Therapeutic Targeting of TGFβ Ligands in Glioblastoma Using Novel Antisense Oligonucleotides Reduces the Growth of Experimental Gliomas

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Abstract

Purpose: Transforming growth factor (TGF)-β is expressed at high levels by glioma cells and contributes to the malignant phenotype of glioblastoma. However, its therapeutic targeting remains challenging. Here, we examined an alternative therapeutic approach of TGFβ inhibition using two novel phosphorothioate-locked nucleic acid (LNA)-modified antisense oligonucleotide gapmers, ISTH1047 and ISTH0047, which specifically target TGFβ1 and TGFβ2.

Experimental Design: We characterized the effects of ISTH1047 and ISTH0047 on TGFβ1/2 expression, downstream signaling and growth of human LN-308, LN-229, and ZH-161 cells as well as murine SMA-560 glioma cells in vitro. Furthermore, we assessed their target inhibition and effects on survival in orthotopic xenogeneic and syngeneic rodent glioma models in vivo.

Results: Both antisense oligonucleotides specifically silenced their corresponding target and abrogated SMAD2 phosphorylation in several glioma cell lines. Moreover, inhibition of TGFβ1 or TGFβ2 expression by ISTH1047 or ISTH0047 reduced the migration and invasiveness of LN-308 and SMA-560 glioma cells. Systemic antisense oligonucleotide administration to glioma-bearing mice suppressed TGFβ1 or TGFβ2 mRNA expression as well as the expression of the downstream target PAI-1 in orthotopic gliomas. Glioma-bearing mice had significantly prolonged survival upon systemic treatment with ISTH1047 or ISTH0047, which was associated with a reduction of intratumoral SMAD2 phosphorylation and, in a fully immunocompetent model, with increased immune cell infiltration.

Conclusions: Targeting TGFβ expression with the novel LNA antisense oligonucleotides ISTH1047 or ISTH0047 results in strong antiglioma activity in vitro and in vivo, which may represent a promising approach to be examined in human patients with glioma.

Introduction

Glioblastoma is the most lethal primary tumor of the central nervous system in adults. The current standard of care for newly diagnosed glioblastoma includes surgical resection followed by radiotherapy and chemotherapy with the alkylating agent temozolomide (TMZ). However, the median survival of patients with glioblastoma is still limited to approximately 16 months with a 5-year survival rate of less than 10% within clinical trials (1). A key mediator of the malignant phenotype of glioma cells is transforming growth factor (TGF)-β. It has pleiotropic functions and exists as three different mature ligands, TGFβ1, TGFβ2, and TGFβ3 (2). All are involved in the phosphorylation of SMAD2 and SMAD3 proteins, which are responsible for transcriptional regulation of genes involved in tumor infiltration and epithelial-to-mesenchymal transition, but also immunosuppression and angiogenesis (3, 4). Elevated levels of TGFβ as well as the activation of the TGFβ/SMAD cascade correlate with high tumor grade and in some cases with poor prognosis in patients with glioblastoma (5, 6). Gliomas generate an immunosuppressive tumor microenvironment through the secretion of a variety of cytokines, including TGFβ that can impede antitumor immune responses (3). Furthermore, TGFβ signaling enhances the invasiveness and migratory ability of glioma cells and maintains a drug-resistant stem cell phenotype (7, 8). Consequently, targeting the TGFβ pathway represents an attractive therapeutic approach. In this regard, various strategies have been explored in vitro and in vivo, including gene transfer of TGFβ scavengers such as decorin (9), furin protease inhibitors, which interfere with the processing of TGFβ (10) or indirect TGFβ signaling targeting through integrin inhibition (11). The most advanced approaches comprise TGFβ receptor (TGFβR)-1

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Antisense oligonucleotides (ASOs) are single-stranded, biochemically modified deoxyribonucleotide molecules that are 15 to 25 nucleotides in length. They are designed as complementary sequences to a target gene’s mRNA, thereby specifically inhibiting gene expression (18). The concept of ASO-mediated gene silencing has emerged as a potentially powerful alternative or concomitant treatment to conventional cancer therapy.

There is a plethora of genes related to cancer progression or therapeutic resistance that cannot be targeted efficiently with antibodies or small molecules but possibly with ASOs. Because of their targeted mode of action, ASOs may be less toxic than many conventional antitumor therapeutics (19). One of the most clinically advanced TGF-β-targeting approaches involved the locoregional application of AP12009 (trabedersen), a generation ASO with a sequence complementary to human TGF-β2 (ISTH0047), targeting TGF-β2 (sequence TCGATGCGCTTCCG), and ISTH0047, targeting TGF-β3 (sequence CAAAGTATTTGGTCTCC), represent 14-mer and 17-mer, full LNA-modified ASO pampers (Supplementary Fig. S1). The specificity of each ASO was confirmed via the NCBI BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to align oligonucleotides to human and murine TGF-β1, or TGF-β3, sequences (Supplementary Fig. S2). Digoxigenin (DIG)-labeled ISTH01047 was also provided by ISARNA Therapeutics GmbH. Recombinant human TGF-β1/2 was purchased from R&D Systems. SD-208 was provided by Scios and galunisertib (LY2157299) by Eli Lilly Co.

