Supplementary Figures

Figure S1. Structure of dPG-NH<sub>2</sub>.

Figure S2. dPG-NH<sub>2</sub>-miR-34a polypex inhibits migration of GBM cells (A172) towards serum and migration of endothelial cells (Human umbilical vein endothelial cells, HUVEC) towards conditioned media from GBM cells (U-87 GBM CM).
Figure S3. Polyanion Competition Assay: dPG-NH₂-miR-34a polyplex (N/P 9) was incubated in the presence of 0.1, 0.15, 0.17, 0.2, 0.22 and 0.25 IU of heparin. Solutions were mixed well, incubated for 15 minutes and then loaded into a 2% agarose gel and run for 30 minutes at 100 V. Free miRNA was run as a reference.

MRI analysis of our intracranial U-87 GBM model clearly shows extravasation of Gd-DTPA (Gadolinium diethylenetriamine pentaacetic acid), confirming the fact that the BBB is compromised in this GBM model (Figure S4). Additional support for the BBB crossing by our polyplex is provided by the ex vivo image that shows Cy5 fluorescence in the tumor in the brain and not the rest of the healthy brain (Figure 7B). Furthermore, evidence of impaired BBB integrity was previously shown on the same U-87 MG model by a modified Miles assay using Evans Blue which binds in the circulation to albumin and extravasates from leaky vessels (Satchi-Fainaro et al., Cancer Cell 2005).

Figure S4. MRI scans of a SCID mouse bearing a mCherry-labeled U-87 GBM intracranial tumor were performed using a 7 Tesla MRI (Bruker Biospec). For BBB integrity evaluations, images were collected before and following administration of the contrast medium Magnetol® (Gadolinium-DTPA (Gd-DTPA) 0.5 M, 0.1 mmol/kg) (Soreq Radiopharmaceuticals, Israel), as described below.
Supplementary data

All tissue culture reagents were purchased from Biological Industries Ltd (Beit Ha Emek, Israel), unless otherwise indicated. Chemical reagents, including salts and solvents, were purchased from Sigma-Aldrich Israel (Rehovot, Israel). Boyden chambers 8 μm were from Transwell-Costar Corp. Hema 3 Stain System was from Fisher Diagnostics. EGM-2 medium was from Cambrex, USA and endothelial cells growth supplement (ECGS) from Zotal, Israel. 2-Iminothiolane was purchased from Sigma-Aldrich (Germany). Maleimide derivative of indodicarbocyanine (IDCC) dye was provided by mivenion GmbH. Sephadex G-25 Superfine was purchased from GE Healthcare (Germany). For buffers and sample preparations, ultrapure water (resistivity ~ 18 MΩ cm⁻¹, pH = 5.6 ± 0.2) was used. Sodium phosphate buffers (10 mM) and (50 mM, 5 mM EDTA) were used for the dye coupling reaction.

dPG-NH₂ synthesis: Dendritic polyglycerol with average molecular weight of 10 kDa (PDI = 1.8) was prepared following literature procedures [40]. To convert 90% of all hydroxyl groups on dPG into amines, the hydroxyl groups were first activated using methanesulfonyl chloride. Mesyl groups were substituted by azides in a successive step. Finally, reduction of the azides by triphenylphosphine via Staudinger reduction resulted in formation of the desired amines. For optimal purification, extensive dialysis was carried out after each reaction step. Quantification of functionalization degree was accomplished by ¹H NMR spectroscopy. ¹H NMR (300 MHz, CD3OD): δ = 3.31–2.40 functionalized PG groups), 4.01–3.21 (PG); ¹³C NMR (75 MHz, CD3OD): δ = 83.0–65.5 (PG), 55.5–43.6 functionalized PG groups).

