless cell index (Cl), derived as a relative change in measured electrical impedance caused by migrated cells, reflects the invasive behavior of the cells.

Matrigel invasion assay

Invasion of glioma cells in vitro after indicated pretreatments was assessed in Boyden chamber assays (BD Biosciences) as described previously (19).

Quantitative RT-PCR

qRT-PCRs were done as described in ref. 20. Primer sequences are provided in Supplementary Data.

Proliferation measurement

Proliferation was also assessed in the xCELLigence RTCA DP system (Roche Diagnostics) by pretreating the cells with either vehicle, CD95L (50 U/ml), APG101 (20 µg/mL), or both in combination and seeding 7,000 cells per well in quadruplicate. Growth curves were analyzed using the RTCA software 1.2. The dimensionless Cl is derived as a relative change in electrical impedance caused by proliferated cells.

Animal experiments

Experiments were performed according to the rules of the German Animal Welfare Act and were licensed by the Regierungspräsidium Karlsruhe (governmental authority, animal application number: 35-9185.81/G-91/10). The mouse has been shown previously to be a relevant species for pharmacological and toxicological investigations of APG101 (11).

Orthotopic syngeneic mouse model. Glioma cells (5 × 10³ murine SMA-560) were stereotactically implanted into the right brain hemisphere of deeply anesthetized VM/Dk mice (in-house breeding facility, German Cancer Research Center, Heidelberg, Germany) at a depth of 3 mm. Postsurgical care included the provision of pain medication.

Treatment protocols. Animals were divided into different experimental treatment groups as indicated (n = 14; n = 3/6 for histology/MRI and n = 11 for survival analysis). APG101 was administered twice weekly from postoperative days 5 to 20 by intraperitoneal injection at 25, 50, or 100 µg/kg bodyweight. Control animals received vehicle (PBS) intraperitoneal injections. Local cranial irradiation at 6 Gy was applied on postoperative day 4, using 15 MeV electrons from a standard Linac radiation source (Siemens).
Lymphocyte depletion was done by single intraperitoneal injections of anti-CD4 (clone GK1.5, 1 mg/mouse) and anti-CD8 (clone 2.43, 0.5 mg/mouse) antibodies (Bio X Cell) 2 days after implantation of tumor cells. Depletion efficacy was monitored via flow cytometric analysis of submandibular vein blood on days 5, 12, and 18 after postimplantation.

**Survival analysis.** Eleven mice per group were used for survival analysis according to Kaplan–Meier. Mice were sacrificed by an overdose of anesthetic at the onset of hind leg paraparesis or hemiparesis (grade 2 neurologic symptoms).

**MRI.** Tumor volume was determined by MRI from the T1 contrast-enhanced regions of the brain in 6 mice per treatment group on day 9, 14, and 18 after tumor cell inoculation. MRI was performed using a 1.5 Tesla whole-body MR scanner (Siemens Symphony) in combination with a radio-frequency small-animal coil for excitation and signal reception. Tumors were located on T2w turbo spins echo images (echo time (TE), 109 ms; relaxation time (TR), 4,000; field of view, 40 × 40 mm; matrix, 128; voxel size, 0.3 × 0.3 × 1 mm³). T1w imaging was performed using a high-resolution spin-echo sequence (TE, 600 ms; TR, 14 ms; field of view, 40 × 40; matrix, 192; voxel size, 0.2 × 0.2 × 1 mm³) 3 minutes after intraperitoneal administration of Gd-DTPA contrast agent (Magnevist, Bayer Schering Pharma, 0.5 mmol/mL).

**Histology and IHC.** For histologic assessments, 3 mice per group were sacrificed on postoperative day 19, brains were removed, cryosectioned (8 µm) and hematoxylin/eosin stained. Mapping of MMP activity was achieved by in situ gelatin zymography as described before (Supplementary Data; ref. 21). MMP activity on cryosections was quantified by measuring the median gray scale of the MMP-GFP fluorescence with ImageJ [Schneider, 2012 #30] and normalizing the arbitrary units to the untreated control images. The difference of fluorescence intensity was expressed in percent.