Real-time quantitative PCR

Total RNA was isolated with the NucleoSpin System (Macherey-Nagel) and cDNA was retrotranscribed using Invitrogen Superscript II reverse transcriptase (ThermoFisher Scientific). For real-time quantitative PCR, cDNA amplification was quantified using SYBR Green chemistry on the 7300 Real-time PCR System (Applied Biosystems). The conditions for the PCR reactions were 40 cycles, 95°C/15 seconds, 60°C/1 minute. Human and mouse HPRT1 transcript levels were used as a reference for relative quantification of mRNA expression levels via the △CT method (25). The following specific primers were used: human HPRT1 fwd: 5'-TGA GGA TTT TGT AAG GGT GT-3'; human HPRT1 rev: 5'-GAG CAC ACA GAC GGC TAC AA-3'; human TGFβ1 fwd: 5'-GCC CTG TGG CTT CTA GTG C-3'; human TGFβ1 rev: 5'-CGT GTC CAG GCT CCA AAT G-3'; human TGFβ2: 5'-AACTACA CTG TCC CTT GCT G-3'; human TGFβ3: 5'-TGT GGA GGT GCC ATC AAC TCT T-3'; human TGFβ3: 5'-ATG ACC AAC TGC CCC TAT CA-3', human TGFβ4: 5'-AGG ACA GCC ACT TGC TGT TG-3'; mouse HPRT1 fwd: 5'-TGT CTG ACC TGC TGT AGT AC-3'; mouse HPRT1 rev: 5'-TTG ATG TCC CCC GTT GAC TGC-3'; mouse TGFβ1 fwd: 5'-TTT ATG TCC CCC GTT GAC TGC-3'; mouse TGFβ1 rev: 5'-GCC CTG TGG CTT CTA GTG C-3'; mouse TGFβ2: 5'-GCC TTA GTT TGG ACA GGA TCT G-3'; mouse TGFβ2 fwd: 5'-TCT ACA TGG CTT GCT G-3'; mouse TGFβ2 rev: 5'-CCC TGG TAC TGT TGT AGA TGG A-3'; mouse TGFβ3 fwd: 5'-AGCATCCACTGTCCATGCA-3', mouse TGFβ3 rev: 5'-TTTCTCTTCCTGTACCCGCGT-3'; mouse plasminogen activator inhibitor (PAI)-1: 5'-GTC GGG AAA GGG TTT ACT TTA CC-3', mouse plasminogen activator inhibitor (PAI)-1 rev: 5'-GAC ACG CCA TAG GGA GAG AAG-3'. For expression inhibitors such as SD-208 (12), galunisertib (13), or LY2109761 (14) and the pan-TGFβ antibody 1D11 (15). Despite promising preclinical data, these agents failed to show a clear clinical benefit in patients with glioblastoma (16, 17). Therefore, the clinical development of most of these drugs has been stopped because of the lack of activity, most likely due to insufficient target inhibition or dose-limiting toxicity (16).

Translational Relevance

Transforming growth factor (TGF)-β has been attributed an important role in glioblastoma progression and may be a key player in tumor invasion, angiogenesis, and immunosuppression. Targeting the TGFβ pathway has been regarded a promising therapeutic strategic, but the approaches used to date have failed due to insufficient inhibition of the TGFβ pathway or dose-limiting toxicity. In this study, we directly inhibited TGFβ1 or TGFβ2 expression with novel second-generation antisense oligonucleotides (ASOs). We demonstrate that ASO-mediated TGFβ inhibition reduces the invasive potential of glioma cells, while increasing the number of tumor-infiltrating immune cells. These novel TGFβ-targeting ASOs reach the tumor upon systemic administration and prolong survival of both immunocompetent and immune-deficient mice bearing orthotopic gliomas. Therefore, this study represents a major improvement in TGFβ-targeted therapy, with high promise for clinical translation in human patients with glioma.

Materials and Methods

Reagents and cell lines

The human malignant glioma cell lines LN-308 and LN-229 were kindly provided by Dr. N. de Tribolet. The mouse glioma cell line SMA-560 was derived from a spontaneous astrocytoma in a VM/Dk mouse (23) and provided by Dr. D. Bigner. The glioma cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Biochrom) and 2 mmol/L L-glutamine (Gibco Life Technologies). The glioma-initiating cell (GIC) line ZH-161 was established after informed consent and approval of the Swiss ethical committees and characterized in detail (24). ZH-161 cells were grown in Neurobasal medium supplemented with 2 μl/ml µM B-27 without vitamin A, 2 mmol/L L-glutamine (Gibco Life Technologies), fibroblast growth factor (FGF)-2, and epidermal growth factor (EGF; 20 ng/ml each, PeproTech). All cells were grown in a humidified 37°C incubator with 5% CO2.

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analysis of human targets in the oligonucleotide-treated xenograft mouse model, the following human-specific primers, which do not recognize the mouse TGFβ1, TGFβ2, or TGFβ3 isoforms, were used: TGFβ1 fwd: 5'-CAATTCCCTGGC-GATACCTCAG-3'; TGFβ3 rev: 5'-GCCAACACTCGGTGAGCAT-CAA-3'; TGFβ2 fwd: 5'-CAGGACTCAGTCGACCTTTGGA-3'; TGFβ2 rev: 5'-CCTCCGGCTCAGGATGCTT-3'; TGFβ3 fwd: 5'-AACCGGTAGTACCCCCAGC-3'; TGFβ3 rev: 5'-CCGACT-CGGTGTTTCTCCTG-3'; PAI1 fwd: 5'-CAGAAAATGGAAGATCGAGGTTGAAC-3'; PAI1 rev: 5'-GGAGGCTCTGTTCCATGGA-3'.