Coupling of mal-IDCC dye to dPG-NH₂ 90%: 20 mg (2.2 µmol) of dPG-NH₂ 90% (240 µmol amine groups, 1 eq) was dissolved in 1 mL solution of sodium phosphate buffer (50 mM, 5 mM EDTA, pH 7.4). 0.26 mL of (1.6 mg/mL in the same buffer) solution of 2-
iminothiolane (0.42 mg, 3.0 µmol, 1.4 eq) was added to dPG-NH₂ 90% and stirred for 20 min. Afterwards, 2.45 mg (3 µmol, 1.4 eq) Mal-IDCC dye in 0.6 mL of the same buffer solution was added to the reaction mixture and stirred for 2 h. The mixture was taken and loaded on Sephadex G-25 superfine column for purification using PB 10 mM. Purification step was repeated three times to separate the free dyes from the conjugates. The resulting fractions were collected and desalted by frequent addition of nanopure water followed by centrifugation (3 x cycles, 6000 rpm, 10 min) using centrifugal filters (MWCO 3 kDa). The blue solutions were collected and lyophilized at -60°C for 24 h to yield a blue powder. The coupling of the dye to dPG-NH₂ 90% was analyzed on reverse phase thin layer chromatography (TLC).

Coupling of Fluoresein isothiocynate (FITC) to dPG-NH₂ 90%: dPG-NH₂ 90% (240 µmol amine groups, 1 eq) was dissolved in 4 mL PBS (10 mM, pH 7.4). Then 0.8 mg (2 µmol) FITC was dissolved in 300 µL DMSO and added dropwise to dPG-NH₂ 90% solution. The resulting orange mixture was stirred for about 2 hours at room temperature, protected from light. Coupling of the dye to dPG-NH2 90% was checked using TLC. Mixture was transferred into a dialysis tube (4-6 kDa MW cutoff) and dialyzed against miliQ water for 24 hours in the dark. Concentration was controlled using filtering tubes and freeze-drying of dye solution aliquots.

Dynamic light scattering (DLS) and zeta potential determination were performed using a ZetaSizer Nano ZS instrument with an integrated 4 mW He-Ne laser (λ=633 nm; Malvern Instruments Ltd., Malvern, Worcestershire, UK). dPG-NH₂-miR-34a polyplexes were prepared by dissolving 0.25 mg or 0.05 mg of dendrimer and the indicated molar ratio of miR-34a in 1 mL DDW for the hydrodynamic radius measurement or the zeta potential measurement respectively. Samples were incubated for 20-30 minutes at room temperature, then PBS was added from X10 stock to a final buffer concentration of 15 mM, pH=7.4 to the zeta potential sample. All measurements were performed at 25°C using
polystyrol/polystyrene (10×4×45 mm) cell for DLS analysis and folded capillary cell (DTS 1070) for zeta-potential measurements. Results are representative of 3 repeats

Scanning electron microscope (SEM): Polymer (0.1 mg/ml) was mixed with miRNA at the indicated molar ratios and incubated at room temperature for 20 minutes. Samples were dropped on a Si wafer and air-dried for 30 minutes. SEM images were taken by Quanta 200 FEG Environmental SEM (FEI, Oregon, USA) at high vacuum and 10.0 KV.

Electrophoresis mobility shift assay (EMSA): The optimal ratio for the polyplex formation was studied by EMSA. In brief, 100 pmol of miR-34a was incubated with dPG-NH₂ at 1:0.1, 1:0.2, 1:0.5, 1:1 and 1:2 molar ratios of carrier to miRNA (equivalent to N/P ratios of 0.4, 0.9, 2.5, 4.5 and 9) for 15 min at room temperature (RT). Mobility of free and nanocarrier-complexed miRNA was then analyzed by agarose gel electrophoresis. N/P ratios were calculated according to standard formulas reported in the literature [42].