**Staining of APG101 in murine tumors.** Brain slices were fixed in 4% paraformaldehyde, washed, and blocked in PBS/0.5% goat serum/0.03% Tween 20. Afterward, slices were incubated with rabbit anti-human IgG-Alexa488 (A11013, Invitrogen) for 1 hour. Slices were washed twice afterward and mounted in Vectashield Mounting Medium with DAPI (Vector Laboratories). Pictures were acquired and quantified on a Zeiss Axio Observer Z1 (Carl Zeiss).

**Staining of CD95L on human brains.** Sections cut to 3 µm were incubated and processed on a Ventana BenchMark XT® immunostainer (Ventana Medical Systems). The sections were incubated with primary antibody at 37°C for 32 minutes. Incubation was followed by Ventana standard signal amplification, UltraWash, counterstaining with one drop of hematoxylin for 4 minutes, and one drop of bluing reagent for 4 minutes. For visualization, ultraView Universal DAB Detection Kit (Ventana Medical Systems) was used.

**Staining of CD95L on SMA-560 tumors.** SMA tumor sections (3 µm) fixed in 4% PFA were dewaxed followed by high temperature antigen demasking in 10 mmol/L citrate buffer pH 6.0 at 99°C for 25 minutes (Target Retrieval Solution, S1699 DakoCytomation GmbH), followed by incubation in blocking solution (Zytomed). Sections were incubated with rabbit CD95L antibodies (Dianova) diluted in antibody diluent (Zytomed) 60 minutes at room temperature, washed twice in PBS, incubated with Zytocohm Plus AP Polymer anti-rabbit (Zytomed) for 30 minutes at room temperature, and rinsed twice for 5 minutes in PBS. Thereafter, sections were incubated with substrate (Liquid Permanent Red, DakoCytomation GmbH). The reaction was stopped with dest. H₂O. The sections were counterstained with hematoxylin (Merck).

**Flow cytometric analysis.** T lymphocytes. Isolation of peripheral blood mononuclear cells (PBMC) was done as described before (22). PBMCs were isolated from submandibular vein blood by red blood cell lysis using 1 ml of ACK lysis buffer, containing 150 mmol/L NH₄Cl, 10 mmol/L KHCO₃ and 100 mmol/L Na₂EDTA, and incubation for 15 minutes. Cells were washed with PBS, blocked with rat anti-mouse CD16/32 (9, 0.5 µg/well, eBioscience), and stained with FITC-conjugated rat anti-mouse CD3 (17A2), pacific blue–conjugated rat anti-mouse CD4 (RM4-5, both BioLegend), and APC-conjugated rat anti-mouse CD8 (53-6.1, eBioscience; all 0.5 µL/well) and measured via a BD FACSCanto II (BD Biosciences).

**Apoptosis analysis.** Apoptosis of SMA-560 and Jurkat cells was measured by washing cells in Annexin V–binding buffer (Biozol Diagnostika) and containing with Annexin V–FITC (BioVision Inc.) and DAPI (Sigma-Aldrich) after treatment with CD95L (50 U/mL), APG101 (20 µg/mL), or both for 4 hours.

**Immunoblot.** Immunoblot was done as described before (18). Antibodies were rabbit anti-phospho-GSK3β (Ser9, Cell Signaling Technology), rabbit anti-GSK3β (27C10, Cell Signaling Technology), and goat anti-GAPDH (LAH1064, Linaris). Anti-phospho-GSK3β antibody was probed first. Anti-GSK3β antibody was probed after incubating membrane with stripping buffer (100 mmol/L HCl, 100 mmol/L β-mercaptoethanol) for 2 hours and verifying complete removal of anti–phospho-GSK3β antibody by probing with secondary HRP-labeled antibody only.

