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RGD peptide conjugation results in enhanced antitumor activity of PD0325901 against glioblastoma by both tumor-targeting delivery and combination therapy

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ABSTRACT

Glioblastoma (GBM) is the most aggressive tumor type in the central nervous system. Both tumor-targeting drug delivery and combination therapy of multiple therapeutic agents with distinct mechanisms are important for GBM treatment. We combined these two strategies and developed a new platform of peptide-drug conjugate (RGD-PEG-Suc-PD0325901, W22) for tumor-targeting delivery using a combination of PD0325901 (a MEK1/2 inhibitor) and RGD peptide. In the present study, the combination of PD0325901 and RGD peptide strongly inhibited U87MG model *in vitro* and *in vivo*. This inhibition contributed to synergistic suppression of cell proliferation by blocking ERK pathway activity and cell migration. Modified by conjugation strategy, their conjugate W22 enhanced PD0325901 delivery to GBM cells by receptor mediated cellular internalization. W22 showed great superiority in targeting to U87MG xenografted tumors and strong anti-tumor efficacy based on ERK pathway inhibition and tumor-targeted delivery *in vitro* and *in vivo*. Moreover, W22 was stable in serum and able to release PD0325901 in the enzymatic environment. These data indicated that the RGD-PEG-Suc-PD0325901 conjugate provided a strategy for effective delivery of PD0325901 and RGD peptide into the GBM cells and inhibition of tumor growth in a synergistic manner.

1. Introduction

Gliomas account for about 50% of primary tumors of the central nervous system (CNS) and are the most aggressive brain tumors with high potential of metastasis (Louis et al., 2007). Glioblastoma multiforme (GBM, also called glioblastoma) is the most common and biologically aggressive subtype of high-grade gliomas (Aldape et al., 2003). Due to their infiltrating nature, surgery and radiotherapy which represent "local" treatments are unable to completely address the tumor. Consequently, post-surgical chemotherapy regimens have been considered to be essential for the treatment (Owonikoko et al., 2014). Although a broad range of chemotherapy agents are successfully applied, the median overall survival period is still less than 15 months for most of GBM

Abbreviations: CNS, central nervous system; GBM, glioblastoma multiforme; BBB, blood-brain-barrier; ECM, extracellular matrix; RTK, receptor tyrosine kinases; EGFR, epidermal growth factor receptor; PDGFR, platelet-derived growth factor receptor; IGFR, insulin-like growth factor receptor; MEK, mitogen activated protein kinase kinase; ERK, extracellular signal-regulated kinase; RGD, c(RGDyK) peptide; RGD-PEG4, c(RGDyK)-PEG_4-NH_2; RGD-Gly5, c(RGDyK)-Gly_5-NH_2; FITC, 3′,6′-dihydroxy-5-isothiocyanato-3H-spiro[isobenzofuran-1,9′-xanthen]-3-one; DIPEA, *N,N*-diisopropylethyl amine; DMF, *N,N*-dimethyl-formamide; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; RP-HPLC, reversed-phase high peformance liquid chromatography; TFA, trifluoroacetic acid; ESI, electrospray ionization; Rt, retention time; PBS, phosphate-buffered saline; HTRF, homogenous time-resolved fluorescence; EDTA, ethylenediaminetetraacetic acid; SRB, sulforhodamine B; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gels; PVDF, polyvinylidene fluoride; PI, propidium idodide; EdU, 5-ethynyl-2′-deoxyuridine; H&E stain, hematoxylin and eosin stain; MEKi, MEK inhibition; Con., concentration.

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patients (Anton et al., 2012). Recently, advanced therapeutic strategies have been focused on using tumor-targeting delivery system with a combination of multiple therapeutic agents in one formulation for achieving the synergetic effects, improving drug efficacy, reducing toxic effects of the anti-cancer agents, and simplifying dosing regiments (Kemp et al., 2015; Yang et al., 2014).

Compared to other tumor types, angiogenesis plays pivotal roles in the progression of malignant GBM, and it involves endothelial cell proliferation, migration, reorganization of extracellular matrix (ECM) and tube formation (Caffo et al., 2013; Wong et al., 2009). Today, many angiogenesis mediators are important targets in brain tumor therapy and diagnosis (D'Abaco and Kaye, 2007; Stromblad and Cheresh, 1996). The integrin $\alpha_v\beta_3$ receptor, one of a class of adhesion molecules family that serves for tumor cells to adhere to ECM, is particularly important in GBM angiogenesis (Desgrosellier and Cheresh, 2010). $\alpha_v\beta_3$ integrin is reported to be over-expressed on the surface of tumor cells and angiogenic vessels, and its expression correlates with the progress of GBM. Its cyclic RGD penta-peptide antagonist, Cilengitide (EMD Pharmaceuticals, Raleigh-Durham, NC, USA) which targets tumor microenvironment, has shown promising anti-tumor and anti-angiogenic effects in various types of cancer, especially GBM (Kurozumi et al., 2012). In clinical studies, patients treated with Cilengitide, alone or in combination with chemotherapy, had showed improvement in both response rate and overall survival time. And RGD-based strategies to target integrin $\alpha_{v\beta3}$ have been reported efficient in GBM diagnosis and treatment (Danhier et al., 2012; Ruan et al., 2015; Hu et al., 2015; Gao et al., 2014).

