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Chemotherapy sensitization of glioblastoma by focused ultrasound-mediated delivery of therapeutic liposomes

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1 **Abstract**

2 In glioblastoma, the benefit from temozolomide chemotherapy is largely limited to a
3 subgroup of patients (30-35%) with tumors exhibiting methylation of the promoter region of
4 the O⁶-methylguanine-DNA methyltransferase (*MGMT*) gene. In order to allow more
5 patients to benefit from this treatment, we explored magnetic resonance image-guided
6 microbubble-enhanced low-intensity pulsed focused ultrasound (LIFU) to transiently open
7 the blood-brain barrier and deliver a first-in-class liposome-loaded small molecule *MGMT*
8 inactivator in mice bearing temozolomide-resistant gliomas. We demonstrate that a
9 liposomal O⁶-(4-bromophenyl)guanine (O⁶BTG) derivative can efficiently target *MGMT*,
10 thereby sensitizing murine and human glioma cells to temozolomide *in vitro*. Furthermore,
11 we report that image-guided LIFU mediates the delivery of the stable liposomal *MGMT*
12 inactivator in the tumor region resulting in potent *MGMT* depletion *in vivo*. Treatment with
13 this new liposomal *MGMT* inactivator facilitated by LIFU-mediated blood-brain barrier
14 opening reduced tumor growth and significantly prolonged survival of glioma-bearing mice,
15 when combined with temozolomide chemotherapy. Exploring this novel combined
16 approach in the clinic to treat glioblastoma patients with *MGMT* promoter-unmethylated
17 tumors is warranted.

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1. Introduction

Glioblastoma is the most aggressive type of glial neoplasm and therefore classified as a World Health Organization (WHO) grade IV tumor. It represents the majority of all gliomas in adults, being the most frequent primary grade IV tumor of the brain [1]. After establishing the diagnosis by surgical resection or biopsy, the current treatment strategy for newly diagnosed glioblastoma patients aged 70 or younger includes radiotherapy and temozolomide (TMZ) alkylating chemotherapy [2].

Although the current standard of care for patients with newly diagnosed glioblastoma has improved with the addition of TMZ [3], the overall survival benefit remains moderate and mainly restricted to a subpopulation of patients with MGMT promoter-methylated tumors [4]. Due to its clinical relevance, present clinical trials are stratifying patient populations according to their MGMT promoter methylation status [5]. Most glioblastoma patients harbor a tumor with an unmethylated MGMT promoter leading to *MGMT* gene expression [6] and subsequent ability to repair the O⁶-methylguanine (O⁶-meG) cytotoxic lesions caused by TMZ. Since no other pharmacological agent has been demonstrated to prolong overall survival in glioblastoma patients, a major research focus has been to overcome MGMT-mediated TMZ resistance. This can be achieved by targeting MGMT with pseudosubstrates leading to its depletion. Agents like O⁶-benzylguanine act as substrate analogues to O⁶-meG lesions: they bind MGMT and subject it to ubiquitin-mediated degradation [6]. However, the clinical use of O⁶-benzylguanine yielded disappointing results mainly due to its dose-limiting systemic myelotoxicity when combined with TMZ [7].

Similar MGMT inactivators (*e.g.* O⁶BTG), were developed for central nervous system and other solid tumors, in attempts to overcome this limitation but resulted in similar outcomes [8], precluding further clinical development.

The formulation of MGMT inactivators into liposomal nanocarriers can potentially improve their pharmacokinetic properties, biodistribution profiles and decrease systemic dose-

1 limiting adverse effects [9]. Liposomes are submicron-sized spherical nanocarriers
2 consisting of an aqueous core confined by a phospholipid bilayer that can protect the
3 encapsulated compound from plasma inactivation [10]. Poly(ethylene glycol) (PEG)-
4 coating of these nanocarriers reduces the clearance by the mononuclear phagocyte
5 system, thereby increasing their systemic circulation half-life [11]. Therapeutic agents
6 loaded in liposomes may represent a promising therapeutic strategy against glioblastoma,
7 especially if they are combined with methods mediating their delivery beyond the blood-
8 brain barrier (BBB) at the tumor site.

9 Albeit the BBB is not completely intact in the tumor region, macromolecular therapeutic
10 agents may still not reach gliomas in sufficient concentrations [9]. LIFU in combination with
11 systemically administered microbubbles can facilitate localized BBB opening under MR
12 imaging (MRI) guidance by exerting mechanical stress to the brain endothelium leading to
13 reversible disruption of cellular tight junctions [10]. Importantly, feasibility studies have
14 demonstrated ultrasound-mediated BBB opening [11, 12] and transport of anti-cancer
15 drugs (e.g. doxorubicin and TMZ) in animal models [13-16]. These promising pre-clinical
16 data led to the initiation of a phase I trial where the safety and efficacy of microbubble-
17 enhanced LIFU combined with doxorubicin treatment will be assessed (NCT02343991).

18 We previously reported on a set of small molecule O⁶BTG derivatives and their liposomal
19 entrapment properties, which are potent MGMT inactivators *in vitro* [17]. Consequently, we
20 decided to assess the *in vivo* efficacy of the most promising agent and hypothesized that
21 liposome-based drug delivery in combination with non-invasive MRI-guided LIFU (Fig. 1)
22 may open a new horizon for the use of MGMT inactivators against glioblastoma and allow
23 for a sensitization to TMZ chemotherapy.

Figure 1. Schematic representation of MRI-guided LIFU-mediated liposome delivery across the BBB. A dedicated rodent focused ultrasound (FUS) system integrated into a 4.7 T MR imaging system (MRI) allowed for graphical user interface (GUI)-controlled positioning of the FUS transducer and execution of low-intensity pulsed focused ultrasound (LIFU) sonications. Microbubbles (1) and liposomal MGMT inactivator (2) were intravenously injected into anaesthetized and stereotactically fixated (not shown) mice. Thirty seconds after onset of infusion, the tumor region as identified on T2-weighted MRI was sonicated to induce BBB opening and liposome influx to the tumor site. Safe sonication levels were ensured by driving the radio frequency (RF)-amplifier *via* an in-house developed passive cavitation detector (PCD) controller.