Immunoblot

Whole protein lysates were generated by lysing the cells with lysis buffer P (26). Denatured whole protein lysates and concentrated supernatants (30 μg/lane) were separated on 10% to 15% acrylamide gels. After transfer to nitrocellulose (Bio-Rad), blots were blocked in 5% milk-TBST and incubated overnight at 4°C with primary antibodies specific for total SMAD2 (clone 86F7, Cell Signaling Technology), phosphorylated SMAD2 (pSMAD2; clone 138D4, Cell Signaling Technology), total SMAD3 (Cell Signaling Technology), phosphorylated SMAD3 (pSMAD3; clone C25A9, Cell Signaling Technology), total AKT (Cell Signaling Technology), phosphorylated AKT (pAKT; Cell Signaling Technology), and β-actin (clone sc-1616, Santa Cruz Biotechnologies). The membranes were then washed in TBST and incubated for 1 hour at room temperature with horseradish peroxidase (HRP)-coupled secondary antibodies: anti-rabbit or anti-goat (clones sc-2004 and sc-2033, Santa Cruz Biotechnologies). Protein bands were visualized by enhanced chemiluminescence (Pierce/Thermo Fisher).

Animal studies

All procedures were performed in accordance with the Cantonal Veterinary Office Zurich and Federal Food Safety and Veterinary Office. For the xenograft models, Crl: CD1 Foxn1 nude mice were purchased from Charles River Laboratories. For the syngeneic studies, VM/Dk mice were bred in house. Mice of 6 to 10 weeks of age were used in all experiments. Mice were anesthetized using an intraperitoneal three-component injection consisting of fentanyl, midazolam, and medetomidin (26). For intracranial tumor cell implantation, the mice were fixed under a stereotactic device (Stoelting) and a burr hole was drilled in the skull 2-mm lateral and 1-mm posterior to the bregma. A Hamilton syringe needle was introduced to a depth of 3 mm. LN-308, LN-229 or ZH-161 human glioma cells (10^5), or SMA-560 murine glioma cells (5 × 10^3) in a volume of 2-μL PBS were injected into the right striatum of Crl: CD1 Foxn1 nude or VM/Dk mice, respectively. C3_ISTH0047 (20 mg/kg), ISTH1047 (10 mg/kg), or ISTH0047 (20 mg/kg) were injected subcutaneously for five consecutive days and then twice weekly. Mice were monitored daily for neurologic symptoms according to Cantonal Veterinary Office Zurich guidelines and euthanized when exhibiting grade 2 symptoms: grade 0, no visible impairment; grade 1, reduced activity, slight imbalance, balance and coordination impairments; grade 2, decreased to no activity, 15% weight loss compared with peak weight measured on tumor inoculation day, slight paralysis of left legs and moderate signs of pain (26). Treatment was started at day 5 after tumor cell inoculation for VM/Dk mice and at day 25 (LN-308) or day 10 (LN-229, ZH-161) after tumor cell injection for Crl: CD1 Foxn1 nude animals. For the assessment of oligonucleotide delivery to the brain, digoxigenin (DIG)-labeled ISTH1047 (10 mg/kg) was subcutaneously injected for two consecutive days and the mice were PBS perfused 48 hours after the last injection. For liver enzyme analyses, blood was collected from LN-308-bearing mice on the day the first animal became symptomatic and sent for aspartate transaminase (AST) and alanine transaminase (ALT) quantification at the Laboratory for Veterinary Medicine.

Statistical analysis

Data were derived from experiments performed at least twice in triplicates with similar results. Quantitative data are presented as mean ± standard deviation (SD). Statistical significance was assessed using one-way ANOVA using Bonferroni correction as a post hoc test. All statistical analyses were performed using Prism 5 (GraphPad Software) at P < 0.05 or P < 0.01. Additional methods are described in the Supplementary data.

Results

ISTH1047 and ISTH0047 decrease TGFβ1/2 expression and abrogate TGFβ signaling in glioma cells in vitro

