Correction

NEUROSCIENCE, CHEMISTRY

The authors note that the manuscript title “Inhibition of brain tumor growth by intravenous poly(β-L-malic) acid nanobioconjugate with pH-dependent drug release” should instead appear as “Inhibition of brain tumor growth by intravenous poly(β-L-malic acid) nanobioconjugate with pH-dependent drug release.” Additionally, on page 18143, right column, third full paragraph, first sentence, the phrase “poly(β-L-malic) acid” should appear as “poly(β-L-malic acid).” The article initially appeared online with the incorrect title and phrase and has since been corrected.

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Inhibition of brain tumor growth by intravenous poly(β-L-malic acid) nanobioconjugate with pH-dependent drug release

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Effective treatment of brain neurological disorders such as Alzheimer’s disease, multiple sclerosis, or tumors should be possible with drug delivery through blood–brain barrier (BBB) or blood–brain tumor barrier (BTB) and targeting specific types of brain cells with drug release into the cell cytoplasm. A polymeric nanobioconjugate drug based on biodegradable, nontoxic, and nonimmunogenic polymeric acid as a universal delivery nanoplatform was used for design and synthesis of nanomedicine drug for i.v. treatment of brain tumors. The polymeric drug passes through the BTB and tumor cell membrane using tandem monoclonal antibodies targeting the BTB and tumor cells. The next step for polymeric drug action was inhibition of tumor angiogenesis by specifically blocking the synthesis of a tumor neovascular trimer protein, laminin-411, by attached antisense oligonucleotides (AONs). The AONs were released into the target cell cytoplasm via pH-activated trileucine, an endosomal escape moiety. Drug delivery to the brain tumor and the release mechanism were both studied for this nanobiopolymer. Introduction of a trileucine endosome escape unit resulted in significantly increased AON delivery to tumor cells, inhibition of laminin-411 synthesis in vitro and in vivo, specific accumulation in brain tumors, and suppression of intracranial glioma growth compared with pH-independent leucine ester. The availability of a systemically active polymeric drug delivery system that passes through the BTB, targets tumor cells, and inhibits glioma growth gives hope for a successful strategy of glioma treatment. This delivery system with drug release into the brain-specific cell type could be useful for treatment of various brain pathologies.

Brain gliomas are among the most aggressive and lethal cancers. At present, efficient drugs for treatment of gliomas are very limited (1). A hallmark of current cancer treatment is inhibition of tumor angiogenesis (2, 3). Antiangiogenic inhibitors of VEGF receptors and VEGF-independent inhibitors combined with chemotherapy have shown some promise, but the patients’ survival in clinical trials was not significantly changed (4). We documented overexpression of tumor-specific vascular basement membrane protein laminin-411 in glioblastoma and its association with tumor recurrence and decreased patients’ survival time (5, 6). Laminin-411 consists of three different polypeptide chains, and it was hitherto impossible to efficiently block its synthesis in vivo by existing technologies. In our nanoconjugate, two antisense oligonucleotides (AONs) against laminin α4 and β1 chains were covalently attached and delivered through the blood–brain tumor barrier (BTB). Although the microvascular BTB is more permeable than the blood–brain barrier (BBB), it still retains BBB characteristics (7, 8), hampering drug delivery to brain tumors. Antibodies to certain cell surface proteins, including transferrin receptor (TIR), can pass the BBB and BTB by endothelial transcytosis and then direct carrier systems with attached drugs into tumor cells by receptor-mediated endocytosis (9–13).

Polymer conjugates introduce other possibilities to treat cancers by targeting specific tumor markers and providing precise inhibitor delivery to the tumor with minimum side effects (3, 14, 15). The endocytosed carriers, including polymers, are routed to the endosomal pathway, and their design must ensure the drug escape from the endosomes into the cytoplasm to avoid lysosomal degradation. Cytoplasmic delivery using cell penetrating peptides (CPPs) acting directly on cell membranes is a possible mechanism for drug trafficking across the plasma membrane (16), but CPPs are not specific to the cell type. Receptor-targeted cytoplasmic delivery through endosome disruption is a safe and efficient approach avoiding potentially cytotoxic permeation through the plasma membrane. It can be achieved by a pH-sensitive endosomemembrane-disrupting moiety that takes advantage of the low pH (5.0–6.5) in the endosome/lysosome compartments.