1 **2. Materials and methods**

2 *2.1. Reagents and cell lines*

3 The mouse glioma cell line SMA-497 was derived from a spontaneous astrocytoma in a
4 VM/Dk mouse [18] and provided by Dr. D. Bigner (Duke University, Durham, NC). SMA-
5 497 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10%
6 fetal calf serum (Biochrom, Minneapolis, MN) and 2 mM L-glutamine (Gibco Life
7 Technologies, Paisley, UK). The glioma-initiating cell (GIC) line ZH-161 was established
8 after informed consent and approval of the local ethic committees and previously
9 characterized [19]. The cells were maintained in Neurobasal medium supplemented with 2
10 µl/ml B-27 without vitamin A, 2 mM L-glutamine (Gibco Life Technologies), fibroblast
11 growth factor (FGF)-2, epidermal growth factor (EGF) (20 ng/mL each, Peprotech, Rocky
12 Hill, PA). All cells were grown in a humidified 37 °C incubator with 5% CO₂. BG8235
13 microbubbles were kindly provided by Bracco Suisse SA (Geneva, Switzerland) and show
14 similar characteristics as Bracco's BR-38 microbubbles [20]. TMZ was kindly provided by
15 Schering-Plough (Kenilworth, NJ) and prepared in stock solutions (100 mM) in
16 dimethylsulfoxide (DMSO). Isolated human erythrocytes were supplied by Blutspende
17 Zürich (Zurich, Switzerland).

18 *2.2. O⁶BTG-C18 synthesis and liposome formulation*

19 O⁶BTG-C18 was synthesized and loaded into liposomes to form LP-O⁶BTG-C18 as
20 described previously [17]. Briefly, O⁶BTG (Cayman Chemical, Ann Arbor, MI) was
21 incubated with 1-bromooctadecane (abcr GmbH, Karlsruhe, Germany) and lithium hydride
22 (abcr GmbH) in dimethylformamide for 3 h at 80 °C. After purification by silica gel column
23 chromatography, O⁶BTG-C18 was added at an initial molar drug-to-lipid ratio of 4 mol% to
24 a mixture of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, Lipoid GmbH,
25 Ludwigshafen am Rhein, Germany), cholesterol (Sigma Aldrich, St. Louis, MI) and 1, 2-
26 distearoyl-*sn*-glycero-3-phosphoethanolamine-poly(ethylene glycol 2000) (DSPE-PEG,

1 Lipoid GmbH) dissolved in chloroform at a molar ratio of 72.5:20:7.5. Liposomes were
2 formed after rehydration of the lipid film and immediately extruded to obtain vesicles with
3 sizes of 75 nm. 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-
4 chlorobenzenesulfonate salt (ThermoFisher Scientific, Waltham, MA). (DiD)-labelled
5 liposomes were prepared as follows. Briefly, appropriate volumes of DOPC, CHOL, DSPE-
6 PEG, and DiD chloroform stock solutions were mixed to obtain a molar lipid ratio of
7 52:40:7.5:0.5, respectively. After evaporating the organic solvent and complete drying
8 under vacuum overnight, the lipid film was rehydrated with HEPES-buffered saline (final
9 lipid concentration: 7.8 mM). Following seven freeze/thaw cycles, liposomes were
10 extruded 10 times with a Lipex 10 mL extruder (Lipex Biomembranes, Burnaby, BC,
11 Canada) through 0.05 μ m pore sized polycarbonate membranes (Sterlitech Corporation,
12 Kent, WA) and were stored at 4 °C until further use. The fluorescence intensity was
13 determined with an Infinite M200Pro plate reader (Tecan, Maennedorf, Switzerland) at λ_{ex}
14 = 600 nm and λ_{em} = 668 nm. Hydrodynamic diameters and
15 polydispersity index (PDI) were measured by dynamic light scattering (DLS, intensity-
16 average) using a Delsa Nano C particle analyzer (Beckman Coulter, Brea, CA)
17 (Supplementary Table 1).

18

19 2.3. Immunoblot

20 Whole protein lysates were generated by lysing the cells with Lysis buffer P [21].
21 Denatured whole protein lysates or concentrated supernatants (30 μ g/lane) were
22 separated on 10-15% acrylamide gels. After transfer to nitrocellulose (Biorad, Hercules,
23 CA), blots were blocked in Tris-buffered saline with Tween[®] 20 (TBST) (Sigma Aldrich)
24 containing 5% milk and incubated overnight at 4 °C with primary antibodies specific for
25 human (MT 23.2, ThermoFisher Scientific) or mouse (MAB3299, R&D Systems,
26 Minneapolis, MN) MGMT and β -actin (clone sc-1616, Santa Cruz Biotechnologies, Santa

Cruz, CA). The membranes were then washed in TBST and incubated for 1 h at room temperature with horseradish peroxidase (HRP)-coupled secondary antibodies: anti-mouse, anti-rat or anti-goat (clones sc-2004 and sc-2033, Santa Cruz Biotechnologies). Protein bands were visualized by enhanced chemiluminescence (ThermoFisher Scientific).

2.4. *Clonogenic survival assays*

Clonogenic survival assays were performed by seeding 50 cells per well in a 96-well plate for SMA-497 cells. Cells were exposed to DMSO, O⁶BTG-C18 dissolved in DMSO or LP-O⁶BTG-C18 24 h after seeding. Medium was removed 24 h later and TMZ was added at increasing concentrations. Growth of the SMA-497 cells was followed by observation for 15-20 days in FCS-containing medium. Cell density was assessed by crystal violet staining of adherent cells. Spherogenicity assays were performed by seeding 150 ZH-161 cells per well in a 96-well plate. Cells were exposed to DMSO, O⁶BTG-C18 dissolved in DMSO or LP-O⁶BTG-C18 24 h after seeding. TMZ was added at increasing concentrations and cell growth was followed by observation for 15-20 days. Sphere forming ability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT) cell proliferation assay for ZH-161 cells.

2.5. *Hemolysis assay*

LP-O⁶BTG-C18 was evaluated for its hemolytic activity on isolated human erythrocytes. Leukoreduced and filtered human erythrocytes derived from healthy donors were delivered in saline, adenine, glucose and mannitol (SAGM) nutrient solution and stored at 4 °C until usage. The hemolysis assay was performed by mixing equal volumes of erythrocyte suspension and free O⁶BTG-C18 dissolved in PBS containing polysorbate 80 (Sigma-Aldrich) (25% v/v, isotonic) or LP-O⁶BTG-C18 in PBS at final O⁶BTG-C18 concentrations of either 31 or 310 µg/mL. Hundred % hemolysis was determined by preparing a sample of

ultrapure water instead of liposomes. Additionally, control samples of PBS and Triton X-100 (10 µg/ml) (Sigma-Aldrich) were mixed together with erythrocyte suspension as negative and positive controls, respectively. The samples were incubated for 1 h at 37 °C while gently shaking. Then, erythrocytes were separated by centrifugation at 1000 x g for 5 min and the amount of released hemoglobin in the supernatant was determined by spectrophotometry at 578 nm within a microtiter plate using an Infinite M200Pro plate reader (Tecan).

