Journal of Controlled Release 264 (2017) 45–54

Contents lists available at ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

Injectable nanomedicine hydrogel for local chemotherapy of glioblastoma after surgical resection

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ARTICLE INFO

Keywords:
Lipid nanocapsules
Gemcitabine
Hydrogel
Nanomedicine
Glioblastoma
Local delivery

ABSTRACT

Glioblastoma (GBM) treatment includes, when possible, surgical resection of the tumor followed by radiotherapy and oral chemotherapy with temozolomide, however recurrences quickly develop around the resection cavity borders leading to patient death. We hypothesize that the local delivery of Lauroyl-gemcitabine lipid nanocapsule based hydrogel ($GemC_{12}$-LNC) in the tumor resection cavity of GBM is a promising strategy as it would allow to bypass the blood brain barrier, thus reaching high local concentrations of the drug. The cytotoxicity and internalization pathways of $GemC_{12}$-LNC were studied on different GBM cell lines (U251, T98-G, 9L-LacZ, U-87 MG). The $GemC_{12}$-LNC hydrogel was well tolerated when injected in mouse brain. In an orthotopic xenograft model, after intratumoral administration, $GemC_{12}$-LNC significantly increased mice survival compared to the controls. Moreover, its ability to delay tumor recurrences was demonstrated after perisurgical administration in the GBM resection cavity. In conclusion, we demonstrate that $GemC_{12}$-LNC hydrogel could be considered as a promising tool for the post-resection management of GBM, prior to the standard of care chemo-radiation.

1. Introduction

Glioblastoma (GBM) is the most aggressive and lethal brain tumor in adults. It is a grade IV astrocytoma characterized by rapid proliferation, high infiltration capacity, chemoresistance and ability to quickly form recurrences, even after multiple surgery and treatment [1]. GBM can be divided into IDH-wildtype GBM (90%) which arises in an acute *de novo* manner without previous lower grade pathology or symptoms, or into IDH-mutant GBM (10%) which derives from the progressive evolution and transformation of lower grade astrocytomas and normally affects younger patients [2]. In both cases, maximal safe surgical resection of the accessible primary tumor is the first and most important step in the management of these tumors, but it can only be applied to 65–75% of GBM patients [3,4]. Following resection, GBM patients are generally treated with standard treatment regimens which include radiotherapy plus concomitant and adjuvant oral chemotherapy with the alkylating agent Temozolomide (TMZ) [5]. However, recurrences develop at the resection border margins (90% of cases) or in other regions of the brain within two years leading, in most of the cases, to death [6,7]. Indeed, despite the efforts of the scientific community, the prognosis for GBM patients remains poor (median survival < 15 months), 2- and 4- year survival rates are 27% and 10% respectively and the long-term survivors are nearly inexistent [8,9].

Limitations in the effectiveness of current standard of care treatments are amplified through the formation of GBM recurrences due to several hurdles. The anatomical location of the tumor interferes with a complete surgical resection while the presence of the blood-brain barrier (BBB) limits the number of cytotoxic drugs that can effectively reach the tumor site at therapeutic concentrations. In addition, GBM cells widely diffuse into the brain parenchyma, and their tendrils are often undetectable by imaging techniques. Moreover, cancer stem cells with high tumorigenic ability, self-renewal potential and strong resistance to radio and chemotherapy have been recognized in gliomas [10–13]. As chemoradiation can have an impact on the wound healing process, GBM patients generally follow the standard radio- and chemotherapy regimen several weeks after surgery, once the wound has healed [14]. During this time gap, the residual tumor cells can proliferate around the resection cavity borders. Further difficulties in

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http://dx.doi.org/10.1016/j.jconrel.2017.08.019
Received 10 August 2017; Accepted 18 August 2017
Available online 19 August 2017

treatment are brought about by the high heterogeneity of GBM cells combined to their innate and acquired chemoresistance, reducing the efficacy of TMZ. Indeed, only one third of GBM patients are responsive to alkylating agents [13,15,16].