Cell culture: U-87 MG, T98G, U251, U373 and A172 human glioblastoma cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza, Switzerland. Cancer cells were grown in DMEM supplemented with 10% FBS, 100 mg/mL Penicillin, 100 U/mL Streptomycin, 12.5 U/mL Nystatin, and 2 mM L-glutamine (Biological Industries, Bet Ha Emek, Israel). HUVEC were cultured in EGM-2 medium (Lonza, Switzerland). All cells were grown at 37°C in 5% CO₂.

psiCHECK luciferase reporter assay: psiCHECK™-2-based (Promega) construct was prepared for the evaluation of the target activity of miR-34a. One copy of a consensus target sequence, of miR-34a was cloned into the multiple cloning site located downstream of the Renilla luciferase translational stop codon in the 3'-UTR region. The resulting vector was termed psiCHECK™2-miR34. U-87 MG, U251 and U373 cells (1x10⁶) were seeded in 10 cm
dishes and incubated in 37±1°C, 5% CO₂ incubator for 24 h. Each cell-containing plate was transfected with 4 μg psiCHECKTM2-miR34, using 4 μl Lipofectamine™ 2000 reagent (Invitrogen). Following 5-hours incubation, cells were re-plated in a 96-well plate at final concentration of 5x10³ cells per well. Sixteen hours later, cells were transfected with dPG-NH₂-miR-34a, dPG-NH₂-NC-miR or left untreated. Seventy two hours following miRNA transfection, Renilla and FireFly Luciferase activities were measured in each of the miRNA transfected samples, using Dual-Luciferase® Assay kit (Promega) according to manufacturer procedure. Renilla Luciferase activity value was divided by Firefly Luciferase activity value for each sample (normalization). Renilla luciferase activity is finally expressed as the percentage of the normalized activity value in each tested sample relative to the normalized value obtained in cells transfected with psiCHECK™-2-miR34 plasmid only but with no dPG-NH₂-miRNA polyplex.

Cells viability (XTT) assay: Cells were plated in a 96-well plate at final concentration of 5x10³ cells per well. Sixteen hours later, cells were transfected with dPG-NH₂-miR-34a, dPG-NH₂-NC-miR or left untreated. Seventy two hours following miRNA transfection, viable cells were monitored by XTT cell proliferation kit (Biological Industries, Beth Ha Emek, Israel).

Oligonucleotide sequences:

miR-34a mimic and negative control miRNA (NC-miR) were purchased according to the following sequences (BioSpring GmbH, Germany):

MiR-34a guide strand: 5Ph/UGGCAGUGCUUAGCUUGGUUG, passenger strand: CAAUCAGCAAGUAUACUGCCCU; Negative control (NC) guide strand: 5Ph/TGGACTCTGTAGAAAGGAGTATG, passenger strand: TACTCCTTTATCAGACTCCATA.

Primers:
C-MET: F - CAGTGGTGGGAGCACAATAA, R - TGT AAA GTT CCT TCC TGC TTC A;
CDK6: F - TCACGAACA GAC AGA GAA ACC, R - CTC CAG GCTCTGGAACCT TAT C;
Notch1: F - CCCACAAGGTGTCTTCCAG, R – AGGATCAGTGCGCTCGTG;
Bcl-2: F - GGC CAG GGT CAG AGT TAA ATA G, R - GGA GGTTCACAG ATGTCTTC TC;
Actin: F – CCAACCCGAGAATAA, R - CCAGAGGCGTACAGGATAG.

IL-6: F - GGCACTGGCAGAAACACCC, R - GGCAAGTCTCCTCATGATCC;
TNF-α: F - CCCAGGCAGTCAGATCATCTTC, R - TCAGCTGAGGTTTGCTACAA;
GAPDH: F - ATTCACCCATGGCATAATC, R - GGATCTCGCTCCTGGAAGATG.