Several pH-dependent escape devices were designed, such as polyethyleneimine (PEI), which is protonated during endosome acidification (17). These polymers, however, are of limited use due to their cytotoxicity (18). Instead, nontoxic lysogenic peptides, such as Glu-Ala-Leu-Ala (GALA) (19, 20) derived from certain viruses or bacteria, are used for endosome escape of micelles and liposomes (21) or are covalently bound to nanoconjugates to deliver water-soluble proteins, antibodies, and nucleic acids (9, 22).

The nanoconjugate based on poly(β-L-malic acid) (PMLA) that is introduced here meets all criteria for nanomedicine drugs (23) and is a promising candidate for future clinical use, alone or in combination with other treatments for brain cancer. This polymeric drug is designed for i.v. brain tumor treatment using a targeted pH-dependent endosome escape unit. A similar delivery system might be used to treat other brain pathological conditions.

Results

Synthesis of Polymeric Acid-Based Nanoconjugates. Nanoconjugates of varying composition were synthesized. A schematic of a conjugate with all functional groups is shown in Fig. L4, and a simplified chemical formula in the Fig. S1. NHS-activated carboxylates of polymeric acid were first conjugated with H2N-Leu-Leu-Leu-OH (LLL), H2N-Leu-Leu-Leu-NH2, H2N-Leu-ethylcysteine (LOEt), 2-


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mercapto-1-ethylamine. The newly introduced sulf hydrol groups were converted to disulfides with thiolated morpholino AON and to thio ethers with maleimidated mAbs and optionally with male imidated fluorophore Alexa Fluor 680. The composition of synthesized nanoconjugate and pendant group functions are shown in Fig. 1B. The composition was confirmed by analytical tests to completely correspond to the synthetic strategy. The calculated molecular weight values compared within 15% with experimental absolute molecular weights by light scattering, which also confirmed the absence of particle aggregation. Sizes by dynamic light scattering ranged from 6.6 nm for PMLA to 18 ± 2 nm for P/LLL/AON/Hu/Ms and 22 ± 2 nm for P/LOEt/AON/Hu/Ms. The ζ potentials (pH 7.0) ranged from −27 ± 1 mV for polymeric acid, −9.4 ± 0.7 mV for P/LLL/AON/Hu/Ms to −5.2 ± 0.4 mV for P/LOEt/AON/Hu/Ms; the potentials of LLL containing conjugates significantly shifted in the presence of liposomes but only a marginal shift was seen for P/LLL. This indicated that most P/LOEt was on the liposomes, but most P/LLL was in the free solute state. Measurements were performed with 200 μg/mL P/LLL or P/LOEt and in the presence of 160 μM lipid (liposomes).

**pH-Dependent Membrane Disruption for Nanoconjugate Escape from Endosomes.** The TR-targeting nanoconjugates were released from endosomes/lysosomes through constitutive or pH 5.0–5.5 activated membrane disruption. Two versions of membrane disruption units were used: pH-sensitive P/LLL (PMLA conjugated with 40% LLL) and pH-independent P/LOEt (PMLA conjugated with 40% LOEt) (24, 25). At pH 5 both nanoconjugates were membrane disruptive, with similar concentration dependence (Fig. 2A). When AONs and mAbs were added to P/LLL/AON/IgG and P/LOEt/AON/IgG nanoconjugates, the leakage remained high (80–90%) (Fig. 2B), indicating that membrane disruption activity survived conjugation of AONs and mAbs. At physiological pH 7.4, P/LLL conjugates were inactive, whereas membrane disruption by P/LOEt conjugates remained (Fig. 2C). P/LOEt activity was high over the investigated pH range, but P/LLL activity was only high at pH 5.5 and abruptly dropped at pH 6 (Fig. 2D). The pH dependence mirrored the range of acidification in late endosomes/lysosomes. This justified using P/LLL conjugates for endosome-specific escape. In contrast to P/LOEt conjugates, the specific acidification requirement for P/LLL conjugates would not allow cell membrane disruption at neutral pH.