Based on the chosen sequence, ISTH1047 and ISTH0047 were designed as ASOs specifically inhibiting TGFβ1 or TGFβ2 (Supplementary Figs. S1 and S2). First, we examined the biological effects of both oligonucleotides in human LN-308 and mouse SMA-560 glioma cells in vitro. ISTH1047 downregulated TGFβ1 protein levels up to 96 hours after transfection in both cell lines, whereas ISTH1047 reduced TGFβ2 protein levels for up to 96 hours in LN-308 cells and 72 hours in SMA-560 cells (Fig. 1A and B). Increasing concentrations of both oligonucleotides reduced the expression of their corresponding TGFβ ligand target at mRNA level, reaching the highest inhibition efficiency at 50 nmol/L or more in both cell lines (Supplementary Fig. S3A and S3B). Moreover, TGFβ1 or TGFβ2 mRNA levels were significantly reduced up to 72 hours after transfection in human LN-308, LN-229, and ZH-161 glioma cells as well as the murine glioma cell line SMA-560. TGFβ1 mRNA levels were not significantly affected after ASO transfection in LN-308 or SMA-560 cells, but ISTH0047 exposure increased TGFβ2 levels in LN-229 cells and reduced TGFβ2 mRNA levels in ZH-161 cells (Fig. 1C). We observed a reduction of intracellular SMAD2 phosphorylation with increasing concentrations of ISTH1047 or ISTH0047 in LN-308 and SMA-560 cells. ISTH1047 diminished pSMAD2 phosphorylation at 50 nmol/L, whereas ISTH0047 exerted strongest effects at 100 nmol/L (Supplementary Fig. S3D). Total SMAD2 levels were not altered upon ISTH1047 or ISTH0047 transfection in either cell line (Supplementary Fig. S3C and S3D). The decrease of pSMAD2 levels occurred at 48 hours after transfection (Fig. 1D). Similarly, pSMAD3 levels were reduced in both LN-308 and SMA-560 cells. To assess the effect of TGFβ1 or TGFβ2 inhibition on alternative TGFβ signaling pathways, we examined the phosphorylation levels of AKT 48 hours after ASO exposure. pAKT was reduced.
Figure 1.
ISTH1047 and ISTH0047 interfere with TGFβ signaling. A and B, LN-308 (A) or SMA-560 (B) glioma cells were exposed to ISTH1047 or ISTH0047 at 50 nmol/L for 24 hours and TGFβ1/2 protein levels in the cell culture supernatant were determined by ELISA at 48, 72, or 96 hours after transfection. C, LN-308, LN-229, ZH-161, or SMA-560 glioma cells were exposed to ISTH1047 or ISTH0047 at 50 nmol/L for 24 hours and TGFβ1/2/3 mRNA levels were assessed by RT-PCR 48 hours after transfection. D and E, LN-308 or SMA-560 glioma cells were exposed to ISTH1047 or ISTH0047 at 50 nmol/L for 24 hours. pSMAD2 (D), pSMAD3, total SMAD3, pAKT, and total AKT (E) levels were assessed by immunoblot at 48 hours after transfection. F, LN-229 or ZH-161 glioma cells were exposed to ISTH1047 or ISTH0047 at 50 nmol/L for 24 hours. pSMAD2, total SMAD2, pSMAD3, total SMAD3, pAKT, and total AKT levels were assessed by immunoblot at 48 hours after transfection. C3 ISTH0047 at 50 nmol/L was used as scrambled sequence oligonucleotide control (Ctrl). Exogenous TGFβ1/2 (5 ng/mL) was used to induce pSMAD2 in ZH-161. The TGFβ receptor I kinase inhibitor SD-208 (1 μmol/L) was used as an additional control for TGFβ pathway inhibition. Results are expressed as means and SD (n = 3), statistical analysis was performed with one-way ANOVA and Bonferroni post hoc testing (*, P < 0.05; **, P < 0.01).
following exposure to ISTH1047 or ISTH0047 only in LN-308 cells (Fig. 1E). In line with these findings, exposure to ISTH1047 or ISTH0047 reduced pSMAD2 and pSMAD3 expression in LN-229 cells and the GIC line ZH-161. No consistent effect of ASO-mediated TGF\(_{\beta}\)-inhibition on pAKT levels was observed in these cells (Fig. 1F).

Oligonucleotide-driven TGF\(_{\beta}\) inhibition impairs glioma cell migration and invasion

We went on to examine the biological effects of ISTH1047 and ISTH0047 on the viability and clonogenicity of glioma cells. A concentration of 50 nmol/L of either oligonucleotide alone had no impact on cell viability (Supplementary Fig. S4A and S4B). Moreover, in clonogenic survival assays, transfection with either oligonucleotide had no effect in LN-308 cells, although ISTH1047 reduced colony formation of SMA-560 cells (Supplementary Fig. S4C and S4D). Migration and invasion into the surrounding tissue can be induced by TGF\(_{\beta}\) and are among the hallmarks of glioblastoma. Exposure to ISTH1047 reduced migration of LN-308 and SMA-560 cells in Transwell-Boyden chamber assays. A similar inhibition of glioma cell migration was observed with ISTH0047. Conversely, addition of exogenous TGF\(_{\beta}1/2\) resulted in increased glioma cell migration (Fig. 2A and B; Supplementary Fig. S5). To assess whether the two oligonucleotides impair invasiveness of glioma cells, we performed spheroid invasion assays into a three-dimensional collagen I gel. Spheroid invasion was significantly reduced following TGF\(_{\beta}1\)- or TGF\(_{\beta}2\)-specific oligonucleotide transfection for up to 96 hours in LN-308 cells, whereas exogenous TGF\(_{\beta}1\) stimulation increased invasiveness at 96 hours (Fig. 2C; Supplementary Fig. S6A). In SMA-560 cells, ISTH1047 transfection blocked spheroid formation in agar. Targeting TGF\(_{\beta}2\) using ISTH0047 led to a significant reduction in spheroid invasion, whereas stimulation with exogenous TGF\(_{\beta}2\) increased the invasion of SMA-560 cells (Fig. 2D; Supplementary Fig. S6B).