**Single-photon emission CT images.** APG101 (batch 080415-06) was labeled with 123I using the chloramine T method. Imaging was done at 4, 24, and 48 hours after intravenous tracer administration using a dual-head gamma camera system (GE Hawkeye) equipped with medium-energy collimators. Sixty-four 40-second projections were acquired over 360° using a 64 × 64 matrix. The radius of rotation was minimized according to the patient’s size. A zoom factor of 1.78 was chosen. Images were reconstructed by filtered back-projection using a Butterworth filter (FC = 0.5 Nyquist, order = 8). First-order attenuation correction (0.12/cm) was performed using the method described by Chang (23).

**MRI.** MRI examination was performed on 3T MR scanners (Magneton Trio, Siemens). The MRI protocol comprised a T1-weighted magnetization prepared rapid gradient echo (MPRAGE) sequence (TR = 1,900 ms, TE = 3.05 ms, TI = 900 ms, FOV = 172.5 mm, slice = 1 mm, reconstruction in the sagittal, coronal, and axial plane) before and after administration of...
0.1 mmol/kg of Gadovist (Bayer Schering) an axial T2-w TSE sequence (TR 2,500 ms, TE 100 ms, slice thickness 5 mm), and an axial FLAIR sequence (TR 8,000 ms, TR 120 ms, TI 120 ms).

Statistical analysis

In vitro data are expressed as mean ± SEM. Statistical significance was assessed by Student t test (two-tailed with unequal variance). Values of P < 0.05 were considered significant. All in vitro experiments were independently performed at least three times in triplicate or more. Animal survival data were plotted by the Kaplan–Meier method and analyzed by the log-rank test.

Results

APG101 enriches in the contrast-enhancing part of glioblastoma patients

In patients with glioblastoma, gene expression of CD95 correlated with a reduced overall survival (Fig. 1A). The very first clinical phase II trial targeting the CD95/CD95L signaling axis with the CD95L-binding fusion protein APG101 (Fig. 1B) recently proved that targeted inhibition of the CD95 pathway in glioblastoma patients prolongs survival and serves as a novel and promising treatment approach (13), which may be transferrable to other tumor diseases as well. The molecular weight of

Figure 1.

APG101 enriches in the contrast-enhancing part of a glioblastoma patient. A, Kaplan–Meier survival plot showing survival of 486 glioblastoma patients with low and upregulated CD95 expression levels according to queries of The Cancer Genome Atlas (TCGA). B, Schematic drawing of the molecular composition of the fusion protein APG101. C, SPECT images obtained 48 hours after application of 123I-APG101 in compassionate use patient 4 are depicted in axial slices showing pronounced and homogeneous tracer uptake in the fronto-parietal glioblastoma. D, SPECT-CT images of fusion images in axial, sagittal, and coronal direction and corresponding MRI taken at the same day reveal more widespread uptake of 123I-APG101 than enhancement of Gd-DTPA, which is only present at the outer, nonnecrotic tumor margins.
APG101 (not taking into account the glycosylation) is 84 kDa; given the size of APG101, it was questionable whether the molecule would cross the blood–brain barrier to enter the tumor tissue. By administering 123I-labeled APG101 for SPECT analysis in a patient with recurrent glioblastoma treated on compassionate use basis, APG101 enriched in the solid tumor (Fig. 1C). SPECT using 123I-labeled APG101 showed high tracer uptake with a predominant localization in the same parts of the tumor that demonstrated gadolinium uptake (Fig. 1D).

APG101 acts on human and murine glioma cells

To further narrow down the site and mode of action of APG101, we analyzed APG101’s efficacy in an immunocompetent mouse model. We were particularly interested in potential tumor cell–independent effects mediated by inhibition of CD95/CD95L–induced signaling in stromal cells. We thus probed the syngeneic astrocytoma cell line SMA-560 derived from VM/Dk mice (24). In vitro, stimulation with CD95L increases invasiveness of glioma cells, irrespective of human or murine origin. Importantly, because the human and murine receptor–ligand pairs are cross-reactive, this effect can be inhibited by cotreatment with APG101, independent of the host species (Fig. 2A). Stimulation of SMA560 cells, which are CD95 positive as determined by qPCR with CD95L in vitro leads to an inhibitory phosphorylation of GSK3β (ser9) of CD95L/APG101-treated SMA-560 cells. C, Boyden chamber invasion data of SMA-560 expressed as percentages of invaded cells, treated as indicated, relative to untreated control cells set to a 100% (mean ± SD, representative results for n = 3). D, Flow cytometric analysis of DAPI/Annexin-FITC stained SMA-560 cells 4 hours after CD95L treatment. E, Proliferation of SMA-560 following treatment with CD95L and/or APG101. n = 3, one representative example shown.

