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# Prolonged survival upon ultrasound-enhanced doxorubicin delivery in two syngenic glioblastoma mouse models

Zsofia Kovacs [a,b,e], Beat Werner [a,e], Anahita Rassi [d,e], Jörn Oliver Sass [d,e], Ernst Martin-Fiori [a,e], Michele Bernasconi [b,c,e,*]

[a] Center for MR-Research, University Children's Hospital Zurich, Zurich, Switzerland
[b] Oncology Department, University Children's Hospital Zurich, Zurich, Switzerland
[c] Experimental Infectious Diseases and Cancer Research, University Children's Hospital Zurich, Zurich, Switzerland
[d] Clinical Chemistry and Biochemistry, University Children's Hospital Zurich, Zurich, Switzerland
[e] Children's Research Center, University Children's Hospital Zurich, Zurich, Switzerland

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ABSTRACT

Glioblastoma multiforme (GBM) is the most common and most aggressive malignant primary brain tumor in humans with a very poor prognosis. Chemotherapeutical treatment of GBMs is limited by the blood–brain barrier (BBB). This physical and metabolic barrier separates the blood from the brain parenchyma and prevents the entry of toxins but also of potentially useful chemotherapeutics from the blood into the brain. Microbubble-enhanced focused ultrasound (MB-FUS) has been proposed to disrupt locally and reversibly the BBB to facilitate diffusion of drugs from the micro vasculature into brain tissue. The present study investigates the feasibility and the safety of such an approach in two syngenic mouse models of GBM (GL261 and SMA-560). Local doxorubicin (DOX) concentration in MB-FUS sonicated normal brain tissue as well as in brain tumor tissue was increased as compared to the unsonicated control tissue in the contralateral hemisphere. Moreover, ultrasound mediated BBB disruption, in combination with DOX therapy, resulted in a significant increase of survival and in a slower disease progression in the two syngenic GBM mouse models. In conclusion, our results confirm that MB-ultrasound might ultimately be an effective technology to improve the therapy of GBM, and they provide for the first time evidence that combining MB-FUS with DOX treatment is effective in syngenic mouse models for GBM which can serve as preclinical models to study the impact of immune system on the therapeutic application of MB-FUS chemotherapy.



## 1. Introduction

Glioblastoma multiforme (GBM), the most common and the most malignant primary brain tumor type, is characterized by a widely infiltrative phenotype, which precludes complete surgical resection. GBM's invariably aggressive biological behavior leads to dismal clinical outcome. Despite multimodal treatment that includes surgery, radiation therapy, and chemotherapy with alkylating agents, the median survival time after diagnosis reaches only about 15 months [1,2]. Temozolomide (TMZ), a second-generation orally administered DNA alkylating agent, is the only drug that, in combination with radiotherapy, has increased the survival of GBM patients in the last decades, 5-year survival from 2% to 10% [3]. However, in about half of the patients therapeutic efficacy of TMZ is abrogated by the expression of $O^6$-metylguanine-DNA methyltransferase (MGMT) [4]. Therefore, there is still an urgent clinical need to improve efficacy of therapies against GBM.

One of the major obstacles to an effective chemotherapy of GBM is represented by the blood–brain barrier (BBB) that hinders the penetration of most of the therapeutic compounds into the central nervous system (CNS) and thus prevents drugs from acting on GBM cells that infiltrate the tumor penumbra and can not be removed surgically [5]. Safe methods to modulate the permeability of the BBB could significantly increase the spectrum of available drugs against brain tumors and at the same time allow to achieve therapeutic effects at lower systemic doses with reduced side effects. This in turn could lead to the revival of anticancer drugs that have been suspended from clinical use because the high systemic concentrations needed to cross the BBB were associated with unacceptable adverse side effects. Doxorubicin (DOX) is a widely used antitumor antibiotic possessing significant activity against a variety of human malignancies, including leukemias, lymphomas, sarcomas, and carcinomas such as those of breast and lung [6–13]. However, the use of free DOX is limited by its severe side effects, including nephrotoxicity and cardiotoxicity [14]. In addition, the efficacy of free DOX is

* Corresponding author at: Experimental Infectious Diseases and Cancer Research, August Forel Strasse 1, CH-8008 Zurich, Switzerland. Tel.: +41 44 634 88 06.
*E-mail address:* michele.bernasconi@kispi.uzh.ch (M. Bernasconi).