TGF\(_{\beta}1/2\) inhibition in murine glioma models in vivo

Because of the strong anti-TGF\(_{\beta}\) activity of the two ASO molecules observed in vitro, we aimed at exploring target down-regulation in vivo using the xenogenic LN-308 as well as the syngeneic SMA-560 model. To this end, we determined whether systemically administered ISTH1047 or ISTH0047 reach the tumor site and inhibit TGF\(_{\beta}1\) or TGF\(_{\beta}2\) expression in experimental gliomas. DIG-labeled ISTH1047 was detected specifically in the tumor region upon systemic administration in mice bearing LN-308- or SMA-560-derived gliomas (Fig. 3A–E). Moreover, systemic administration of ISTH1047 or ISTH0047 to mice with intracranially growing gliomas resulted in a significant down-regulation of TGF\(_{\beta}1\) or TGF\(_{\beta}2\) mRNA expression levels, respectively, in the tumor-bearing hemisphere without significantly altering TGF\(_{\beta}3\) levels (Fig. 3F). To examine whether the observed target downregulation had an effect on TGF\(_{\beta}\)-dependent downstream signaling, we assessed the expression of PAI1, a classic
TGFβ response gene (27). Indeed, PAI1 mRNA levels were significantly downregulated in gliomas derived from LN-308 or SMA-560 cells following systemic treatment with ISTH1047 or ISTH0047 (Fig. 3C). Expression levels of all assessed targets were upregulated in the tumor-bearing hemisphere compared with the normal counterpart, apart from TGFβ3 and TGFβ1 levels in the SMA-560 model. TGFβ2 levels were also reduced after ISTH0047 treatment in the normal hemisphere in both glioma models (Fig. 3F and G).

After confirmation of ASO delivery and target inhibition in experimental gliomas, we assessed effects on survival. Systemic treatment with either ASO prolonged survival of mice with orthotopically growing xenogenic LN-308– or syngeneic SMA-560–derived gliomas (Fig. 4A and G). Tumor volumes were reduced in mice receiving ISTH1047 or ISTH0047 treatment in both models (Fig. 4B and H). In LN-229–bearing mice, although only ISTH0047 exerted a significant survival benefit, neither treatment reduced the tumor volume at an early time point of analysis (Fig. 4C and D; Supplementary Fig. S7). On the contrary, in the ZH-161 model, we observed a significantly reduced tumor burden in mice treated with ISTH0047 that exhibited a nonsignificant trend for better survival compared with control-treated mice (Fig. 4E and F). Furthermore, treatment with ISTH1047 or ISTH0047 reduced tumoral SMAD2 phosphorylation in LN-308–, LN-229–, and SMA-560–bearing mice (Fig. 5). The number of invasive glioma satellites was also decreased in gliomas of mice treated with TGFβ3-targeting ASO (Supplementary Fig. S8).

For a detailed evaluation of host cell infiltration in the tumor, we assessed the frequencies of different immune cell populations after ISTH1047 or ISTH0047 treatment in the syngeneic, immune-competent SMA-560 glioma model. We observed a significant increase in the CD45+ leukocyte population in ISTH0047-treated mice, which correlated with an increased infiltration by CD3+ T cells (Fig. 6A and B). Although CD45+/CD3+ cells exhibited an increase by trend only in ISTH1047-treated mice, the cytototoxic CD8+ T-cell population was significantly enriched in the tumors of mice treated with either ISTH1047 or ISTH0047 (Fig. 6C). CD4+ T cells and CD11b+ macrophage/microglial cells were not more frequent in the tumor upon ASO treatment (Fig. 6D and E).

In terms of blood vessel density, ISTH0047 reduced the number of CD31+ endothelial cells (Supplementary Fig. S9). finally, treatment with either oligonucleotide alone was well tolerated without significant weight loss. Blood analyses of mice treated with ISTH1047 demonstrated increased ASAT and ALAT levels, without any clinically apparent adverse symptoms (Supplementary Fig. S10).

Figure 3. ASOs are detected at the tumor site upon systemic administration and inhibit TGFβ1 or TGFβ2 expression in orthotopically growing gliomas in vivo. LN-308 (0²) or SMA-560 (0³) tumor xenografts were treated with either ISTH1047 or ISTH0047, respectively. A–E, Twenty-five or 5 days after tumor inoculation for Crl: CD1 Foxn1 or VM/Dk mice, respectively, systemic treatment with DIG-ISTH1047 (30 mg/kg) was initiated for 5 consecutive days. Two days following the last injection, brains from LN-308 (A) or SMA-560 (B) glioma-bearing mice were collected and histologically assessed for DIG expression in the tumor region using an anti-mouse DIG antibody. Representative images of DIG fluorescence staining in LN-308 (A) or SMA-560 (B) tumors are shown (DIG, green; nuclei staining with DAPI, blue). Contralateral normal brain hemisphere was used as an additional control (C). Mouse IgG was used as a negative control (D). Mean fluorescence intensity was quantified using the Bitplane Imaris software (E). Results are expressed as means and SD (n = 3); statistical analysis was performed with one-way ANOVA and Bonferroni post hoc testing (*, P < 0.05; **, P < 0.001). F and G, Twenty-five or 5 days after tumor inoculation for Crl:CD1 Foxn1 or VM/Dk mice, respectively, systemic treatment with C3_ISTH0047 (Ctrl) (20 mg/kg), ISTH1047 (30 mg/kg), or ISTH0047 (20 mg/kg) was initiated for 5 consecutive days. Two days following the last injection, tumor-bearing and non–tumor-bearing (normal) hemispheres from LN-308– or SMA-560–glioma-bearing mice were collected and assessed for TGFβ1, TGFβ2, TGFβ3, or PAI1 mRNA expression using RT-qPCR. Human-specific TGFβ1, TGFβ2, TGFβ3, or PAI1 primer sequences were used to assess target gene expression in LN-308–derived tumors. Results are expressed as means and SD (n = 4); statistical analysis was performed with one-way ANOVA and Bonferroni post hoc testing (P < 0.05; *, control vs. treatment in tumor tissue; **, control vs. treatment in normal tissue; †, normal tissue vs. tumor tissue).