In the last decades, many strategies have been adopted to increase the therapeutic efficacy and survival rate of GBM patients (*e.g.* gene therapy, immunotherapy, targeted therapy, nanomedicines, ultrasounds, etc.) [17–22]. Among them, the local delivery of chemotherapeutic drugs in the tumor resection cavity has shown a promising role [23–25]. This approach aims at increasing the local concentrations of the drugs, subsiding systemic side effects, while also reducing the lapse of time between resection and the chemotherapy which in turn prevents the growth of the remaining cancer cells, often responsible of recurrences. Gliadel®, a carmustine-loaded biodegradable wafer, is the most-successful and the only local delivery implant currently approved by the FDA for GBM [26,27]. Its use has shown modest effect in prolonging the overall survival of GBM patients but tumor recurrences have been reported in the majority of treated cases. To improve the sustained intracerebral drug release and overcome limitations such as local side effects, poor drug penetration depth and implant dislodgements, many researchers are currently focusing on the local delivery of cytotoxic drugs through different delivery systems (*e.g.* foams, films, membranes, hydrogels) [25,28]. Our group is mainly focused on craniotomy-based drug delivery *via* anti-cancer loaded hydrogels [29,30]. These injectable and adaptable systems can be implanted or injected into the resection cavity immediately after surgery and can guarantee a sustained release of the drug in the surrounding brain tissue over time. Some hydrogels are also administrable intratumorally in non-operable GBM tumors [31]. Several aspects need to be considered when developing an effective anticancer drug loaded hydrogel for the local treatment of GBM. Firstly, choosing a drug that does not interfere with the mechanisms of action or the chemoresistance pathways of TMZ, and could have radiosensitizing and/or synergic properties with the standard treatments is of importance. Secondly, the release profile of the drug from the hydrogel should be controlled and sustained over time. Finally, the system should be injectable, degradable and well tolerated. It should have mechanical properties compatible with the brain tissue and possibly adapt to the resection cavity and adhere to the brain parenchyma [25].

Recently, we proposed the use of an innovative hydrogel uniquely formed of lipid nanocapsules (LNC) and Lauroyl-gemcitabine ($GemC_{12}$) for the local treatment of GBM [29]. This injectable nanomedicine hydrogel presents mechanical properties adapted for brain implantation and allows a sustained release of the drug over 1 month *in vitro*. *In vivo*, this system is well tolerated during one week in mouse brain and reduces tumor growth in a subcutaneous human GBM model, when compared to free drug.

In this paper, we hypothesize that $GemC_{12}$-LNC nanomedicine hydrogel could improve the GBM recurrences management when injected in the tumor resection cavity immediately after surgery. Therefore, (i) the *in vitro* cytotoxicity and cellular uptake, (ii) the *in vivo* mid- and long-term tolerability in mouse brain, and (iii) the antitumor efficacy of the hydrogel after intratumoral injection in an orthotopic human xenograft GBM model and after local administration in the resection cavity in an orthotopic resection model were investigated.

2. Materials and methods

2.1. Formulation of $GemC_{12}$ lipid nanocapsules hydrogel ($GemC_{12}$-LNC)

The gel formulation $GemC_{12}$-LNC was prepared using a phase-inversion method previously reported in the literature [32]. Briefly, 0.093 g of $GemC_{12}$ (synthesized as previously described [33]), 1.24 g of Labrafac® (Gattefosse, France) and 0.25 g of Span 80 (Sigma-Aldrich, USA) were weighed and stirred in a water bath at 50 °C with 200 μL of acetone (VWR Chemicals, Belgium) until complete dissolution of the drug. The acetone was then allowed to evaporate and 0.967 g of Kolliphor® (Sigma-Aldrich, Germany), 0.045 g of Sodium Chloride (VWR Chemicals, Belgium) and 1.02 g of injectable water (Braun, Germany) were added to the formulation. Three cycles of heating and cooling were performed under magnetic stirring (500 rpm) between 40 and 70 °C. During the last cooling cycle, at the temperature corresponding to the phase-inversion zone, 2.12 g of injectable water was added and the formulation stirred for one more minute. The formulations were then inserted into insulin syringes (BD Micro-Fine™ needle 0.30 mL, Ø 30 G; Becton Dickinson, France) before the gelation process occurred, and stored at 4 °C until further use. The unloaded LNC were obtained using the same method without adding the active compound. For the fluorescent-labeled LNC, 83.4 μL of the fluorescent DiD fluorophor (1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindodicarbocyanine 4-Chlorobenzenesulfonate salt, Thermo Fischer Scientific, USA; 1 mg/mL solution in absolute ethanol), were added to the first step of the formulation process, which was then carried on as previously described protected from the light. All the formulations were obtained under aseptic conditions.