Cancer cells migration assay: Cells migration was evaluated using transwells chambers with pore size of 8 mm (Costar Corp., Corning, NY, USA). U-87 MG and A172 cells were treated with dPG-NH2-miR-34a, dPG-NH2-NC-miR (200 nM miR) or left untreated. Forty eight hours later, cells (1x10^5/ well) were seeded at the upper chamber of 10 µg/mL fibronectin-coated 24-well transwells. Following 2 h of incubation, 10% FBS-containing media or serum-free media were added to the lower chamber. Cells were allowed to migrate to the lower chamber for additional 4 h, followed by fixation using methanol and staining (Hema 3 stain system, Fisher HealthCare). The stained migrated cells were imaged using a Nikon TE2000E inverted microscope integrated with Nikon DS5 cooled CCD camera. Migrated cells from the captured images were counted using NIH imageJ software. Migration was normalized to untreated cells towards 10% FBS.

HUVEC migration assay: Cells migration was evaluated using transwells chambers with pore size of 8 mm (Costar Corp., Corning, NY, USA). HUVEC (2x10^5 cells) were added to the upper chamber of 10 µg/mL fibronectin-coated 24-well transwells chambers. Following 2 hours of incubation, conditioned media from A172 cells (pretreated with dPG-NH2-miR-34a, dPG-NH2-NC-miR or no treatment), serum-free media (negative control) or 10% FBS-
containing media (positive control) were added to the lower chamber. Cells were allowed to migrate to the lower chamber for an additional 4 h, followed by fixation using methanol and staining (Hema 3 stain system, Fisher HealthCare). The stained migrated cells were imaged using a Nikon TE2000E inverted microscope integrated with Nikon DS5 cooled CCD camera. Migrated cells from the captured images were counted using NIH ImageJ software.

Conditioned media preparation: Conditioned media (CM) for HUVEC migration assay was collected from confluent 100 mm tissue culture plates of A172 GBM cells following 48 hours incubation following treatment with dPG-NH₂-miR-34a, dPG-NH₂-NC-miR, or no treatment. Prior to the experiments, CM was filtered through 0.45 mm syringe filter to remove cells and debris.

Peripheral blood mononuclear cells (PBMCs) isolation: Blood sample obtained from healthy human donors was purchased from the blood bank of Sheba Medical Center (Ramat Gan, Israel), diluted with sterile PBS and split to 10 x 50 mL tubes (30 mL/ tube). Ficoll (10 mL) was slowly and gently dispensed at the bottom of the tube forming 2 well defined layers. Blood solutions were centrifuged at 20°C, 300 g (1400 rpm) for 30 min with deceleration 0. Light PBMCs ring were removed from all 10 tubes’ interphase into a one new 50 mL tube. PBMCs were split to 4 tubes (12 mL/ tube), PBS was added up to 50 mL and tubes were centrifuged at 250 g (1100 rpm) for 10 min. PBMCs pellets were then resuspended in approximately 40 mL RPMI 1640 growth medium (with 10% FBS, L-Glutamine, PSN).

MRI analysis: Tumor progression was assessed by gadolinium (Gd)-diethylenetriamine pentacetate (DTPA)–enhanced T1-weighted MRI (Bruker, Biospin). The MRI scans were performed under inhalational 1%–2% isoflurane (Nicholas Piramal) anesthesia in 98% oxygen. Mouse was scanned in a 7T/30 spectrometer (Bruker, Biospin) using a quadrature head coil and a 400-mT/m gradient system, under inhalational 1%–2% isoflurane (Nicholas Piramal) anesthesia in 98% oxygen. The MRI protocol included Gd-
DTPA–enhanced T1-weighted MRI (repetition time = 800 ms and echo time = 12 ms). After the first T1 scan, mouse was i.p. injected with 150 µL of 0.1-M Gd-DTPA (Soreq Radiopharmaceuticals). Images were analyzed by mricro and Bru2Anz softwares.

Statistical methods: Data are expressed as mean ± standard deviation (s.d.) for in vitro assays or ± standard error of the mean (s.e.m.) for in vivo assays. Statistical analysis for two sets of data was performed using an unpaired t-test. Significance was defined as $P < 0.05$. 