2.6. *Cryo-transmission electron microscopy (cryo-TEM) imaging*

Samples of 5 µL at a concentration of 4 mol% were added to a copper grid covered by holey carbon film (Quantifoil Multi A Micro Tools GmbH, Jena, Germany). Each sample was snap-frozen into a cryobox of liquid ethane (Carl Zeiss NTS GmbH, Oberkochen, Germany) and immediately transferred into the pre-cooled cryo-electron microscope (Philips CM 120, Eindhoven, Netherlands). Images were captured using a 2k CMOS Camera (F216, TVIPS, Gauting, Germany) (Supplementary Fig. 1).

2.7. *Animal studies*

All procedures were performed in accordance with the Cantonal Veterinary Office Zurich and Swiss Federal Food Safety and Veterinary Office (Permission number 085/2014). For the studies involving glioma-bearing mice, VM/Dk mice were bred in house. Mice of 6-10 weeks of age were used in all experiments. Before all intracranial procedures, mice were anaesthetized using an intraperitoneal 3 component injection consisting of fentanyl (0.05 mg/kg; Fentanyl, Kantonsapotheke, Zurich), midazolam (5 mg/kg; Dormicum®, Roche Pharma AG, Reinach) and medetomidine (0.5 mg/kg; Dorbene®, Graeb AG, Bern) mixed in 0.9% NaCl. For intracranial tumor cell implantation, the mice were fixed under a stereotactic device (Stoelting, Wood Dale, IL) and a burr hole was drilled into the skull 2 mm lateral and 1 mm posterior to the bregma. A Hamilton syringe needle was introduced

1 to a depth of 3 mm. SMA-497 murine glioma cells (5×10^3) in a volume of 2 μ L PBS were
2 injected into the right striatum of VM/Dk mice. For the *in vivo* MGMT depletion studies, 10
3 days after tumor inoculation, the LIFU protocol (section 2.7.2) was applied and animals
4 were perfused with PBS 48 or 72 h after treatment. Tumor-bearing and contralateral
5 hemispheres and liver were extracted. Tissues were dissociated with a scalpel and lysed
6 in RIPA lysis buffer containing protease (P-8340, Sigma) and phosphatase 2/3 (P-0044; P-
7 5726, Sigma) inhibitor cocktails. MGMT protein levels were assessed by immunoblot as
8 described in the 'Immunoblot' section. For survival studies, 5 or 6 days after tumor
9 inoculation, the LIFU protocol was applied and 2 days later, TMZ (10 mg/kg) or DMSO
10 control was orally administered for five consecutive days (Supplementary Fig. 2). TMZ was
11 dissolved in sterile water containing 0.1% DMSO. Mice were sacrificed when they reached
12 grade 2 neurological symptoms [22]. For hematological analyses, blood was collected from
13 three pre-randomized mice three days after TMZ treatment and analyzed at the laboratory
14 for veterinary medicine (Zurich, Switzerland). For liposome extravasation studies, the LIFU
15 protocol was applied in healthy C57BL/6J (Charles River Laboratories, San Diego, CA) or
16 VM/Dk mice intravenously injected with DiD-labeled liposomes (13 μ mol lipid/kg) and 2 h
17 after treatment animals were perfused with PBS and 20% paraformaldehyde (PFA). Brains
18 were fixed for 24 h in 20% PFA and then cryoprotected in 30% sucrose solution for further
19 48 h. Fixed brains were mounted in Cryochrome medium (ThermoFisher Scientific) and
20 cut in sections of 8 μ m. Processed brain sections were mounted on coverslips using the
21 Dako fluorescent mounting medium (S3023, Agilent, Santa Clara, CA) and analyzed for
22 DiD fluorescence (644/665 nm excitation/emission) and brain autofluorescence (430/490
23 nm excitation/emission) detection under the Axio Imager microscope (Zeiss, Oberkochen,
24 Germany). Whole organ fluorescence imaging was performed using the CRI's Maestro In
25 vivo Fluorescence Imaging System 2.2 (Cambridge Research & Instrumentation, Inc.,
26 Woburn, MA). DiD fluorescence accumulation was quantified as DiD fluorescence signal

1 ratios of the LIFU-treated brain regions versus the corresponding untreated regions of the
2 contralateral hemispheres using area fraction analyses [23] in the ImageJ analysis
3 software (<https://imagej.nih.gov/ij/>, Open Source). Ratio values of higher than 1 indicate
4 increased DiD fluorescence intensity/accumulation in the LIFU treated areas. Hematoxylin
5 and Eosin (H&E) staining of cryosections was performed as previously [23]. For
6 immunofluorescence staining of blood vessels, cryosections were fixed, blocked, and
7 stained with primary antibody specific for CD31 (clone MEC 13.3, BD Biosciences,
8 Franklin Lakes, NJ) , followed by the rabbit anti-rat IgG-Alexa Fluor 488-coupled
9 secondary antibody (Thermo Scientific, Waltham, MA) and mounted in Vectashield
10 Mounting Media with 4', 6'-diamino-2-phenylindole (DAPI) (Vector laboratories, Inc.,
11 Burlingame, CA). Confocal images were acquired using a Leica SP5 confocal
12 microscope (Wetzlar, Germany).

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14 2.7.1. *Magnetic resonance imaging (MRI)*

15 Anesthesia was induced using 3% isoflurane (Abbott, Cham, Switzerland) in a 4:1
16 air/oxygen mixture. The tail vein was cannulated for administration of contrast agents.
17 Body temperature was kept at 36.0 ± 0.5 °C using a hot water circuit integrated into the
18 animal support (Bruker, Billerica, MA). Data were acquired on a Bruker BioSpec 47/30
19 (Bruker) small animal MR system operating at 4.7 T. The system was equipped with a 2×2
20 phased-array mouse head surface coil (Bruker). Tumor size was measured using T2-
21 weighted MRI starting on day ten after tumor implantation, and then every three days with
22 the following parameters: repetition time (TR) = 300 ms, echo time (TE) = 3.854 ms, matrix
23 size = 384×384, field of view (FOV) = 20×20 mm² (resolution = 0.052×0.052 mm²), slice
24 thickness = 0.5 mm. Tumor volume was quantified using the Volumest plugin in the
25 ImageJ analysis software (<https://imagej.nih.gov/ij/>, Open Source). BBB opening was
26 confirmed by Gd-DOTA-enhanced T1-weighted MRI acquired with the following

parameters: 3D FLASH sequence, TR = 25 ms, TE = 3.884 ms, flip angle 30°, number of average = 15, matrix size = 40x60x40, FOV = 12x20x14 mm³ (resolution = 0.300x0.333x0.350 mm³), Gd-DOTA 50 µl intravenously (Guerbet, Paris, France). Gd-DOTA enhancement is shown as maximal intensity projection on a 3D-rendered mouse brain template using 3dSlicer (<https://www.slicer.org>, Open Source).