Leucine and its derivatives may form strong hydrophobic interactions allowing assembly into hydrophobic patches and/or can interact directly into lipid membrane bilayers and provoke their disruption (26, 27). This may be the mechanism of membrane interaction with studied nanopolymers. However, LLL carries a charged terminal carboxylate that cannot be accommodated into a lipophilic environment unless carboxylate is neutralized by protonation. By acid–base P/LLL titration, this protonation followed a pKα of 5.5, and this pH dependence paralleled the leakage dependence in Fig. 2D. P/LOEt did not contain this ionizable group and did not show pH dependence. When LLL was replaced by LLL–NH2 with a nonionizable terminal amide group instead of carboxylate, pH dependence and lisosome leakage became similar to LOEt.

The simplest reaction mechanism implies that polymer and liposome bind before the lysis occurs. The concentration dependence in Fig. 2A–C may indicate similar binding affinities for P/LLL and P/LOEt to the liposome membrane at pH 5.0 and a very low affinity for P/LLL at pH 7.4 due to the carboxylate charge. P/LOEt and P/LLL liposome membrane affinities dramatically diverged as indicated by confocal microscopy (Fig. 2E) and ζ potential (Fig. 1C). P/LOEt readily bound to the liposome membrane at neutral pH 7.4, but P/LLL amounts were too low to be detected (Fig. 2E). The ζ potential of P/LOEt did not change as a function of pH either in the presence or absence of liposome (Fig. 1C). The liposome ζ potential is near zero, and its contribution is negligible. In the presence of liposome, ζ potential of P/LOEt increased from −13 to −5. This change accounts for various parameters that affect the electro phototherapeutics underlying ζ potential measurement and may indicate that the majority of P/LOEt was sticking to the liposome after membrane disruption and leakage. Minor amounts of free P/LOEt would not be detectable by this technique. In contrast, P/LLL ζ potential is pH-dependent (Fig. 1C), the pH sensitivity correlating well with pH-dependent liposome leakage activity. The inefficiency of P/LLL in staining liposomes could indicate that small amounts of P/LLL bound to the liposome were below the Zetasizer detection limit.

Overall, nanoconjugates with LOEt readily bound to lipid membranes and in addition to their disruption gave rise to side effects at physiological pH that are not seen with LLL conjugates. This “stickiness” at physiological pH could induce cell toxicity and also systemic depletion of the circulating nanocojugate after i.v. injection, reducing its availability at the tumor site.

**Cell Viability and Increased Suppression of Target Laminin-411 Synthesis with pH-Dependent Endosome Escape Unit.** Using P/LLL- and P/LOEt-containing nanoconjugates, cell viability was tested on the human glioma cell line U87MG. P/LLL was nontoxic at all concentrations (≤2 mg/mL), but P/LOEt decreased cell viability at
as low as 0.15 mg/mL (Fig. 3A, Left). At 0.5 mg/mL P/LOEt, cells shrunk and rounded up within 2 h, whereas no such changes were seen with P/LLL (Fig. 3A, Center). Similar data were obtained with human U87MG and rat RG62 gliomas and human breast cancer MDA MB-231 cells. By FACS analysis, the shrunk and rounded up within 2 h, whereas no such changes were seen with P/LOEt (50 μg/mL) as a function of pH. Only P/LLL membrane disruption activity is pH-dependent following an apparent pKₐ 5.5. (E) Binding of P/LOEt and P/LLL to liposomes at neutral pH. Confocal microscopy showing colocalization of P/LOEt and giant artificial liposomes. P/LLL and P/LOEt were conjugated with rhodamine (red). Giant liposomes were labeled with NBD [N(1)-(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)propane-1,3-diamine] (green). (Upper) Large amounts of P/LOEt stuck to the vesicle membrane; liposomes and P/LOEt colocalized at pH 7.4 (yellow). (Lower) Binding of rhodamine-labeled P/LLL to liposomes could not be detected. Concentrations of conjugates were 20 μg/mL. The data correlate well with ζ potential measurement (Fig. 1C).