Discussion

TGFβ signaling represents a central hub orchestrating various biological functions that are involved in the malignant phenotype of glioblastoma. TGFβ1, TGFβ2, and TGFβ3 are significantly upregulated compared with normal brain tissue, and the expression of TGFβ3 correlates with the expression of the target gene PAI1 (6). Therefore, a ligand-based approach to directly inhibit TGFβ1 or TGFβ2 with ASOs represents an attractive therapeutic strategy. The promising but inconclusive results of a trial assessing the local administration of a first-generation ASO targeting human TGFβ3 mRNA (20) led to the development of two second-generation LNA-modified ASOs, ISTH1047, and ISTH0047 targeting TGFβ1 and TGFβ2, respectively (Supplementary Fig. S1), which can be administered systemically. ISTH1047 and ISTH0047 are second-generation ASOs that benefit from the addition of LNA modifications, which increase their RNA binding affinity and confer enhanced nuclear resistance versus first-generation molecules (28). Here, we characterized the antiglioma activity of these novel ASOs in human and mouse glioma models in vitro and in vivo.

TGFβ1 or TGFβ2 levels were significantly reduced in various human and mouse glioma cells upon exposure to either ASO at low concentrations (Fig. 1A–C; Supplementary Fig. S3A and S3B), indicating that both molecules result in strong target inhibition. In contrast, API2009 achieved a significant TGFβ3 reduction only in the micromolar range (29). Because a biologically meaningful TGFβ inhibition should lead to a reduction of canonical SMAD-dependent signaling, we initially examined this pathway in several glioma cell lines. Both ASO reduced SMAD2 phospho-Smad2 levels in vitro (Fig. 1D), denoting a potential benefit of these novel ASOs in targeting TGFβ/SMAD signaling against adenosine triphosphate (ATP)-competitive inhibitors in vitro. In line with our findings, another approach targeting TGFβ ligands using adenosival transfer of soluble TGFβR1 and II resulted in an almost complete reduction of pSMAD2 levels in glioma cells (30). Indirect approaches against the TGFβ/SMAD2 signaling axis, e.g., via integrin inhibition, have also proven to inhibit glioma progression in preclinical models (11, 31). Integrin αvβ3 is upregulated in high-grade gliomas and plays a crucial role in TGFβ1 activation and subsequent SMAD2 signaling. αvβ8 inhibition decreased both TGFβ2 RNA (20) and TGFβ2 bioavailability inducing almost complete SMAD2 dephosphorylation in LN-308 and LN-229 glioma cells (11). In the current study we show that blocking either TGFβ1 or TGFβ2 expression using ASO approaches, results in a similarly strong reduction of pSMAD2 levels, suggesting that direct inhibition of a
single isoform is sufficient to abrogate TGFβ/SMAD2 signaling (32, 33). Although the exact mechanism for this remains elusive, we conclude that depleting a single TGFβ isoform may impair autoregulatory feedback loops resulting in strong reduction of pSMAD2 levels, despite the presence of other isoforms (26, 34, 35). Although similar suppression was detected at the level of another canonical target, pSMAD3, less activity was seen when exploring pAKT as a representative noncanonical target of TGFβ, yet, noncanonical signaling in response to TGFβ is less prominent in our models (Fig. 1E and F). We have recently revealed an unexpected role for TGFβ3 in the regulation of downstream TGFβ signaling despite high expression of the other two isoforms in glioblastoma (26). Here, blocking TGFβ3 or TGFβ3 expression influenced TGFβ3 expression only in LN-229 glioma cells (Fig. 1C). This was also the cell line that exhibited the least potent pSMAD2 reduction after exposure to ISTH1047 or ISTH0047 in vitro (Fig. 1F).
Figure 5. ISTH1047 or ISTH0047 treatment reduces tumoral SMAD2 phosphorylation. A–F, LN-308 (10⁴), LN-229 (10⁵), or SMA-560 (5 × 10³) glioma cells were injected stereotactically into the right hemisphere of Crl: CD1 Foxn1 or VM/Dk mice, respectively. Twenty-five (LN-308), 10 (LN-229) or 5 days (SMA-560) after tumor inoculation, systemic treatment with C3_ISTH0047 (Ctrl; 20 mg/kg), ISTH1047 (10 mg/kg), or ISTH0047 (20 mg/kg) was initiated for 5 consecutive days and then administered twice weekly. Brains from LN-308 (A, B), LN-229 (C, D), or SMA-560 (E, F) glioma-bearing mice were extracted for histologic analyses from three prerandomized animals per group when the first mouse developed clinical symptoms to assess pSMAD2 levels. Representative images of pSMAD2 stainings of LN-308 (A), LN-229 (C), or SMA-560-derived (E) tumors are shown (pSMAD2, green; nuclei staining with DAPI, blue). Mean fluorescence intensity (B, D, F) was quantified using the Bitplane Imaris software. Results (B, D, F) are expressed as means and SD (n = 2–3); statistical analysis was performed with one-way ANOVA and Bonferroni post hoc testing (*, P < 0.05).
supporting the notion that TGF\(\beta_3\) may compensate for the loss of other isoforms to regulate TGF\(\beta\) signaling (26). We therefore hypothesize that the effects of TGF\(\beta\) isoform-specific inhibition on canonical and noncanonical pathways may be cell dependent and do not solely rely on the basal expression of either TGF\(\beta_1\) or TGF\(\beta_2\) alone, but also on the potential availability of counter-regulatory pathways.