2.7.2. *MRI-guided low intensity-pulsed focused ultrasound (LIFU) application*

The intervention protocol for LIFU-mediated opening of the BBB on anesthetized mice consisted of animal preparation with clean shave of the skull region to be exposed to ultrasound, insertion of a tail vein catheter, positioning and fixation on an animal bed, acoustic coupling of skull and transducer and MRI-guided definition of target tissue volume. Mice were intravenously injected with O⁶BTG-C18 or LP-O⁶BTG-C18 at 7.2 mg/kg, corresponding to 310 µmol lipid/kg. LIFU-mediated BBB permeabilization was achieved approximately 2 minutes after liposome administration, using a dedicated MR-compatible rodent FUS system (IGT, Pésac, France) attached to a 4.7 T rodent MRI system (Bruker) that allowed for computer-controlled positioning of the LIFU transducer and parametrization of LIFU application. The LIFU protocol consisted of standard isoflurane gas anesthesia and subsequent infusion of 50 µL (16 x10⁶ bubbles) softshell BG8235 microbubbles (Bracco Suisse SA) at a rate of 1 µL/s for a duration of 50 s in total. Thirty seconds after onset of the microbubble infusion, the right tumor-bearing hemisphere was sonicated at the tumor injection site as identified on T2-weighted MR images. Ultrasound was applied using a 6-element annular array transducer (Imasonic, Besançon, France) carrying a passive cavitation detector and was delivered in bursts of 10 ms at a repetition frequency of 1 Hz for a total duration of 180 s. LIFU pressure was controlled by an automated feedback loop that analyzed the passive cavitation detection (PCD) signatures and drove LIFU pressure below inertial cavitation threshold (0.28 – 0.55 MPa).

Using real-time feedback from locally induced cavitation allowed to compensate for acoustic effects caused by physiological and geometrical differences between individual animals and setups (Supplementary Fig. 3). BBB opening was confirmed by gadolinium (Gd-DOTA)-enhanced (Sigma-Aldrich) MRI.

2.7.3. Quantification of O⁶BTG-C18 in serum

Blood was extracted 1, 3, and 6 h after tail vein injection of free or LP-O⁶BTG-C18. At the indicated time, blood was collected *via* cardiac puncture in Microvette[®] tubes (Sarstedt, Sevelen, Switzerland) containing a clotting activator to obtain serum. The samples were gently mixed for 5 min, left for 30 min to clot at room temperature and then centrifuged for 5 min at 10'000 x *g*. The supernatant was collected and immediately frozen in liquid nitrogen and stored at -80 °C until further usage. Samples were spiked with an appropriate amount of the internal standard, O⁶BTG-C18 (N7). O⁶BTG-C18 was purified *via* acetone protein precipitation followed by hexane liquid-liquid extraction and the drug amount was analyzed with liquid chromatography combined with a linear ion trap mass spectrometer (LC–MS) (LTQ XL, ThermoFisher Scientific) using a reverse-phase XBridge BEH C18 column (130 Å, 5 µm, 4.6 mm x 250 mm, Waters, Milford, MA). The employed method was isocratic using a methanol/water mixture of 97:3 (v/v) and a flow rate of 0.8 mL/min. Drug quantification was carried out using calibration curves of spiked O⁶BTG-C18 standard solutions.

2.8. Statistical analyses

Statistical calculations were done using the GraphPad Prism Version 5 including paired t-test (single comparisons), 1-way ANOVA and Bonferroni post-hoc testing (multiple comparisons) and Log-rank (Martel-Cox) testing for survival analyses. *P* values of ≤ 0.05 (*;#), ≤ 0.01 (**;##) or ≤ 0.001 (***) were considered statistically significant.

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3. Results and Discussion

3.1. *O⁶BTG-C18 reduces MGMT protein levels and sensitizes glioma cells to TMZ exposure*

The current standard of care for newly diagnosed glioblastoma involves tumor resection followed by radiotherapy plus TMZ chemotherapy [2]. However, this regiment confers benefit only to a small subgroup of patients with tumors harboring MGMT promoter methylation. Therefore, there is a substantial need to overcome MGMT-mediated resistance and increase the therapeutic activity of TMZ. Based on the commercially available MGMT depleting drug O⁶BTG, we synthesized several small molecule MGMT inactivators [17]. Through an initial screen of several first-in-class O⁶BTG derivatives we focused on the O⁶BTG-C18 derivate with an N9 modification (Fig. 2A). O⁶BTG-C18 reduced MGMT protein levels in a concentration-dependent manner in murine SMA-497 and patient-derived ZH-161 glioma cells (Fig. 2B). In line with previous data [17, 24], substitution in the N9 position did not substantially affect the MGMT-depleting activity of O⁶BTG in glioma cells. The liposomal (LP)-O⁶BTG-C18 nanoparticles had a mean hydrodynamic diameter of approximately 72 nm (Supplementary Table 1). Cryo-TEM images of the vehicle liposomes (control) and LP-O⁶BTG-C18 confirmed the spherical unilamellar structure of the nanoparticles [17] and indicated that no drug precipitate was present (Supplementary Fig. 1). We confirmed the biological activity of these stable LP-O⁶BTG-C18 particles by their MGMT-depleting activity as soon as 24 h after exposure with the effect lasting up to 72 h post-treatment in glioma cells (Fig. 2C). MGMT depletion had the expected effect, as pre-exposure of free O⁶BTG-C18 or LP-O⁶BTG-C18 sensitized SMA-497 or ZH-161 cells to increasing TMZ concentrations; with the EC₅₀ decreasing 6-fold for SMA-497 cells and 3-fold for ZH-161 cells (Fig. 2D,E). Importantly, LP-O⁶BTG-C18 did not exert any hemolytic effect on isolated human erythrocytes (Fig. 3), rendering this formulation suitable for further

in vivo investigation. This is in contrast to the control formulation which had to be prepared with a surfactant in order to dissolve O⁶BTG-C18, which induced significant red blood cell lysis *in vitro*.

Figure 2. Free and liposomal O⁶BTG-C18 deplete MGMT in glioma cells. **A.** O⁶BTG-C18 was synthesized *via* nucleophilic substitution using bromoalkane Br-C18 and lithium hydride as base in dimethylformamide at 80°C. 75-nm sized LP-O⁶BTG-C18 was composed of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, cholesterol and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-poly(ethylene glycol) in a molar ratio of 72.5:20:7.5. **B, C.** Murine SMA-497 or patient-derived sphere-forming ZH-161 glioma cells were exposed to increasing concentrations of free O⁶BTG-C18 (**B**) or 5 μ M LP-O⁶BTG-C18 (**C**) and MGMT protein levels were assessed by immunoblot. **D, E.** SMA-497 (**D**) or ZH-161 (**E**) cells were seeded for clonogenicity or spherogenicity assays and exposed to 5 μ M of O⁶BTG-C18 or LP-O⁶BTG-C18 for 24 h. TMZ was added at increasing concentrations and the cells were allowed to grow for 20 days. Clonogenicity or spherogenicity was determined by crystal

1 violet or MTT staining, respectively. * = DMSO vs O⁶BTG-C18; # = DMSO vs LP-O⁶BTG-
2 C18. Results are expressed as means and SD (n=3), statistical analysis was performed
3 with one-way ANOVA and Bonferroni post-hoc testing with * or # $p \leq 0.05$, ** or ## $p \leq$
4 0.01 and *** $p \leq 0.001$.