Survival of glioma-bearing mice is prolonged by APG101

Next, different APG101 doses were studied in vivo. Cranial MRI-based tumor volumetry revealed a dose-dependent inhibition of tumor growth with maximal effects shown for 100 mg/kg body-weight APG101 (Fig. 3A and B). Importantly, APG101 treatment also resulted in a dose-dependent survival advantage of up to 18 days at 100 mg/kg APG101 (P < 0.05) over control-treated mouse models.
mice (Fig. 3C). Tissue analyses of APG101-treated tumors confirmed that APG101 enriched within the murine tumor and the surrounding brain (Supplementary Fig. S1A and S1B). Importantly, in situ zymography revealed a marked reduction in activity of invasion-related MMP at 100 mg/kg (Fig. 3D). This indicates inhibition of CD95 signaling by APG101 in the tumor microenvironment is resulting in profound inhibition of glioma-derived MMP production as a potential mode of action. Accordingly, freshly prepared human microglial cells showed a marked change in MMP gelatinolytic activity independent from \textit{mmp-2} or \textit{mmp-9} mRNA expression in this ex vivo model (Supplementary Fig. S2A–S2D).

**Efficacy of APG101 is independent of tumor-infiltrating T lymphocytes**

Previous studies suggested a tumor-specific expression of CD95L on endothelial cells, which is related to a selective infiltration of T lymphocytes in different tumors that in turn affects tumor growth (15). We identified CD95L-expressing endothelial cells also in human and murine glioblastoma samples (Fig. 4A and B). Hence, the question arose whether expression of CD95L in glioblastomas likewise affects T-cell infiltration. In vitro APG101 is indeed capable of preventing CD95L-triggered apoptosis of T cells (Supplementary Fig. S3). To address the relevance of tumor-infiltrating T lymphocytes for the efficacy of APG101 in vivo, we depleted CD4 and CD8-positive cells in VM/Dk mice using anti-murine CD4/anti-murine CD8 antibodies. Single treatment with anti-CD4 and anti-CD8 antibodies caused a depletion of respective T-cell populations 24 hours after treatment that lasted for at least 20 days. In the next step, we depleted T cells in VM/Dk mice 72 hours after intracranial implantation of syngeneic SMA-560 tumor cells. The experimental procedure is detailed in Fig. 4C. Sustained T-cell depletion was confirmed via FACS analysis on days 5, 12, and 18 after tumor cell engraftment (Fig. 4D). Nevertheless, MRI-based measurements of tumor volumes did not reveal a relevant reduction of the efficacy of APG101 after depletion of T cells (Fig. 4E).

**Figure 3.** APG101 inhibits tumor growth and prolongs survival in glioma-bearing mice. A, Comparative T1-weighted MRI based volumetry of murine SMA-560 tumors (left; mean ± SD, \(n = 6\)) and histologic analysis of coronally cryosectioned and hematoxylin/eosin-stained mouse brains (right) of untreated control and APG101 (25, 50, 100 mg/kg bodyweight) treated VM/Dk-mice on postoperative days (POD) 9, 14, and 18 (scale bar, 1 mm). B, Representative T1-weighted, contrast agent-enhanced MRI images of orthotopically implanted SMA-560 glioma in five different planes. Tumors appeared as hyperintense areas within the brain (scale bar, 5 mm). C, Survival data of glioma-bearing mice treated twice weekly intraperitoneally as indicated are plotted by the Kaplan–Meier method and analyzed by log-rank test (\(n = 11\); treatment group; \(\hat{P} < 0.05\)). D, Mapping of MMP activity by in situ gelatin zymography using cryopreserved brain slices of tumor-bearing VM/Dk-mice (untreated or treated with 100 mg/kg). Inhibition of MMP activity by EDTA served as a negative control; fluorescent counterstaining of cell nuclei was carried out by using 0DAPI (scale bar, 50 μm). The difference of MMP-GFP fluorescence to control animals is expressed in percent (%).
APG101 enhances the efficacy of radiotherapy