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hampered by multi-drug resistance originating from the P-glycoprotein, as well as from systemic factors [15,16]. To overcome these problems DOX has been encapsulated in liposomes such as in the FDA-approved Doxil [17,18], which is used to treat metastatic breast cancer [19] and glioblastoma [20,21] due to its favorable pharmacological profile and preserved efficacy in extreme metabolic conditions. Although Doxil strongly reduced the cardiotoxicity of DOX [17], other side effects occurred in patients after DOX treatment, like mucositis and palmar–plantar erythrodysesthesia or hand–foot syndrome due to the local accumulation of the liposomes in skin capillaries [22]. The prevalence of these side effects limits the maximum tolerated dose of Doxil to 50 mg/m$^2$, compared with 60 mg/m$^2$ for free DOX [23]. In order to increase the tumor specific effect with lower injected doses, targeting of DOX-loaded vehicles to the tumors with specific antibodies or ligands [24–26], or by incorporating DOX in stimuli responsive carriers such as pH-, temperature- or ultrasound-responsive nanocarriers has been tried [27–29], and resulted in enhanced drug release and increased life span in preclinical models.

MRI-guided focused ultrasound in combination with *intravenously* (*i.v.*) injected microbubbles, is a drug-independent technology that has been shown to induce localized transient BBB disruption (BBBD) [30–32]. Liposomal DOX has been locally delivered into the brain tumor tissue after MB-FUS-mediated BBB opening, improving outcomes of glioma treatment in mice [33] and rats [34,35]. The majority of MB-FUS studies targeting the brain used encapsulated DOX. However, a major caveat of co-injecting DOX-liposomes and microbubbles is the fact that DOX-liposomes can still extravasate and accumulate in other tissues, like the skin capillaries [36–38].

In this study we investigate the therapeutic impact of microbubble enhanced low intensity ultrasound in combination with free DOX as a preclinical feasibility study in two syngeneic mouse brain tumor models that do not require a deficient immune system: the well-characterized GL261 glioblastoma with B6 genetic background [39] and the highly invasive SMA-560 astrocytoma model with VM/Dk genetic background [40]. These models mimic the growth of GBM in an immunocompetent environment and replicate two scenarios with different degrees of invasiveness.

We demonstrate the feasibility of local DOX delivery into healthy mouse brain tissue in two genotypes as well as in the tumor tissue of two different GBM mouse models. In addition, we evaluate the effect of local delivery of free DOX through ultrasound on the therapy efficacy and mice survival.

## 2. Materials and methods

### 2.1. Cell lines

The two murine glioma cell lines, GL261 [39], and SMA-560 [40] were a kind gift of Prof. Michael Weller (Department of Neurology, University Hospital Zurich, Switzerland). Both cell lines were maintained in Dulbecco's modified Eagle's medium that was supplemented with 10% fetal bovine serum (Gibco–Invitrogen, Basel, Switzerland), 1% L-glutamine and 1% penicillin–streptomycin (Sigma-Aldrich, Buchs, Switzerland).

### 2.2. Microbubbles

BG6895 microbubbles (Bracco Suisse, Geneva, Switzerland) were used in all experiments. These are formulated as a cake produced aseptically by lyophilization of PEG4000 solution containing small amounts (116 μg) of neutral phospholipids, distearoylphosphathidylcholine and a pegylated phospholipid (DPPE-MPEG5000) and palmitic acid similarly to a previously described procedure [41]. Bubble sizes range from <1 μm to approximately 6 μm. Most of the bubbles are less than 2 μm, and there are essentially no bubbles above 6 to 8 μm. For injection, the microbubbles are reconstituted from the cake by adding 5 ml of saline.