5
6 **Figure 3. LP-O⁶BTG-C18 does not induce hemolysis of human erythrocytes.** O⁶BTG-
7 C18 dissolved in polysorbate 80 solution or LP-O⁶BTG-C18 were mixed together with
8 human erythrocytes in suspension for 1 h at 37 °C. Released hemoglobin in the
9 supernatant was determined by spectrophotometry. Triton X-100 was used as positive
10 control, PBS as a negative control, and polysorbate 80 or empty LP vehicles were used as
11 controls for their respective agents. Hundred % of hemolysis threshold was identified using
12 ultrapure water. Results are expressed as means and SD (n=3).

3.2. MRI-guided LIFU facilitates BBB opening

We developed a LIFU protocol to deliver the loaded liposomes across the BBB in mice using a dedicated experimental platform that allows for non-invasive, MRI-guided, fast, and reproducible BBB opening. Ultrasound pressure was controlled by an automated feedback loop that analyzed the passive cavitation detection signatures and drove LIFU pressure below inertial cavitation threshold (0.28 – 0.55 MPa). Our LIFU system performed an *in situ* titration of the acoustic pressure for each animal prior to the application of the therapeutic pulses. Based on the determined target cavitation dose, a microbubble feedback loop ensured that ultrasound pulses were applied to reliably open the BBB without causing irreversible damage to the brain tissue (Supplementary Fig. 3). This protocol differs from traditional methods [12, 25-27], since it does not titrate pressure through a cohort of test animals to establish a fixed acoustic pressure, but instead determines a safe and adequate pressure for each animal individually using a softshell microbubble feedback loop. Using this novel approach we could compensate for acoustic effects caused by physiological and geometrical differences between animals and experimental conditions. Common techniques to confirm ultrasound-mediated BBB opening include contrast-enhanced T1-weighted MRI, assessment of extravasated Evans blue dye [15], or histological detection of the delivered drug agent [15]. Here, besides conducting *in vivo* gadolinium (Gd-DOTA)-enhanced MRI to confirm BBB opening (Fig. 4A), we generated and delivered stable fluorescent lipophilic carbocyanine derivative (DiD)-labelled liposomes immediately after LIFU application. DiD-labelled liposomes were visualized *ex vivo* 2 h after LIFU treatment, whereas Gd-DOTA enhancement MRI was performed in real-time right after the LIFU-mediated BBB opening. Together with the strong positive contrast enhancement and rapid body elimination, the smaller size of Gd-DOTA [28] might explain the more pronounced extravasation observed compared to DiD-labelled liposomes

(Fig. 4A). Similar to quantum dot-loaded cationic liposomes [29], DiD-labelled liposomes were detected *ex vivo* specifically in the sonication area (Fig. 4B-E). In agreement with previous studies, blood vessel staining revealed no apparent damage on vasculature structure after LIFU treatment (Supplementary Fig. 4), suggesting that LIFU does not cause irreversible damage to the BBB [11, 12]. Quantification of DiD fluorescence in the sonicated brain areas versus untreated regions of the contralateral hemispheres revealed significantly increased levels of DiD-liposome accumulation in the LIFU-treated region (Fig. 4F). These data demonstrate that controlled LIFU-mediated BBB disruption can efficiently and selectively facilitate the delivery of liposomal formulations into the target regions under MRI guidance.

Figure 4 legend on next page.

Figure 4. LIFU-mediated BBB opening facilitates liposomal nanoparticle delivery. A-F. Healthy mice were intravenously injected with DiD-labelled liposomes (0.3 µg/kg). LIFU was applied targeting a specific region in the right hemisphere (white arrowheads) with the contralateral non-LIFU treated hemisphere serving as a control (blue arrowheads). 3D reconstruction of enhancing region in T1-weighted MRI after intravenous administration of Gd-DOTA following LIFU application overlaid to schematic mouse brain is shown. The color-coded region indicates the extent of Gd-DOTA extravasation due to LIFU-mediated BBB disruption. DiD fluorescence and enhancing MRI volumes are depicted (**A**). DiD extravasation at the sonication site was assessed on perfused brains *ex vivo* either macroscopically (**B**) or using histological sections (**C-E**) by fluorescence microscopy. The 430/490 nm excitation/emission was used as a control for brain autofluorescence (AF). White squares depict the location of sonication and DiD-labelled LP extravasation. Scale bar = 200 µm. 10x magnification in white square (**B, E**). Representative images of one mouse (n=4). **F.** DiD fluorescence accumulation was quantified as DiD fluorescence intensity ratio of the LIFU-treated or non-LIFU exposed brain regions of the right hemisphere versus the corresponding untreated regions of the contralateral hemisphere. The dashed line indicates the baseline ratio for no change. Results are expressed as means and SD (n=4), statistical analysis was performed with paired t-test with ** $p \leq 0.01$.

3.3. *LP-O⁶BGT-C18 in combination with LIFU exerts potent in vivo MGMT-depleting activity in glioma-bearing mice*

Next, we assessed the pharmacokinetics and *in vivo* activity of LP-O⁶BTG-C18 in SMA-497 glioma-bearing mice after intravenous application. Liposomal entrapment resulted in a substantial reduction in the clearance of O⁶BTG-C18, corresponding to an almost 20-fold increase in area under the serum concentration versus time curve, up to 6 h post-injection compared to the control formulation (Fig. 5A). We then went on to assess degradation-inducing MGMT protein depletion after systemic administration of LP-O⁶BTG-C18 in SMA-497 glioma-bearing mice. The increase in systemic exposure combined with an increased delivery across the LIFU-permeabilized BBB allowed for a potent reduction in MGMT protein levels 48 and 72 h after treatment in orthotopically growing gliomas (Fig. 5B). Free O⁶BTG-C18 reduced MGMT protein levels after LIFU treatment only after 72 h (Fig. 5B). This less prominent effect for the O⁶BTG-C18 control formulation indicates that a prolonged circulation time is important for sufficient deposition of the agent at the tumor site. Moreover, the fact that LP-O⁶BTG-C18 required 48 h to exert its potent MGMT depleting activity after assisted LIFU guidance into the tumor area suggests that a potential sustained O⁶BTG-C18 release from the liposomal formulation might be in effect. Neither agents formulated with liposomes nor in solution had an effect on MGMT protein levels without LIFU application (Fig. 5B), indicating that BBB opening is required to enhance their activity at the tumor site. Our data are in line with other therapeutic approaches attempting to reduce MGMT protein levels in TMZ-resistant gliomas via the delivery of MGMT-targeting siRNA oligonucleotides either by continuous administration using osmotic pumps [30], or direct tumoral injections [31]. However, our approach has the advantage of utilizing a derivative of an MGMT inactivator combined with a non-invasive delivery method already considered for clinical use [7, 8]. Liver MGMT levels were used as additional control and remained unaltered after treatment (Fig. 5C), suggesting that the

sonication of the tissue in the brain area may facilitate the diffusion and cellular uptake of LP-O⁶BTG-C18. Moreover, colloidal formulations (control micelles and liposomes) are known to be mainly taken up by the mononuclear phagocyte system rather than hepatocytes [32].