Because radiotherapy, as a fundamental component of glioblastoma therapy, induced the mRNA expression of proinvasive CD95L (Supplementary Fig. S4), inhibition of CD95/CD95L signaling with APG101 was combined with radiotherapy in a clinical paradigm. SMA-560 murine astrocytoma-bearing VM/Dk mice were treated with radiotherapy at 6 Gy in a single fraction, which corresponds to a clinically meaningful fractionated dose and APG101 at 100 mg/kg twice weekly intraperitoneally or with either treatment alone. Both administration of APG101 alone and treatment with APG101 in combination with irradiation resulted in a significant reduction of tumor growth rates compared with irradiation treatment alone (Fig. 5A). In addition, neurologic symptom-free survival of tumor-bearing VM/Dk mice, which received APG101 in addition to radiotherapy, was markedly increased compared with radiotherapy alone (Fig. 5B). In line with previous experiments in other glioma cell lines (17) and clinical observations, SMA-560-derived tumors exhibit increased invasiveness and formation of tumor satellites upon radiation exposure, which was confirmed on MRI and by histologic examinations (Fig. 5C). Importantly, treatment with either APG101 alone or in combination with radiotherapy and APG101 prevented the formation of these infiltrative tumor satellites. Moreover, APG101 treatment reduced baseline and radiation-enhanced MMP activity (Fig. 5D). In addition, no adverse effects were observed in the combined treatment group.

**Discussion**

CD95 (APO-1/Fas) is a prototype transmembrane death receptor of the TNF superfamily, which regulates tissue homeostasis mainly in the immune system by induction of caspase-dependent apoptosis (6, 7). Extracellular binding of its ligand CD95L leads to recruitment of several adaptor proteins into the DISC in the cytoplasm of target cells. These findings stimulated efforts to induce cell death in gliomas and other tumors by various CD95 stimuli, recently by an agonistic super-CD95 ligand (25). However, many cancer cells are resistant toward CD95-mediated apoptosis (26). Also, systemically used CD95-activating antibodies caused severe side effects through induction of apoptosis of hepatocytes in the liver (27).

Several lines of evidence indicate that CD95 also transmits nonapoptotic signals. Depending on tissue type and conditions, nonapoptotic functions of the CD95 receptor/ligand
system comprise, for example, liver regeneration, neuronal development, inflammation, and even promotion of tumorigenicity (8, 28, 29): In various tumors, CD95 activity is associated with a prosurvival and/or migratory signaling summarized in ref. 30 and a knockdown of either CD95 or its ligand reduced tumor growth rates in vitro as well as in xenografted mice (8). Likewise, for glioma, induction of apoptosis by CD95 was restricted to long-term cultures and has never been achieved in clinically relevant models (31). In contrast, activation of nonapoptotic CD95 signaling pathways led to invasive glioma growth. Upon CD95L binding, activation of the Src family member Yes and PI3K resulted in the formation of a PI3K activation complex and subsequent phosphorylation of GSK3β resulting in enhanced expression of pivotal glioblastoma invasion-related MMPs (11). The shift of paradigm to inhibit rather than to stimulate CD95 signaling in glioma was further supported by in vitro as well as in vivo findings showing a significant inhibition of glioma cell migration and invasion by using a preclinical CD95L-neutralizing antibody (11, 32). The molecule designed for clinical application (14) was termed APG101 (asunercept; Fig. 1B). After phase I data in healthy volunteers did not show any relevant adverse effects (14), a phase II clinical trial in patients with first and second recurrences of a glioblastoma demonstrated that combining a second radiotherapy with weekly infusions of APG101 resulted in an increased progression-free survival compared with the group of patients that received radiotherapy only. Also, survival was prolonged specifically in the group with lower methylation level at the CpG2 site in the CD95L promoter (13).