Exposure of glioma cells to ISTH1047 or ISTH0047 did not affect cell viability (Supplementary Fig. S4), although SMA-560 glioma cells transfected with the TGF\(\beta_1\)-specific ASO displayed reduced clonogenic survival, also resulting in lack of spheroid formation for assessment of invasive potential in vitro (Supplementary Fig. S4D). Ashley and colleagues also reported growth-suppressive properties of TGF\(\beta_1\) inhibition in SMA-560 cells and the induction of apoptosis, indicating a potential survival dependency of these cells specifically related to TGF\(\beta_1\) (35).

Reduced migration and invasion of glioma cells upon TGF\(\beta_1\)/SMAD singling blockade has been widely shown (12, 14, 36). Correlating with the greater pSMAD2 downregulation after ASO-mediated TGF\(\beta_1\) or TGF\(\beta_2\) inhibition (Fig. 1D; Supplementary Fig. S3C and S3D), these agents also exhibited more pronounced inhibitory effects on the migratory and invasive potential of glioma cells compared with TGF\(\beta\)RI kinase inhibition (Fig. 2). The high binding affinity of LNA-modified ASO to cell-surface proteins, such as heparin, can potentiate their cellular uptake via absorptive endocytosis and consequent target inhibition (37, 38), and combined with the irreversible nature of ASO-mediated target silencing (39), may explain the superiority of these agents over small-molecule ATP-competitive TGF\(\beta\)R kinase inhibitors (12, 40) observed here.

Because drug delivery to gliomas is one of the limiting factors of current novel therapeutic approaches administered systemically (41), we assessed whether these second-generation ASOs reach the tumor site after systemic treatment in orthotopic murine glioma models in vivo. Indeed, systemic subcutaneous administration of DIG-labeled ISTH1047 resulted in a strong oligonucleotide accumulation in the tumors with minimal deposition in the normal contralateral hemisphere (Fig. 3E). Even though in vivo oligonucleotide uptake pathways are currently poorly understood, LNA modifications are known to improve the pharmacokinetic properties of ASOs, facilitating prolonged tissue half-life and more efficient cellular uptake compared with AP12009 or other first-generation ASOs (18). The only other approach to directly inhibit TGF\(\beta\) ligand activity includes the intravenous administration of the pan-TGF\(\beta\)–neutralizing antibody 1D11, which also appears to selectively accumulate in the tumor (15).

Systemic treatment of glioma-bearing mice with either of the two ASO molecules resulted in a ligand-specific downregulation and
reduction of the SMAD-dependent target gene PAI1 in the tumor-bearing hemisphere (Fig. 3F and G). Given that TGFβ1 and TGFβ3 expression strongly correlates with PAI1 expression in The Cancer Genome Atlas analyses (6), this profound reduction in TGFβ and PAI1 levels demonstrates that isoform-specific targeting of TGFβ is feasible and interferes with TGFβ signaling in the tumor following systemic administration. TGFβ1/2 levels were upregulated in the tumor compared with normal brain in both glioma models (Fig. 3F), supporting the notion that TGFβ is highly expressed in glioblastoma (5, 6). However, we did not detect any increase in tumoral TGFβ2 compared with normal brain tissue in SMA-560–bearing mice. The inherent low TGFβ3 expression levels of SMA-560 gliomas have been described before (13), which may not allow for the discrimination of such changes at the mRNA level between tumoral and normal tissue. TGFβ2 levels in SMA-560–bearing mice were reduced only after ISTH0047 treatment, further supporting the specificity and efficacy of these ASOs in the glioma setting (Fig. 3F). Reduced TGFβ1, or TGFβ3 levels resulted in a significant reduction of tumor invasiveness in vivo (Supplementary Fig. S8), and are in line with our in vitro observations of reduced invasive potential (Fig. 2C and D), enhancing the importance of the TGFβ/SMAD pathway as a major mediator of glioma cell invasion (12, 42, 43).