Figure 5. LP-O⁶BTG-C18 depletes tumoral MGMT levels after LIFU-mediated delivery in glioma-bearing mice. **A-C.** Five x 10³ SMA-497 glioma cells were inoculated stereotactically into the right hemisphere of VM/Dk mice. Ten days after tumor inoculation, mice were intravenously injected with free O⁶BTG-C18 or LP-O⁶BTG-C18 (dose for both formulations: 7.2 mg/kg). Mice were PBS-perfused 1, 3, or 6 h after treatment and serum was collected for determination of O⁶BTG-C18 concentration via liquid chromatography-mass spectrometry. Results are expressed as means and SD (n=3-6). Statistical analysis was performed with one-way ANOVA and Bonferroni post-hoc testing with * *p* < 0.05 (**A**). The tumor region was targeted using MRI and the LIFU sonications were either applied (+LIFU) or not (-LIFU). Mice were PBS-perfused 48 or 72 h after treatment and the tumor-bearing hemisphere (n=3) or liver (n=2) were lysed. MGMT protein levels in brain (**B**) and liver (**C**) samples were assessed by immunoblot. Densitometry analyses of MGMT protein levels normalized to the corresponding β -actin control are depicted. Vehicle injections were used as a control. Results are expressed as means and SD.

1
2 3.4. *LIFU-mediated delivery of LP-O⁶BTG-C18 reduces tumor growth in vivo and*
3 *prolongs survival of mice bearing TMZ-resistant experimental gliomas*

4 Based on these promising findings, we went on to examine the combination of LP-O⁶BTG-
5 C18 and LIFU as a strategy for TMZ sensitization *in vivo*. Male and female mice were
6 distributed equally amongst the treatment groups. The tumor region in the right hemisphere
7 of SMA-497 glioma-bearing mice was targeted for focused LIFU sonication under MRI
8 guidance and Gd-DOTA-enhanced T1-weighted MRI confirmed the BBB opening (Fig. 6A).
9 Our data are in agreement with previous studies [14, 15, 33], showing enhanced Evans blue
10 or gadolinium extravasation after ultrasound sonication in the tumor region, compared to the
11 leaky glioma vasculature itself (Supplementary Fig. 5). LP-O⁶BTG-C18 was administered
12 intravenously immediately before LIFU application and subsequent oral treatment with TMZ
13 significantly reduced the growth rate of SMA-497 gliomas as demonstrated by MRI (Fig.
14 6B,C), and prolonged survival compared to all other treatment groups (Fig. 6D;
15 Supplementary Table 2). In contrast to previous studies [17, 18, 26, 28], our experimental
16 setup allowed to control for the extent of LIFU-mediated BBB opening between the treatment
17 groups, as visualized by Gd-DOTA influx (Supplementary Fig. 6). The comparable grades
18 of BBB opening between the groups receiving LIFU treatment suggest that the survival
19 prolongation can be attributed to the combinatorial treatment regimen and not any potential
20 variation in BBB openings. While a single LIFU-mediated LP-O⁶BTG-C18 dose was
21 sufficient to sensitize gliomas to TMZ, tumor growth inhibition could potentially be
22 propagated by frequent treatments. The pronounced therapeutic efficacy of this novel
23 combinatorial treatment was confirmed in a second, independent study (Supplementary Fig.
24 7). LIFU in combination with LP-O⁶BTG-C18 and TMZ represents the first treatment
25 approach resulting in a clear therapeutic benefit in this highly therapy-resistant glioma
26 model. Experimental SMA-497 gliomas closely recapitulate the highly infiltrative nature of

1 human glioblastoma, and respond neither to radiotherapy nor anti-angiogenic treatment nor
2 transforming growth factor (TGF)- β receptor inhibition [23]. Given their aggressive growth *in*
3 *vivo*, previous studies have failed to show a survival benefit upon treatment with various
4 drugs [23, 34]. Since LP-O⁶BTG-C18 is biologically active as an MGMT inactivator in human
5 ZH-161 glioma cells as well (Fig. 2C,E), assessing the efficacy of this combinatorial
6 approach in patient-derived xenograft glioma models can further aid its clinical translation.
7 Other studies showed that concurrent TMZ administration and ultrasound-induced BBB
8 disruption prolonged the survival of animals bearing MGMT promoter-methylated gliomas
9 [14, 15]. In our experiments, mice treated with LIFU and TMZ also exhibited reduced tumor
10 growth and prolonged survival compared to TMZ alone despite the inherent MGMT-
11 mediated TMZ resistance of SMA-497 gliomas (Fig. 6 C,D; Supplementary Table 2;
12 Supplementary Fig. 7). Although dose-dense treatments did not demonstrate improved TMZ
13 efficacy in patients with glioblastomas expressing MGMT [35], our findings suggest that
14 ultrasound-mediated BBB opening could increase TMZ exposure and anti-tumor activity
15 [15]. Previous studies have shown that TMZ has the potential to deplete MGMT when
16 administered in higher concentrations [36, 37], which may explain the marginal survival
17 benefit observed with TMZ treatment when LIFU was applied without the liposomal MGMT
18 inactivator in our model. We did not observe acute hematological toxicity or other side effects
19 when O⁶BTG-C18 was applied as a liposomal formulation together with TMZ or combined
20 with transient BBB opening or both. The overall white blood cell, red blood cell and platelet
21 count remained unchanged within normal ranges (Supplementary Fig. 8). Since LP-O⁶BTG-
22 C18 was given intravenously to glioma-bearing mice and TMZ was administered orally, our
23 study also provides a better model of the clinical setting compared to previous approaches
24 [38, 39]. Compared to our study, an almost 5-fold higher dose of O⁶BTG was required in
25 other experimental set-ups to sensitize other tumors to alkylating chemotherapy [40, 41].

