Metabolite-based molecular drug delivery by targeting GPR158 in glioblastoma

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Abstract

Glioblastoma multiforme (GBM) is a lethal form of intracranial tumor. One of the obstacles to treat GBM is the blood-brain barrier which limit the transportation of drugs into the tumor site. Here, based on our previous study on metabolitin (MTL) and osteocalcin, we generated a molecular drug delivery system that consisted of metabolitin and small molecules such as fluorescent dye or peptide drugs for diagnosis and treatment. And we designed a GBM diagnostic probe (MTL-ICG) and therapeutic peptide drug (MTL-NBD) that can cross the blood-brain barrier (BBB). In a NIR animal live imaging system, we found MTL-ICG can penetrate cross BBB and label the GBM site. The *in vitro* experiment showed that MTL-NBD had inhibitory effect on GBM cell line (U87-MG). Besides, after orthotopic transplantation of GBM into mouse cortex, treatment of MTL-NBD intravenously showed inhibition trend, which were similar with the effect of NBD, a known anti-tumor polypeptide drug. In addition, we found the GPR158, the receptor of osteocalcin, was also high expressed in grafting site. Taken together, these findings suggest that MTL is a promising cell penetrating peptide targeting GPR158 in GBM, which provide a novel delivery tool for GBM.

Keywords: Metabolitin; Osteocalcin; Glioblastoma multiforme; GPR158; Antagonistic polypeptides; Cell penetrating peptides; Blood brain barrier

33 Introduction

34 GBM is a tumor located in the central nervous system, with headache, vomiting,
 35 epilepsy as the main symptoms. According to the statistics of the American Central
 36 Brain Tumor Registry (CBTRUS), more than three out of every million people in the
 37 United States suffered from GBM from 2012 to 2016 (Ostrom et al., 2019). Primary
 38 GBM accounts for 95% of confirmed GBM cases; secondary GBM accounts for about
 39 5% of confirmed cases (Ohgaki et al., 2004). Among them, the median survival of
 40 patients treated with chemotherapy alone was only 12.1 months, and that of
 41 patients treated with radiotherapy combined with TMZ was only 14.6 months (Stupp
 42 et al., 2005). To date, it is still a challenge to treat GBM, and there are big gap to
 43 understand BGM related signaling pathways, therapeutic drugs and treatment
 44 methods.

45 For GBM treatment drug, as the only standard treatment currently used in clinical
 46 practice, TMZ is a derivative of imidazoltetrazine, a precursor of the anticancer drug
 47 Temorda, which produces cytotoxicity through transformation into an active DNA
 48 alkylation agent (Denny et al., 1994). However, glioma LN-18, T98G and U138 cell
 49 lines have been reported to be consistently resistant to TMZ (Lee, 2016). It takes a
 50 long time to develop new drugs, and it is necessary to find effective drugs for GBM as
 51 soon as possible under the condition of poor prognosis and limited treatment for
 52 GBM. Therefore, some researchers have evaluated the effect of some marketed
 53 drugs on GBM. Studies suggest that psychotropic drugs, such as traditional
 54 antipsychotic chlorpromazine, are promising for the treatment of
 55 chemically-resistant glioma (Oliva et al., 2017). Atypical antipsychotic drug
 56 olanzapine can enhance the inhibitory effect of TMZ on GBM cell lines
 57 (Karpel-Massler et al., 2015). Tricyclic antidepressant promimazine can induce
 58 autophagy in GBM U87-MG cell lines (Jeon et al., 2011). Non-psychiatric drugs such
 59 as bendazole can promote the survival of glioma-bearing mice by inhibiting
 60 microtubule polymerization (De Witt et al., 2017). Clomiphene can inhibit IDH1
 61 mutation of glioma cells, thus significantly inhibiting the growth of glioma in mice

(Zheng et al., 2017). Although there are some achievements in the researches on GBM related pathways and drugs, there are not enough drugs with high efficiency, low toxicity and specific characteristics that can be used in clinical practice. So it is necessary to study other potential signaling pathways and drugs in the development of GBM.