In the current study, there was a predominant location of APG101 in the tumor after treatment of patients with intravenous infusions illustrating that APG101 reached its target. The distribution of APG101 might in part be explained by disruption of the blood–brain barrier, but the more widespread

Figure 5.
APG101 enhances the efficacy of radiotherapy. A, Left, orthotopic growth of SMA-560 glioma in VM/Dk mice treated as indicated (XRT, radiotherapy) monitored by T1-weighted MRI-based volumetry on postoperative days (POD) 9, 14, and 18. Mean tumor volume in each group on POD 9 was set to a 100% (mean ± SD, n = 6/treatment group; *, P < 0.05). Right, representative T1-weighted MRI scans of SMA-560 glioma in three different sectional planes on POD 18 after indicated treatments. The core tumor is marked by an asterisk, and the infiltrating part of the glioma is highlighted by the cross symbol (scale bar, 5 mm). B, Kaplan–Meier plot of glioma-bearing mice treated twice weekly intraperitoneally as indicated (log-rank test; n = 11/treatment group; *, P < 0.05). C, Top, representative cranial T1-weighted MR images of SMA-560 tumor-bearing mice 18 days after focal radiation at 6 Gy alone (XRT), after intraperitoneal administration of 100 mg/kg APG101 or after a combination treatment (APG101 + XRT). The hyperintense bulky core tumor is marked by an asterisk, and the infiltrating less intense part of the glioma is highlighted by the cross symbol (scale bar, 3.5 mm). Bottom, representative images of coronally cryosectioned and hematoxylin/eosin-stained tumors after indicated treatment. Arrows mark radiation-induced tumor satellite formation (scale bar, 1 mm). D, Mapping of MMP activity by in situ gelatin zymography using cryopreserved brain slices of tumor-bearing VM/Dk mice, untreated or treated with radiotherapy at 6 Gy and APG101 at 100 mg/kg or with either treatment alone. Fluorescent counterstaining of cell nuclei was carried out by using DAPI (scale bar, 50 μm). The difference of MMP-GFP fluorescence to control animals is expressed in percent (%).
Although APG101 blocked CD95L-dependent apoptosis of T cells in vitro (Fig. 2A), which has been confirmed in other glioma cell lines employing 3D in vitro and organotypic tissue culture models (33).

We further demonstrate that APG101 blocks CD95L-driven glioma cell invasiveness of human as well as of murine cells in vitro (Fig. 2A), which has been confirmed in other glioma cell lines employing 3D in vitro and organotypic tissue culture models (33).

Interestingly, in murine SMA-560 cells expressing CD95 only in low levels, in vitro (11) activation of CD95 by CD95L was neither translated in a proapoptotic nor proproliferative or proinvasive signal, although GSK3β was phosphorylated at serine 9. This argues for additional tumor micromilieu-derived factors necessary to activate the CD95 axis or a dose-dependent effect preventing an effective activation of the CD95 pathway in vitro due to weak CD95 expression or insufficient GSK3β inhibition. APG101 blocks glioma cell invasiveness also in vivo (Fig. 3) and prolongs the survival of tumor-bearing mice in a dose-dependent way (Fig. 3C). To mimic the patient situation more closely, we decided to use an immunocompetent model consisting of VM/Dk mice and the derived syngeneic tumor cell line SMA-560 (24). These tumors do not only grow invasively, but are sensitive to sublethal radiation dosages. They also show an increase of invasive growth following radiotherapy in vivo (Fig. 5), which has been likewise observed in glioblastoma patients (17). The latter appears to be in part dependent on an increase of CD95L expression following radiotherapy (Supplementary Fig. S4). This irradiation-induced proinvasive phenotype caused by enhanced metalloproteinase activity was subsequently successfully attenuated in our animal studies by the administration of APG101 (Fig. 5D). Importantly, APG101 treatment also led to a reduction of radiation-induced tumor satellite formation and reduced tumor growth also in combination with radiotherapy (Fig. 5A and C), in line with an increased survival time (Fig. 5B). As proposed for nonradiotherapy stimulated invasiveness, APG101 reduced radiotherapy-enhanced gelatinase activity (Fig. 5D), which is seen also in microglial cells (Supplementary Fig. S2D). No adverse effect on the beneficial effect of radiotherapy was observed in mice. We conclude that APG101 can efficiently counteract the increased invasive potential and tumor satellite formation of glioma cells triggered by radiation treatment.