### 2.3. Intracranial implantation

All experimental studies were carried out in the accordance with the protocols approved by the Cantonal Veterinary Office of Zurich.

For the GL261 model 12 week old female B6 (Cg)-Tyrc-2J/J mice (B6-albino) were acquired from The Jackson's Laboratory (Bar Harbor, ME). For the SMA-560 model, VM/Dk mice were bred in-house. $1 \times 10^5$ GL261 or $1 \times 10^3$ SMA-560 cells were harvested and suspended in 2 μl PBS for orthotopic injection. Before intracranial implantation, mice were anesthetized with a combination of Ketamine (100 mg/kg; Ketalar, Parke-Davis, Morris Plains, NJ) and Xylazine (20 mg/kg; Rompun, Bayer HealthCare, Leverkusen, Germany) by *i.p.* administration. The anesthetized mice were immobilized in a stereotaxic instrument (Stoelting, Wood Dale, IL) and a hole was drilled in the skull 1.5 mm posterior to the bregma and 2 mm lateral to the sagittal suture. The needle of a Hamilton syringe (Hamilton, Darmstadt, Germany) was introduced to a depth of 3 mm and the cells were injected into the right striatum.

### 2.4. Focused ultrasound

B6-albino wild type or with GL261 tumors were sonicated transcranially with bursts of focused ultrasound. Mice were kept under gas anesthesia on a warm pad; eye-drops were applied to prevent drying. Focused ultrasound was created by a single-element, spherical FUS transducer (center frequency: 612.5 kHz; focal depth: 50 mm; active diameter: 64 mm; model: H-107_MR, Sonic Concepts, Bothell, WA). A coupling cone filled with distilled and degassed water was attached to the transducer and a latex membrane sealed the opening at the tip of the cone (Fig. 1a). Calibration measurements were made in degassed water using an Onda HNR-0500 hydrophone (Onda, Sunnyvale, CA).

### 2.5. Unfocused ultrasound

As a consequence of the highly invasive nature of SMA-560 astrocytoma and the large size it quickly reaches unfocused ultrasound (UUS) was used to target both the tumor core and the invading SMA-560 tumor cells. VM/Dk mice with SMA-560 tumors were sonicated transcranially with bursts of unfocused focused ultrasound (Fig. 1b), targeting the whole tumor-bearing hemisphere. An unfocused, circular single-element ultrasound transducer (A397S-SU, Olympus NDT Inc., Waltham, MA) was used, which had a nominal element size of 29 mm and a center frequency of 500 kHz. The transducer was fixed on the top of a 30 cm tall coupling column filled with degassed distilled water and sealed with a latex membrane. Calibration measurements were made in degassed water using an Onda HNR-0500 hydrophone. Both transducers were placed on a 3D positioning system and both were driven by Agilent 33220A function generator (Agilent Technologies, Santa Clara, CA) via an ENI 2100L-RF 50-dB broadband power RF-amplifier (Electronics & Innovation, Rochester, NY).

### 2.6. Sonications

Immediately after the injection of DOX the microbubble infusion (60 μl, 1 μl/s) started and 30 s later the ultrasound sonications were applied for 3 min. FUS sonications were delivered at 612.5 kHz with 0.4 MPa acoustic pressure in bursts of 10 ms length at 1 Hz repetition time. UUS sonications were delivered at 500 kHz with 0.4 MPa acoustic pressure in bursts of 10 ms length at 1 Hz repetition time. For focused ultrasound experiments the tumor was located on MR images and mechanically aligned with the previously determined MR coordinates of the acoustic focus. For unfocused ultrasound experiments the transducer was visually centered above the cranial penetration hole from tumor injection.