1 The majority of studies assessing the penetration and efficacy of therapeutic nanoparticles
2 in pre-clinical glioma models utilize relatively large liposomes that largely rely on passive
3 BBB delivery via the enhanced permeability and retention effect [30, 31, 42, 43]. The data
4 presented in this study strongly suggest that O⁶BTG-C18-loaded liposomes require LIFU
5 application to efficiently bypass the BBB and exert potent chemosensitizing effects.
6 Alternative ways to circumvent the BBB assessed in the clinical setting include direct local
7 delivery of chemotherapeutic agents in the form of biodegradable polymer wafers at the time
8 of surgery [44], or the use of convection-enhanced delivery *via* intra-parenchymal micro-
9 catheters [45]. However, these applications carry severe limitations compared to the LIFU-
10 based approach, including invasive surgical placement, the increased risk of local neuro-
11 toxicity, hemorrhage, edema, or infection as well as the limited penetration of the drug [46].

Figure 6. LIFU in combination with systemic LP-O⁶BTG-C18 and TMZ treatment

reduces tumor growth and prolongs survival of glioma-bearing mice. A-D. Five x 10³ SMA-497 glioma cells were injected stereotactically into the right hemisphere of VM/Dk mice. Six days after tumor implantation the LIFU protocol was applied targeting the tumor area via MRI together with LP-O⁶BTG-C18 or control treatment. BBB opening was visualized by T1-weighted Gd-DOTA-enhanced MRI (A). Two days later systemic oral administration with TMZ (10 mg/kg) or DMSO control was initiated for five consecutive days. **B, C.** T2-weighted MRI tumor volume monitoring was initiated ten days after tumor

1 inoculation and then every three days for three pre-randomized animals. Representative
2 MRI images of one mouse per group are shown with the yellow lines depicting the tumor
3 area (n=3). Crosses (†) indicate that the animals had to be sacrificed due to score 2
4 neurological symptoms. Values are expressed as means and SD (n=3), statistical analysis
5 was performed with one-way ANOVA and Bonferroni post-hoc testing with * or # $p < 0.05$.
6 * = TMZ vs LIFU+TMZ or LIFU+TMZ+LP-O⁶BTG-C18; # = LIFU+TMZ vs LIFU+TMZ+LP-
7 O⁶BTG-C18. **D.** Mice were treated as described in (**A**) and sacrificed when they developed
8 grade 2 neurological symptoms (n=4-7 per group). Kaplan-Meier survival curves are
9 shown with median survival depicted in parentheses for each group. Survival curves were
10 analyzed for differences *via* Log-rank (Martel-Cox) test with * $p < 0.05$ or *** $p < 0.001$.
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12
13

14 **4. Conclusion**

15 In this work, we formulated a new liposomal-loaded O⁶BTG derivative (LP-O⁶BTG-C18)
16 that exhibits potent MGMT depletion activity *in vitro* and *in vivo*. Our dataset provides
17 strong evidence for the enhanced therapeutic activity of TMZ, when combined with LIFU-
18 mediated delivery of LP-O⁶BTG-C18 in glioma-bearing mice. Since LIFU is currently in
19 clinical evaluation for various indications such as targeted drug delivery (NCT02343991)
20 and neuromodulation (NCT02522429), this new non-invasive combinatorial approach to
21 treat glioblastoma patients with MGMT promoter-unmethylated tumors holds promise for
22 clinical translation. Preliminary findings from trials in patients with recurrent glioblastoma
23 patients also suggest that LIFU-induced BBB opening may improve chemotherapy efficacy
24 [47, 48]. Ultimately, the present study serves as a proof of concept to advance a new class
25 of non-invasive modalities towards clinical application for brain diseases with an unmet
26 medical need.

1 **Appendix A. Supplementary data**

2 Supplementary data to this article are available online.

3

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12 **Conflict of Interest**

13 The authors declare no conflict of interest.

14

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16 **Highlights**

- 17 • A novel therapeutic liposomal agent inactivates MGMT in glioblastoma
- 18 • MRI-guided LIFU opens the BBB to deploy nanotherapeutics in targeted regions
- 19 • LIFU-driven delivery of liposomal MGMT inactivators reverses glioma
- 20 chemoresistance

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Appendix A. Supplementary data

Supplementary Figure 1

Supplementary Figure 1. Cryo-TEM images of liposomal formulations. Samples of control vehicle (**A**) and LP-O⁶BTG-C18 (**B**) at a drug loading of 4 mol% were added to a copper grid covered by holey carbon film, snap-frozen into liquid ethane and transferred into the pre-cooled cryo-electron microscope. Representative images for each formulation are shown. This figure was reproduced from reference [17] with permission.

Supplementary Table 1. Hydrodynamic diameter and PDI of empty liposomes and LP-O⁶BTG-C18 at drug concentrations of 4 mol%. Results are expressed as means and SD (n=3).

Formulation	Hydrodynamic diameter (nm)	PDI
Empty liposomes	76.0 ± 0.3	0.034 ± 0.013
LP-O ⁶ BTG-C18	71.4 ± 0.5	0.052 ± 0.007

1 **Supplementary Figure 2**

2

3 **Supplementary Figure 2. Treatment plan.** Five x 10³ SMA-497 glioma cells were

4 injected stereotactically into the right hemisphere of VM/Dk mice (T.I.). Six days after

5 tumor inoculation, mice were intravenously injected with vehicle control or LP-O⁶BTG-C18

6 (7.2 mg/kg). The LIFU protocol was applied targeting the tumor area *via* MRI. Two days

7 after tumor LIFU treatment, systemic oral treatment with TMZ (10 mg/kg) or DMSO control

8 was initiated for five consecutive days. T2-weighted MRI tumor volume monitoring was

9 initiated ten days after tumor inoculation and then every three days for three pre-

10 randomized animals.

1 **Supplementary Figure 3**

2

3 **Supplementary Figure 3. Microbubble feedback loop for LIFU power control.** Mice
4 were intravenously infused with 50 μ L softshell BG8235 microbubbles at a rate of 1 μ L/s for
5 a duration of 50 s in total. LIFU was applied using a transducer with a PCD and, was
6 delivered in bursts of 10 ms at a repetition frequency of 1 Hz for a total duration of 180 s.
7 Very short (200 μ s) bursts of ultrasound were emitted and the acquired inertial cavitation
8 dose (ICD) signal from the softshell microbubbles was analysed for specific signatures that
9 are indicative for the threshold where desired stable cavitation turns into unwanted inertial
10 cavitation. Applied LIFU power (**right panel**) was adaptively modulated to keep the instant
11 ICD (**left panel**) within the limits of the operation band (highlighted blue area) over 180 s.
12 This protocol was applied to each individual mouse for LIFU treatment. Representative ICD
13 and LIFU power data for one mouse are shown.