Based on these promising data, we determined the antiglioma activity of both novel antisense molecules in vivo. ISTH0107 or ISTH0047 treatment significantly reduced tumor volumes and ultimately prolonged the survival of LN-308 or SMA-560 glioma–bearing mice (Fig. 4A, B, C, and H). Consistent with the TGFβ and PAI1 mRNA inhibition data, pSMAD2 levels were significantly reduced by ISTH1047 or ISTH0047 in both models (Fig. 5A, B, E, and F). A recent study with the TGFβRI antagonist galunisertib as monotherapy showed a marginal survival benefit in the SMA-560 model, whereas it did not significantly affect tumor size (13). Moreover, the 1D11 pan-TGFβ–neutralizing antibody resulted in an almost complete tumor remission when administered daily in immunocompetent mice bearing subcutaneous gliomas, but the opposite effect was noted when it was given to immunodeficient glioma-bearing mice; additionally, 1D11 failed to show a therapeutic effect in an orthotopic glioma model (15). Our ASO-mediated approach utilized a twice weekly dosage regime after the initial 5-day ASO treatment and led to a striking decrease of pSMAD2 levels and significant tumor size reduction in the xenogenic LN-308-bearing and the syngeneic SMA-560–bearing glioma models. In LN-229–bearing mice, a significant survival benefit was observed only after ISTH0047 treatment without any reduction in tumor burden, which, however, was assessed at a very early point in time which limits the significance of this finding (Fig. 4C and D). LN-229 gliomas exhibit a highly invasive phenotype that could only be mitigated by ISTH0047-mediated TGFβ3 inhibition and not by ISTH1047 (Supplementary Fig. S8). Moreover, pSMAD2 expression was almost eliminated after ISTH0047 treatment, suggesting that TGFβ3 inhibition has a potent effect on the TGFβ pathway in this model (Fig. 5C and D). Therefore, it may be that the survival benefit observed with ISTH0047 in this model is mainly due to the potent reduction of tumor infiltration and invasion (42), further supporting the cell-dependent effects of TGFβ isoform inhibition noted in our in vitro studies. We did not observe a significant survival benefit upon ASO treatment in the GIC model in vivo. However, TGFβ2 inhibition significantly reduced tumor volume and had the better survival outcome compared with TGFβ3 inhibition (Fig. 4E and F). In line with this finding, TGFβ3 is known to be an essential regulator of the self-renewing and tumor-propagating capacity of patient-derived GIC (44). Therefore, a more intensive ISTH0047 treating regime has the potential to exert more prominent survival benefit in GIC tumor mouse models.

Because TGFβ is a multifunctional cytokine with major immunosuppressive properties, we assessed the host cell responses to ASO-mediated TGFβ3 or TGFβ1 inhibition in the syngeneic, immunocompetent SMA-560 glioma model. We noted enhanced immune cell infiltration in the tumor after ASO treatment. More specifically, the cytotoxic CD45+ CD8+ T-cell population was significantly increased after either ISTH1047 or ISTH0047 treatment (Fig. 6C). The enhanced tumor infiltration with cytotoxic T cells suggests that TGFβ depletion allows for an activation of the adaptive antitumor immune response. Interestingly, pSMA22 depletion correlates with the increased tumoral cytotoxic T-cell infiltration, supporting the notion that targeting TGFβ signaling in tumors can restore immune surveillance (13, 45). Finally, ISTH0047 treatment resulted in a significantly reduced number of CD31+ endothelial cells, suggesting that TGFβ2 inhibition may reduce the number of aberrant tumor blood vessels and thereby facilitate tumor vasculature normalization (13). Blood vessel normalization is a process associated with a better trafficking of immune cells in the tumor bed, which may facilitate cytotoxic T-cell infiltration (46). Overall, these data indicate a potential proimmunogenic effect of ASO-mediated TGFβ1/2 inhibition that might assist in the elimination of glioma cells, while reducing their invasive and migratory abilities.

Except for ALAT levels marginal beyond the normal range upon treatment with ISTH1047, no relevant toxicity was observed in the in vivo survival experiments (Supplementary Fig. S10). However, when the two ASO molecules were administered as a combinatorial treatment in the syngeneic SMA-560 glioma model, the mice exhibited severe systemic toxicity, and therefore this treatment arm was not selected for further investigations. For reasons currently undefined, LNA-modified ASOs have been linked with an increased risk of acute kidney injury in preclinical studies when compared with other second-generation ASO variants (47). However, in vivo preclinical toxicology studies tend to overpredict the renal effects of ASOs, as these agents do not appear to exert significant nephrotoxicity when tested in humans (48). Therefore, even though informative, any toxicology studies performed in murine glioma models with ISTH1047 or ISTH0047 do not necessarily predict the situation in patients with glioblastoma. Altogether, these data show that ASO-mediated TGFβ ligand targeting represents an alternative, convenient, and efficient way to block the TGFβ/SMAD pathway, resulting in a significant survival benefit compared with other approaches, in both the xenograft and syngeneic glioma setting in vivo.

The present data set strongly suggest that TGFβ retains an important role in glioblastoma progression and that an efficient targeting approach can potentially improve clinical outcome. We show strong therapeutic activity of two novel second-generation TGFβ–specific ASOs in several independent glioma models which may now allow for clinical evaluation of their therapeutic potential in human patients. Given the immune-modulatory activities of the two ASO molecules, a potential combination of ISTH1047 or ISTH0047 with immune checkpoint inhibitors that are currently explored in clinical neuro-oncology (49, 50), may result in synergistic therapeutic activity and warrants further investigation.
Disclosure of Potential Conflicts of Interest

M. Weller reports receiving commercial research grants from Isarna Therapeutics. M. Janicot is an employee/paid consultant for Isarna Therapeutics. P. Roth reports receiving speakers bureau honoraria from MSD, Bristol-Myers Squibb, Covagen, Virometix, and NovoCure. No potential conflicts of interest were disclosed by the other authors.

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References

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