In this study, we attempted to design a novel GBM probe and anti-GBM polypeptide, which consisted of transmembrane peptide MTL (YLGASVPSPDPLEPT), flexible link (GG) and ICG or NBD (TALDWSWLQTE). And we tested the loading capacity of the MTL *in vitro* and *in vivo*.

71

72 **Materials and Methods**

73 ***The experimental materials***

The peptide MTL-NBD (YLGASVPSPDPLEPTGGTALDWSWLQTE) and positive peptide NBD (RQIKIWFQNRRMKWGGTALDWSWLQTE) (Friedmann-Morvinski et al., 2016) were synthesized by Shanghai Jieptide Biotechnology Co., LTD. Luciferase substrates sourced from GOLD BIO. DMEM, DPBS, 0.25% (W/V) trypsin solution, penicillin-streptomycin and fetal bovine serum (FBS) were sourced from Gibco Life Technologies (USA). Cell counting Kit (CCK8) was purchased from DOJINDO. OCT embedding materials are sourced from SAKURA Tissue-Tek.

81

82 ***In vitro cell survival rate test***

The effect of drugs on cell survival rate were tested by CCK-8 kit. First, peptide MTL-NBD was dissolved with DPBS and diluted to a series of different concentrations of 10 μ M, 20 μ M, 50 μ M, 100 μ M, 200 μ M with DMEM, and peptide NBD was dissolved with 0.05% DMSO DMEM and diluted to 50 μ M. U87 cells were cultured in a 25mL dish with DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 5% CO₂ 37 °C. When the cells grew to 80% of the culture dish, the cells were digested with 0.25% trypsin and made into a single cell suspension. By cell counting, the cell suspension was diluted to 5×10⁴/mL, with 100

91 μL adding into each well of the 96-well plate, 5000 cells per well. The cells were
 92 cultured for 24 hours to make the cells adhere to the wall. The 96-well plates were
 93 washed with DMEM for three times and replaced with fresh DMEM medium. They
 94 were starved for 4 hours. At 0 h, 24 h, 48 h, different concentration of MTL-NBD and
 95 50 μM NBD were added into the cell culture medium. Fresh medium and drugs were
 96 replaced every 24 h. Adding CCK-8 detection solution into 96-well plate at 0 h, 24 h,
 97 48 h and 72 h, with 10 μL per well, and incubating for 2 hours, then they were
 98 detected at 450 nm in detector. The cell survival rate (%) was equal to (drug group-A
 99 control group)/(control group-A blank group) ×100. The control group was added
 100 with PBS, and the blank group had no cells.

101

102 ***In vivo model was established by orthotopic transplantation***

103 Adult female C57 mice or BALB/C nude mice were used in experiment. The mice
 104 were purchased from Beijing Vital River Experimental Animal Technology Co., LTD.
 105 The mice were anesthetized by isoflurane and operated with isoflurane to maintain
 106 anesthesia. Eye ointment was applied to prevent eye desiccation. The mouse was
 107 fixed in the brain stereotatic injection instrument of small animals. After the skin was
 108 disinfected by povidone-iodine, a longitudinal incision about 1 cm was cut along the
 109 middle seam of the head with the sterilized surgical scissors. The point of
 110 intersection of sagittal and coronal fractures of the skull, namely Bregma point, was
 111 used as the origin. In experiment, a hole was drilled at x=-2.5 mm, Y =+1.5 mm,
 112 z=-3.5 mm and the needle was inserted at a uniform speed. After a 3-minute pause,
 113 the needle was raised 0.05mm and 1 μL U87 single cell suspension was injected, with
 114 a total of 1 million cells. The injection lasted for 5 minutes. After the injection, the
 115 needle was stopped for 5 minutes, and then the needle was slowly pulled out. The
 116 wound of the mouse skin was sutured and povidone-iodine was used for disinfection.
 117 The mice were observed until they woke up and put back into the cage.