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1 **Supplementary Figure 4**

2 **Supplementary Figure 4. Blood vessel staining in brain sections from LIFU-treated**
3 **mice. A-B.** Healthy mice were intravenously injected with DiD-labelled liposomes (0.3
4 μg/kg). LIFU was applied targeting a specific region in the right hemisphere. Hematoxylin
5 and Eosin (H&E) staining of LIFU-treated brain area (**A**), black square indicates 40x area
6 magnification on the right side. CD31staining for blood vessels (green) and nuclear DAPI
7 (blue) staining at 60x magnification (**B**). Representative images from one mouse (n=3).

1 **Supplementary Figure 5**

2

3 **Supplementary Figure 5. Gadolinium (Gd-DOTA) enhancement MRI before and after**

4 **LIFU-mediated BBB opening.** Five x 10³ SMA-497 glioma cells were injected

5 stereotactically into the right hemisphere of VM/Dk mice. Six days after tumor implantation,

6 the LIFU protocol was applied targeting the tumor area via MRI. BBB opening was

7 visualized by T1-weighted Gd-DOTA-enhanced MRI before (**A, C**) and after LIFU (**B, D**) in

8 selected mice. Contrast enhancement indicates the extent of Gd-DOTA extravasation as

9 depicted in green dashed lines. Representative sagittal (**A, B**) and axial (**C, D**) MR images

10 of one mouse are shown.

1 **Supplementary Figure 6**

2 **Supplementary Figure 6. LIFU-mediated BBB opening in different treatment groups.**

3 Five x 10³ SMA-497 glioma cells were injected stereotactically into the right hemisphere of
 4 VM/Dk mice. Six days after tumor implantation, the LIFU protocol was applied targeting the
 5 tumor area (depicted in yellow dashed lines) via MRI alone (**A, E**), with TMZ (**B, F**) or LP-
 6 O⁶BTG-C18 (**C, G**) or with TMZ plus LP-O⁶BTG-C18 (**D, H**). BBB opening was visualized
 7 by T1-weighted Gd-DOTA-enhanced MRI. Contrast enhancement indicates the extent of
 8 Gd-DOTA extravasation within the path of targeted LIFU sonication as depicted in blue
 9 dashed lines (E-H). Representative images of one mouse per group are shown.

1 **Supplementary Figure 7**

2

3 **Supplementary Figure 7. Systemic LP-O⁶BTG-C18 in combination with LIFU and TMZ**

4 **treatment reduces tumor growth and prolongs survival in glioma-bearing mice. A.**

5 Treatment plan: Five x 10³ SMA-497 glioma cells were injected stereotactically into the right

6 hemisphere of VM/Dk mice (T.I.). Five days after tumor inoculation, mice were intravenously

1 injected with vehicle control or LP-O⁶BTG-C18 (7.2 mg/kg). The FUS protocol was applied
2 targeting the tumor area *via* MRI. Two days after tumor LIFU treatment, systemic oral
3 treatment with TMZ (10 mg/kg) or DMSO control was initiated for five consecutive days. **B**,
4 **C**. T2-weighted MRI tumor volume monitoring was initiated ten days after tumor inoculation
5 and then every three days for three pre-randomized animals. Representative MRI images
6 of one mouse per group are shown with the yellow lines depicting the tumor area (**B**).
7 Crosses (†) indicate that the animals had to be sacrificed due to score 2 neurological
8 symptoms. Tumor volume measurements are expressed as means and SD (n=3), statistical
9 analysis was performed with one-way ANOVA and Bonferroni post-hoc testing with * or # p
10 < 0.05. * = TMZ alone vs LIFU+TMZ or LIFU+TMZ+LP-O⁶BTG-C18; # = LIFU+TMZ vs
11 LIFU+TMZ+LP-O⁶BTG-C18 (**C**). **D**. Mice were treated as in (A) and sacrificed when they
12 developed grade 2 neurological symptoms. Kaplan-Meier survival curves are shown with
13 median survival depicted in parentheses for each group. Survival curves were analyzed for
14 differences *via* Log-rank (Martel-Cox) test with * p < 0.05 or *** p < 0.001.

1 **Supplementary Table 2. Survival range. Survival curves were analyzed for**
2 **differences via Log-rank (Martel-Cox) test with *p* values as indicated.**

Treatment group	Mean survival [days]	Median survival [days]	<i>p</i> -value ^a	<i>p</i> -value ^b	<i>p</i> -value ^c	<i>p</i> -value ^d	<i>p</i> -value ^e	<i>p</i> -value ^f	<i>p</i> -value ^g
Untreated (n=4)	15	13	-	-	-	-	-	-	-
LP-O ⁶ BTG-C18 (n=4)	15	13.5	0.265	-	-	-	-	-	-
LIFU+LP-O ⁶ BTG-C18 (n=7)	16	14	0.239	0.246	-	-	-	-	-
LIFU (n=4)	14	13.5	0.193	0.371	0.370	-	-	-	-
TMZ (n=4)	16	14.5	0.096	0.108	0.204	0.158	-	-	-
TMZ+LP-O ⁶ BTG-C18 (n=4)	16	14	0.060	0.064	0.0102	0.390	0.531	-	-
LIFU+TMZ (n=7)	19	17.5	0.011	0.015	0.0103	0.010	0.037	0.018	-
LIFU+TMZ+LP-O ⁶ BTG-C18 (n=7)	30	27	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

^acompared to untreated; ^bcompared to LP-O⁶BTG-C18; ^ccompared to LIFU+LP-O⁶BTG-C18;
^dcompared to LIFU; ^ecompared to TMZ; ^fcompared to TMZ+LP-O⁶BTG-C18; ^gcompared to LIFU+TMZ

1 **Supplementary Figure 8**

2

3 **Supplementary Figure 8. LIFU in combination with systemic LP-O⁶BTG-C18 and TMZ**

4 **treatment does not increase hematological toxicity. A-C.** Five x10³ SMA-497 glioma

5 cells were injected stereotactically into the right hemisphere of VM/Dk mice. Five days

6 after tumor inoculation, the mice were intravenously injected with control vehicle or LP-

7 O⁶BTG-C18 (7.2 mg/kg). The LIFU protocol was applied targeting the tumor area via MRI.

8 Two days after tumor LIFU treatment, systemic oral treatment with TMZ (10 mg/kg) or

9 DMSO control was initiated for five consecutive days. Three days after completion of TMZ

10 treatment, blood samples were collected from three pre-randomized animals from each

11 group. White blood cell (A), red blood cell (B) and platelet (C) populations were measured

12 as a percentage of the total cell population. Dotted lines depict the normal range of each

13 analyzed condition as provided by the laboratory for veterinary medicine (University of

14 Zurich, Zurich, Switzerland). Results are expressed as means and SD (n=3), statistical

15 analysis was performed with one-way ANOVA and Bonferroni post-hoc testing with *p

16 <0.05.

1 **Graphical Abstract**

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