Colocalization of the Polymer Platform and Antisense Oligonucleotides in Cytoplasm. To confirm that the polymer platform and AONs entered the endosomal pathway together in U87MG and T98G glioma cells, the nanoconjugate P/LLL/AON/Hu was double-labeled with Lissamine at AON and Alexa Fluor 680 at PMLA. By confocal microscopy (Fig. S2), the PMLA platform (green) and AONs (red) colocalized (yellow) in intracellular entities, which coincided (white) with stained endosomal membranes (blue) (Fig. S2A). The number of green and red vesicles decreased over time during a 3-h incubation period (Fig. S2B). The decrease coincided with diffuse staining around these entities, in agreement with induced leakage by membrane disruption. Statistical analysis of colocalization expressed as Pearson’s correlation coefficients (28) (Fig. S2C) indicated significant colocalization of PMLA and AON and of each of them with endosomes at 0 h. However, at 3 h, endosomal marker and nanoconjugate colocalization decreased.

Imaging Analysis of Drug Distribution and Accumulation in Tumor and Normal Brain Tissues in Vivo. For these experiments, two mAbs were attached to the PMLA platform. Anti-mouse TIR mAb was used for transporting drugs through the mouse endothelial host system, and anti-human TIR mAb served for targeting implanted human tumor cells. Imaging analysis 24 h after i.v. injection showed that nanoconjugate with anti-mouse TIR mAb delivered only a low amount of drug into the intracranial human U87MG tumor (Fig. 4A, Upper Left). With anti-human TIR mAb attached to nanoconjugate, drug accumulation in the tumor increased (Fig. 4A, Upper Center). These data are consistent with the facilitation of polymer passage through BTB by the enhanced permeability and retention (EPR) effect. In the presence of both endothelium- and tumor-targeting mAbs (anti-mouse and anti-human TIR, respectively), P/LLL/AON/Hu/Ms drug predictably showed the highest accumulation of all variants (Fig. 4A, Upper Right).

We next compared brain tumor-specific accumulation of polymeric drugs containing LLL or LOEt endosomal escape units. In both cases, the drug accumulation 24 h after i.v. injection persisted mainly in brain tumor and to some extent in drug-clearing organs, livers and kidneys. The drug was no longer detected after 72 h.

Fig. 2. Membrane disruption activity of P/LLL and P/LOEt and their binding to liposomes. (A) Concentration dependence of P/LLL and P/LOEt membrane disruption activity at pH 5.6 measured by the liposome leakage assay. The degree of leakage refers to complete leakage in the presence of 0.25% (vol/vol) Triton-X 100. (B) Membrane disruption activity for nanoconjugates P/LLL/AON/IgG and P/LOEt/AON/IgG at pH 5. It is not abolished over the range of concentrations by the conjugation of AON and antibody. (C) Retention of membrane-disruptive activity at pH 7.4 by P/LOEt and its loss by P/LLL. (D) Membrane disruption for P/LLL and P/LOEt (each 50 μg/mL) as a function of pH. Only P/LLL membrane disruption activity is pH-dependent following an apparent pKₐ 5.5. (E) Binding of P/LOEt and P/LLL to liposomes at neutral pH. Confocal microscopy showing colocalization of P/LOEt and giant artificial liposomes. P/LLL and P/LOEt were conjugated with rhodamine (red). Giant liposomes were labeled with NBD [N(1)-(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)propane-1,3-diamine] (green). (Upper) Large amounts of P/LOEt stuck to the vesicle membrane; liposomes and P/LOEt colocalized at pH 7.4 (yellow). (Lower) Binding of rhodamine-labeled P/LLL to liposomes could not be detected. Concentrations of conjugates were 20 μg/mL. The data correlate well with ζ potential measurement (Fig. 1C).
24 h, drug-tumor accumulation of P/LLL/AON/Hu/Ms (Fig. 4A, Upper Right) was 1.5 times higher (P < 0.03) than that of P/LOEt/AON/Hu/Ms variant (Figs. 4A, Lower Center, and B). Because both nanoconjugates use exactly the same targeting strategy, their difference in tumor accumulation could be due to their differences in membrane disrupting units LLL or LOEt, which corresponds well to data shown on Fig. 2. Control P/LLL carrying a non-targeting IgG (P/LLL/AON/IgG) accumulated in the tumor 90% less than P/LLL/AON/Hu/Ms with two targeting antibodies (P = 0.0015) (Fig. 4A, Lower Left, and B).