118

119

120 ***Histomorphological detection***

121 For 5 days after brain injection, female C57 mice were sacrificed by neck
122 dissection, and the entire brain was removed, immobilized with 4% PFA for 48 hours,
123 and then dehydrated in 30% sucrose solution for 48 hours. After dehydration, the
124 brains of mice were embedded in OCT and refrigerated at -80°C for more than 4
125 hours. The embedded brain tissue was taken out and balanced at -20°C for half an
126 hour, sectioned in cryostat (Leica CML1950) with a thickness of 40µm. The sections
127 were immunostained with GPR158 antibody and DAPI. The sections were observed
128 under a microscope or a confocal microscope.

129

130 ***In vivo detection under small animal live imaging system***

131 It has been reported that the survival period of tumor-bearing nude mice could
132 reach 28-29 days after injection of 1 million U87 cells into the brain of Balb/C nude
133 mice (median survival period was 28.5 days) (Candolfi et al., 2007). In addition, U87
134 cells with luciferase were injected into the brain, and after injection of luciferase
135 substrate, *in vivo* tumors can be imaged by IVIS system (Lai et al., 2019). For *in vivo*
136 detection, mice were intraperitoneally injected with 150 µL luciferase substrate at a
137 concentration of 15 ng/ml about 10 minutes before imaging. After isoflurane
138 anesthesia, mice were placed into the system. The biotin luminescence imaging
139 mode was selected. The aperture was 1, and the exposure time was 5 minutes.

140 Bioluminescence imaging was performed on IVIS system after brain injection in
141 BALB/C nude mice. The mice were injected 20 mg/kg MTL-NBD polypeptide or 20
142 mg/kg NBD polypeptide intravenously for 4 consecutive days. The nude mice were
143 imaged every two days, and tumor growth rate (tumor growth rate = tumor
144 luminescence intensity after administration/tumor luminescence intensity before
145 administration) was calculated based on the imaging results.

146

147 ***Data Analysis***

148 Statistical differences between data groups were tested by *t test*, and three or

149 more groups were tested by ANOVA (GraphPad Prism 8). $P < 0.05$ was considered as
150 statistic significance.

151

152 **Results**

153 ***Probe design and Imaging of MTL-ICG in mouse brain***

154 For detection purpose, we first design a probe (MTL-ICG) (Fig. 1A). By the live
155 animal system test, we found the MTL could penetrate the BBB, compared with ICG
156 only and PBS group (Fig. 1B), whereas the main organs of different groups showed
157 similar pattern (Fig. 1C).

158

159 ***Tumor imaging using MTL-ICG***

160 The mice were sacrificed in day 5 after transplantation. Frozen sections were
161 prepared for morphological observation. The tumor site was in the cerebral cortex of
162 the mouse, and the U87 cells are clumped with clear boundary and stable
163 morphological characteristics (Fig. 2A). The fluorescent signals could be detected by
164 the live animal system, compared with scrambled peptide group (SCR), ICG only
165 group and PBS group (Fig. 2B), and there were no obvious change in main organs (Fig.
166 2C).

167

168 ***Design of MTL-NBD and in vitro test***

169 U87 cells were co-incubated with MTL-NBD and NBD. The CCK-8 detection was
170 performed to evaluate whether MTL-NBD had an inhibitory effect on GBM *in vitro*.
171 The different concentrations of MTL-NBD can inhibit the growth of U87 cells, similar
172 with the positive drug NBD (Fig. 3B).

173

174 ***Comparison of MTL-NBD and NBD in vivo***

175 Twenty four hours after surgery, nude mice were injected with 20 mg/kg
176 MTL-NBD or 20 mg/kg NBD intravenously every day, and *in vivo* imaging was
177 performed in Day 1, Day 3 and Day 5 (Fig. 4A).. The luminescent intensity of the

178 images was analyzed by software and tumor growth rate was calculated. According
179 to luminescent intensity analysis, MTL-NBD and the positive drug NBD had certain
180 similar inhibitory effect on the growth of brain GBM, with no significant difference
181 (Fig. 4B).

182

183 ***Expression of GPR158 in GBM graft***

184 It has been reported the GPR158 is the receptor for osteocalcin, so we speculated
185 MTL could bind to the GPR158 too. Thus, we tested the localization of GPR158 in the
186 grafts, and we found GPR158 showed high expression in tumor sites (Fig. 5).