Apart from auto-/paracrine CA9 signaling between tumor cells, expression of CA9 by tumor cells as a mode of "counterattack" against infiltrating immune cells with antitumor activity has been controversially discussed (34). In glioblastomas, tumor-infiltrating lymphocytes are abundantly present, but not related to clinical outcome (35). The counterattack concept has recently been refined for many tumors, although not yet for glioblastomas, by the discovery of tumor endothelium-bound CA9 in murine and human tumors, which constitutes a selective barrier and induces apoptosis in effector killer T cells but allows the infiltration of regulatory T cells (15). In the current work, we confirmed glioma endothelium-bound CA9 in human and murine brain tumors (Fig. 4A and B). Considering the proposed barrier function of CA9, we interrogated whether the presence of T cells is of relevance for the antitumor efficacy of APG101. Although APG101 blocked CA9-dependent apoptosis of T cells in vitro (Supplementary Fig. S3), depletion of CD4 and CD8-positive cells in vivo did not diminish the efficacy of APG101 (Fig. 4E). Moreover, we found APG101 to be well distributed among the murine tumors and brain following application and not restricted to the tumor endothelium (Supplementary Fig. S1). Together with APG101’s ability to block MMP activity (Figs. 3D and 5D; Supplementary Fig. S2D) and tumor cell invasiveness (Figs. 2A and 5C), we propose that in gliomas, APG101 is mainly acting directly on the tumor cells and the resident microglia, and its efficacy is probably less dependent on additional lymphocytic cell populations but microglial cells of the tumor microenvironment in the analyzed models. This finding is especially relevant if APG101 is considered to be administered in combination with alkylation chemotherapy in the future, which may cause lymphopenia (36), but may not affect the efficacy of APG101. Thus far, the development has been focused on the combination of APG101 with radiotherapy and might continue with this concept by focusing on patients with glioblastoma harboring an unmethylated MGMT promoter, a large patient group with a so far insurmountable resistance to any medical treatment. However, integrating temozolomide at some stage into the development may be warranted.

Taken together, our findings support the concept of APG101 acting directly on the tumor cells in vivo but also exert relevant activity on the microenvironment, namely microglia, relating the therapeutic efficacy of APG101 mainly to its ability to inhibit tumor cell invasiveness. Moreover, APG101 overcomes radiotherapy-associated invasiveness, which complements the clinical implementation of a combinatorial treatment scheme consisting of radiotherapy and APG101 in the treatment of glioblastoma patients at recurrence (13), which is currently promoted to the first-line therapy in the NCT Neuro Master Match (N2M2) trial (4) aiming to excel the prognosis for patients with unmethylated O6-methylguanine-DNA methyltransferase promoter (37) and therefore unlikely to benefit from the currently established standard of care consisting of irradiation and chemotherapy with temozolomide (1).

**Disclosure of Potential Conflicts of Interest**

P.-N. Pfennig is a senior consultant at HCL Technologies. J. Sykora is the head of histology at Apogenix AG. C. Mez is the head of cellular analytics at Apogenix AG. C. Kunz is a director (Clinical Development) at Apogenix AG. M. Kluge is the preclinical head at Apogenix AG. M. Platten reports receiving a commercial research grant from Bayer and other commercial research support from Pfizer/Merck, has received speakers bureau honoraria from Medac, and has ownership interest in patents. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

**References**


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