Sections of brain tumors harvested 3 h (Fig. 4C) and 6 h after i.v. injection of P/LLL/AON/Hu/Ms with labeled AON and PMLA were analyzed by confocal microscopy. As shown in Fig. 4C, positive signals for both Alexa Fluor 680 (PMLA label) and Lissamine (AON label) were seen mainly in tumor cells and also in tumor vessels positive for von Willebrand factor (vWF) staining. Both moieties showed noticeable codistribution in the cytoplasm of tumor cells (Fig. 4C, Lower). Normal brain regions of the same animals showed little drug accumulation (Fig. 4C, Upper), in agreement with whole brain imaging (Fig. 4A). The data suggest that nanoconjugate is efficiently passing through BTB and internalizing into the tumor cells.

**Significant Suppression of Tumor Growth and Vascularity by P/LLL/AON/Hu/Ms Nanoconjugate.** Systemic multiple treatments of mice bearing intracranial human glioma U87MG with nanoconjugate P/LLL/AON/Hu/Ms blocking laminin-411 synthesis significantly suppressed tumor growth (Fig. 5A). The mean tumor volume was 4 mm$^3$ (P < 0.001 vs. PBS), compared with 18 mm$^3$ (P < 0.01 vs. PBS) after P/LOEt/AON/Hu/Ms treatment and with 47 mm$^3$ in PBS-treated controls. Tumor size reduction by P/LLL/AON/Hu/Ms with pH-dependent LLL escape unit was highly significant, resulting in 90% smaller tumors compared with PBS-treated animals. LLL-containing nanoconjugate was also more than 2-fold more efficient in inhibiting tumor growth than the variant with LOEt. This result fully corroborated in vitro data on laminin-411 inhibition (Fig. 3B). On brain sections of PBS-treated animals, large invasively growing tumors were seen, whereas after P/LLL/AON/Hu/Ms treatment, tumor remnants with significant necrosis were typically found (Fig. 5B). Two conjugates without AON or anti-TfR mAbs were used as controls with no effect on tumor treatment compared with PBS, confirming that AONS against two laminin-411 chains acted as antiangiogenic inhibitors to slow down tumor growth. Morphometric analysis of vascularity on tumor sections revealed largely similar vessel numbers in all treatment groups. However, P/LOEt/AON/Hu/Ms and P/LLL/AON/Hu/Ms treatments usually resulted in smaller vessels than in the PBS group, with a significant reduction of vessel area (P < 0.001 vs. PBS for both nanoconjugates). The vessel area decrease was more pronounced for P/LLL/AON/Hu/Ms (over 50% less than for PBS) than for P/LOEt/AON/Hu/Ms (P < 0.05) (Fig. 5C). On immunostained tumor sections, laminin α4 and β1 chain signal was typically strong in the tumor blood vessel basement membranes in the PBS group, and many large vessels with irregular shape were seen (Fig. 5D). After P/LOEt/AON/Hu/Ms, and especially P/LLL/AON/Hu/Ms treatment, staining intensity was noticeably diminished for both laminins chains (Fig. 5D), and vessels became more similar in size to those in normal brains.