2.2. In vitro cellular studies

2.2.1. Cell cultures

U251, T98-G and U-87 MG glioma cells (ATTC, USA) were cultured in Eagle's Minimum Essential Medium (EMEM; ATTC, USA) while 9L-LacZ cells (ATTC, USA) were cultured in Dulbecco's modified Eagle's Medium with 4.5 g/L glucose, 0.58 g/L L-glutamine and 0.11 g/L sodium pyruvate (DMEM; Gibco, Life Technologies, USA). Medias were supplemented with 10% Fetal Bovine Serum (FBS; Gibco, Life Technologies USA), 100 U/mL penicillin G sodium and 100 μg/mL streptomycin sulfate (Gibco, Life Technologies, USA). Cells were subcultured in 75 cm^2 culture flasks (Corning® T-75, Sigma-Aldrich, USA) and incubated at 37 °C and 5% CO_2.

2.2.2. Cytotoxicity studies (crystal violet assay)

Cytotoxicity assays were performed using crystal violet staining after 48 h of incubation with different concentrations of GemHCl, $GemC_{12}$ or $GemC_{12}$-LNC with or without the hENT1 transporter inhibitor dypiridamole (Dyp; Sigma Aldrich, USA). Cells were seeded at a density of 2.5–5 × 10^3 cells/well depending on the cell type in 96-wells plates and incubated at 37 °C and 5% CO_2. To obtain a cell monolayer and obtain homogenous adhesion of the cells throughout the wells, for U-87 MG cell line wells were previously coated with poly(D)lysine (PDL; 0.1 mg/mL per well; Sigma-Aldrich, USA) and then rinsed with phosphate buffered saline (PBS; Gibco, Life Technologies USA) before being plated and incubated at 37 °C and 5% CO_2[29]. They were then either incubated with Triton X-100 (Sigma-Aldrich, USA), different concentrations of Gemcitabine Hydrochloride (GemHCl; Sigma-Aldrich, USA), $GemC_{12}$, $GemC_{12}$-LNC, unloaded LNC or left untreated. The treatments were dissolved in PBS (GemHCl, $GemC_{12}$-LNC and unloaded LNC) or in Water/Ethanol/Tween® 80 6.9/87.6/5.5 v/v ($GemC_{12}$; [34]) and then suitably diluted in complete culture medium. The concentration of active drug ranged between 0.01 and 25 μM. To study the effect of nucleoside transport inhibitors on drug sensitivity, cells were exposed to Dyp (10 μM) before and during the treatments incubation to inhibit hENT1 transporters [35]. After 48 h of incubation with the treatments, cells were fixed with 10% formalin solution (Merck, Germany) for 20 min and then stained with Crystal violet solution (0.5% in 20% Methanol) for 20 min. The plates were then rinsed with distilled water multiple times, air-dried and observed at the microscope. Methanol was added to the wells and spectrophotometric readings were performed after 30 min at 560 nm with a MultiSkan EX plate reader (Thermo Fisher Scientific, USA). Cells cultured with complete culture medium or Triton X-100 were considered as negative and positive controls, respectively. Results are expressed as relative percentage of living cells compared to the negative control (untreated cells) (N = 3,