187

188 **Discussion**

189 In this study, we demonstrated that MTL could be penetrate BBB and target GBM.
190 -NBD had a significant inhibitory trend on GBM proliferation. The polypeptide
191 NTL-NBD has the advantage for GBM therapy. Due to the composition of natural
192 amino acids, NTL-NBD has low toxicity to animals. And it has good water solubility,
193 namely 200 μ L normal saline or DPBS can completely dissolve 1 mg NTL-NBD. The
194 solvent would not introduce toxicity, so it can safely increase the dosage of NTL-NBD
195 in animal experiments, with an optimistic application prospect. However, the water
196 solubility of positive drug NBD is poor, so DMSO is needed to facilitate the
197 dissolution, with potential toxicity, limiting the further application of NBD. In
198 addition, 10% DMSO DPBS with 1000 μ L as solvent could not completely dissolve
199 1mg of positive drug NBD, and only the mixture with loose precipitation could be
200 obtained, which may affect the pharmacokinetics of NBD *in vivo* and reduce the
201 concentration of NBD in tumor.

202 GBM is divided into isocitrate dehydrogenase-wildtype (GBM, IDH-wildType),
203 isocitrate dehydrogenase-mutant (GBM, IDH-mutant), unshaped (GBM, NOS) and
204 other rare types (Louis et al., 2016). Clinically, the frequency of IDH mutation in
205 primary GBM is low, while the frequency of IDH mutations in secondary GBM is high
206 (Balss et al., 2008). It has been reported that GBM may originate from neural stem

207 cells (NSCs) in human subventricular zone (SVZ) tissues (Lee et al., 2018). After
 208 comprehensive analysis, the main pathway of GBM include receptor tyrosine
 209 kinase/RAS binding protein/intracellular phosphatidylinositol kinase (RTK, RAS/PI(3)K)
 210 signaling pathway, tumor suppressor protein (P53) signaling pathway and retinoblast
 211 gene (RB) signaling pathway (Network, 2013). According to our study, we GPR158
 212 signaling might be involved in development of GBM.

213 In conclusion, *in vitro* and *in vivo* experiments have proved that the MTL-ICG and
 214 MTL-NBD we designed in this study could be used as probe and an inhibitory drug for
 215 GBM, indicating that MTL is a valuable peptide for the diagnosis and treatment of
 216 GBM, with a good application prospect as a new anti-GBM peptide drug. Next, the
 217 future work should figure out the relationship between MTL, GPR158 and GBM.

218

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263 **Figure legends**

264 **Figure 1. ICG probe design and *in vivo* cross BBB test**

265 A) ICG probe design by click Chemistry. B) Live imaging of brain tissue after injection
266 of the probe under 808 excitation light. C) The main organs imaging corresponding to
267 panel B.

269 **Figure 2. Histological morphology after cell stereotatic injection and probe 270 treatment *in vivo***

271 A) Microscopic imaging results of mice brain after stereotactic brain injection,
272 counterstaining with DAPI. B) The near infrared imaging of grafted brain, after the
273 grafted mice were injected MTL-ICG, SCR, ICG only or PBS intraperitoneally for 24
274 hours. C) Main organ imaging of modeling mice brain corresponding to panel B.

276 **Figure 3. Design of MTL-NBD and inhibitory effect of MTL-NBD on U87-MG cell 277 growth *in vitro***

278 A) Desing of peptide MTL-NBD; B) U87-mg cell lines were treated with different
279 concentrations of NTL-NBD and NBD, and the cell survival rate was determined by
280 CCK-8 assay kit. The data were the mean values of the three experiments, tested by
281 two-way ANOVA, $p < 0.0001$.

283 **Figure 4. Inhibition effect of NTL-NBD on GBM growth *in vitro***

284 A) Animal live imaging results after stereotactic injection. B) Comparison of tumor
285 growth rate, according to the luminescent intensity of tumor site.

287 **Figure 5. Detection of GPR158 in grafting site**

288 GPR158 showed high expression in U87 transplanted area.

291 **Figures**

292
293 **Figure 1**
294

295
296 **Figure 2**
297

Figure 3

Figure 4

Figure 5