**Discussion**

A number of approaches for targeted drug delivery have emerged, including encapsulation of cargo into micelles, nanoparticles, nanocapsules, and nanotubes (29). Nanoconjugates as covalent delivery devices received wide interest after the introduction of natural and synthetic polymers and dendrimers (2, 17, 22, 30–34). The problems still shared by most delivery platforms include toxicity, immunogenicity, the absence of real biodegradability (i.e., lack of degradation to CO$_2$ and H$_2$O), and the target cell’s cytoplasm drug delivery. Current drug design strategies are mostly focused on bioavailability and tissue targeting but rarely address drug delivery to specific intracellular compartments (35).

By introducing nanoconjugates, which use PMLA of the slime mold Physarum polycephalum as a platform, we have overcome most of the major drawbacks (9, 24, 30, 35–37). PMLA-based...
nanoconjugates can deliver AON drugs into cells in vitro (38) or upon injection into the tumor mass (30), but their systemic administration with tumor cell-cytosplasm delivery (35) was not explored previously. Gliomas are highly invasive tumors, and only systemic treatment could be really beneficial to treat this very aggressive type of brain cancer. The problems now appear to be largely solved by introducing a pH-dependent endosomal escape unit in drugs administered i.v. for brain cancer treatment has not been reported thus far.

A major problem in glioma treatment is the inefficiency of systemic water-soluble delivery systems due to BTB, which was bypassed in our nanoconjugate by tandem conjugated anti-mouse and anti-human TIR mAbs. The nanoconjugate actively passed the endothelial barrier by a well-established mechanism of transcytosis (11, 40, 41). BTB permeation was additionally facilitated by EPR effect (42). However, alone, transcytosis- and EPR-mediated permeation were not efficient for nanoconjugate accumulation in the tumor (Fig. 4A, Upper Left and Center). The second (anti-human) mAb allowed the polymer to specifically bind to and get internalized by implanted human tumor cells (Fig. 4). The move of nanoconjugate from interstitial space into the tumor cell cytoplasm mediated by anti-human TIR is important for successful accumulation and delivery. For future treatment of brain tumors in humans, two anti-species mAbs are unnecessary. However, the technical possibility of attaching two mAbs to the polymer was important in our study with xenogeneic tumors to prove the mechanisms of transcytosis through mouse endothelium and active receptor-mediated human tumor cell-specific targeting.

After endosome internalization, the LLL escape unit is activated during maturation to late endosomes concomitantly with acidification. The delivery platform and AONs remain conjugated to pH in liposome leakage assay. Its pH-dependent membranolytic activity with an operational pKₐ 5.5 matched acidification during maturation from early to late endosomes. In contrast to the previously used membranolytic unit LOEt, the LLL unit was nontoxic at all concentrations tested (Fig. 3). We explain the LOEt cytotoxicity by its lipophilicity at physiological pH ∼7 that renders it sticky and destructive to cell membranes. In contrast, LLL is not sticky at this pH due to its terminal negative charge. Importantly, the absence of stickiness to membranes may also prevent lipophilic units from binding to cell membranes in vascular cells in vivo and to hydrophobic sites of proteins such as opsonins of the reticuloendothelial system (39), thus contributing to reduction of nonspecific effects on nontarget cells.

The pH-restricted membranolysis is important for an optimal target bioavailability of systemically administered drugs. This indeed was borne out for LLL- versus LOEt-containing nanoconjugate, seen as an increased inhibition of target laminin-411 production in vitro and in vivo (Figs. 3B and 5D), higher tumor accumulation (Fig. 4), and significantly increased antitumor efficacy possibly related to reduced production of laminin-411 chains and decreased angiogenesis (Fig. 5C and D). The dual effect of reducing cytotoxicity and increasing bioavailability to the target cells as opposed to nontarget ones underlies the greatly improved efficiency of the nanoconjugate–LLL drug delivery system. To our knowledge, the application of a pH-dependent endosome escape unit in drugs administered i.v. for brain cancer treatment has not been reported thus far.

Materials and Methods

Cell Viability and Cell Death Assays. Cell viability was quantified using CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) and a Spec

Fig. 5. Efficacy of different pH-dependent or pH-independent endosomal escape units in inhibiting brain tumor growth, vascularity, and target protein expression. P/LLL/AON/HuMs and P/LOEt/AON/HuMs were injected i.v. at 5 mg/kg morpholino AONs to laminin-411 α4 and β1 chains. (A) Tumor size quantification after treatment with P/LLL/AON/HuMs or P/LOEt/AON/HuMs. Both nanoconjugates significantly decreased tumor volume. However, volume decrease for P/LLL/AON/HuMs (LLL) with a pH-dependent unit was significantly greater than for P/LOEt/AON/HuMs (LOEt) with pH-independent unit. (B) H&E-stained sections of tumors treated by either PBS or the lead drug P/LOEt/AON/HuMs (LLL). Two different animals represent each group. In PBS-injected mice (Fig. 3B and 3D), invasive growing intact tumors are seen. In the LLL-treated animals (Fig. 3B and 3D), massive necrosis is visible with some tumor remnants. (C) Morphometric analysis of microvessel area after various treatments. Both P/LOEt/AON/HuMs (LOEt) and P/LLL/AON/HuMs (LLL) significantly reduced vessel area (most pronounced in the latter group), compared with PBS. In the LLL group, the reduction was significantly greater than the LOEt group (P < 0.05). Data are from 25 nonoverlapping fields of view per group (field area = 0.245 μm²) using 20× objective (five per tumor, five tumors per group). Percentage of area occupied by vessels (revealed by laminin β1 chains upon nanoconjugate treatment. In the PBS group, vessels stained brightly for both chains and many large vessels with irregular shapes were seen. Upon treatment with P/LOEt/AON/HuMs (LOEt) and especially P/LLL/AON/HuMs (LLL), tumor staining intensity for both chains was diminished and vessels became smaller, more similar to normal brain vessels. Representative pictures for each group are shown. For each antigen, exposure times were the same among groups.
Tumor Treatment in Vivo. Animals were treated according to the approved Cedars-Sinai Medical Center Institutional Animal Care and Use Committee protocols. Athymic mice (Crl:NCrFoxn1nu homozygous) were from Taconic. Human U87MG glioblastoma cells were stereotactically implanted at 5 × 10⁵ into the right basal ganglia field of mice (n = 8 per group) as reported (24). Nanconjugate treatment began after day 8 by i.v. injecting nanoconjugates at doses of 5 mg/kg AONs on every third day, amounting to eight injections in total. Mice were killed on day 48 after cell inoculation, and tumor volumes were measured using histological sections (43) stained with H&E.

Imaging Analysis in Vivo. On days 20–30 after tumor implantation, 100 μL solution of 3 μM Alexa Fluor 680-labeled nanoconjugates was injected i.v. Mice were euthanized after 24 h. Following blood vessel clearance by intraarterial PBS perfusion for 20 min, brains were removed for fluorescence imaging by Xenogen 200 Living Image System 2.30 (Caliper Life Sciences). Light intensities emitted from equally sized surface areas of tumor and nontumor references were measured. In some cases, tumor/reference intensity ratios were calculated. The reference area was in the brain but distant from the tumor. Nanoconjugates without fluorescent Alexa Fluor 680 (background) gave negative results. Injected free dye was rapidly cleared from the tumor. Nanoconjugates without fluorescent Alexa Fluor 680 (background) gave negative results. Injected free dye was rapidly cleared from the tumor.

Statistical Analysis. Statistical analysis was done using Prism4 statistical program (GraphPad). Data in several groups were compared using ANOVA. Data points represent mean ± SD of triplicates; in some cases in Fig. 2, SD bars were too small to be shown. In some experiments, data are expressed as mean ± SEM. P < 0.05 was considered significant.

Materials, Syntheses, Liposome Leakage Assay, Cell and Tissue Section Immunostaining, Colocalization Studies, and Western Blot Analysis. Materials, syntheses, liposome leakage assay, cell and tissue section immunostaining, colocalization studies, and Western blot analysis are presented in detail in SI Materials and Methods.

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