Multiple signaling pathways are critical for brain tumor progression. Several RTKs (such as EGFR with PDGFR or MET) are frequently over-expressed in GBM which results in ligand-independent constitutive activation of downstream signaling pathways (Tanaka et al., 2013; Chang and Johnson, 2012; Snuderl et al., 2011). As one of the major pathways downstream of RTK activation, the RAF/MEK/ERK signaling cascade mediates cell proliferation of malignant glioma cells (Roberts and Der, 2007). MEK1/2 inhibition (MEKi) have held great promise to block abnormally activated ERK signaling cascade either driven from activation at the receptor level (*e.g.*, by EGFR or HER2) or by mutations at various downstream constituent points (*e.g.*, by KRAS, NRAS or BRAF) (Montagut and Settleman, 2009). More recently, it has been found that in GBM mouse model ERK pathway is involved in the development of high-grade GBM, suggesting MEK1/2 as promising therapeutic targets (Li et al., 2014).

Previously, we have developed novel RGD-MEKI conjugates derived from PD0325901 by integrin $\alpha_v\beta_3$ receptor-mediated endocytosis strategy for melanoma therapy (Li et al., 2013). These conjugates also showed potent and specific activity on U87MG glioblastoma cell model (Hou et al., 2015). Our recent study indicated that co-treatment with c(RGDyK) peptide (an analogue of Cliengitide, RGD peptide), PD0325901 showed significant improvement in anti-tumor effects on U87MG cells in a dose-dependent manner. Thus, we considered that the combination of MEK1/2 inhibitor and RGD peptide with distinct mechanisms would be a potential strategy for the effective treatment of GBM. And co-delivery of two drugs would achieve the synergetic effects of both ERK signaling cascade inhibition and anti-angiogenesis in tumor cells and tissue (tumor blood vessels). Furthermore, this RGD peptide can be used as a targeting cargo to integrin $\alpha_v\beta_3$ for specific delivery of MEK1/2 inhibitor to tumor tissue and cells only. In this study, we developed a new platform of peptide drug conjugate (RGD-PEG4-Suc-MEKI conjugate) for GBM. When introducing a degradable ester bond as linker, the RGD-PEG4-Suc-MEKI conjugate served as a prodrug of PD0325901 and achieved an efficient targeted intracellular delivery of MEK1/2 inhibitor and a combinatorial treatment.

In summary, we have designed a RGD peptide conjugated PD0325901 with a mini-PEG linker to realize efficient co-delivery of RGD peptide and PD0325901 to GBM tumors for combination therapy. RGD peptide was used to deliver PD0325901 to tumor cells and tumor vasculature by integrin-targeted delivery strategy, and also to enhance anti-tumor activity on GBM cells with synergistic effect by inhibition of cell migration. *In vitro* and *in vivo* anti-tumor efficacy studies demonstrated that our new conjugate was highly effective for treatment of GBM in the U87MG tumor model.

2. Materials and methods

2.1. Materials

Cyclic RGD peptides, such as c(RGDyK) (RGD), c(RGDyK)-PEG_4-NH_2 (RGD-PEG4), c(RGDyK)-Gly_5-NH_2 (RGD-Gly5), FITC-c(RGDyK) (FITC-RGD) were purchased from GL Biochem (Shanghai) Ltd. (Shanghai, China), with the purity higher than 95%. Diisopropylethyl amine (DIPEA), *N,N*-dimethyl-formamide (DMF), Sulforhodamine B (SRB) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Trypsin-EDTA, fetal bovine serum (FBS), and Dulbecco's modified Eagle's medium (DMEM) were obtained from Hyclone (Logan, UT, USA). Penicillin and streptomycin were purchased form Wako Pure Chemical Industries Ltd., (Osaka, Japan). Anti-integrin $\alpha_{v\beta3}$ antibody, clone LM609 and FITC-conjugated anti-integrin $\alpha_v\beta_3$ antibody, clone LM609 (FITC-LM609) were purchased form Millipore (MA, USA). Anti-ERK1/2, anti-phospho-ERK1/2(Thr202/Tyr204), anti-phospho-S6 (Ser240/244), anti-GAPDH were purchased form Cell Signaling Technology (MA, USA). All other reagents were of analytical grade and used as received without further purification.

2.1.1. Cell lines and cell culture

Human cancer cell lines used in this study were: glioblastoma, U87MG (integrin $\alpha_v\beta_3$ positive cell line) and U251MG; lung cancer, A549 (integrin $\alpha_v\beta_3$ negative cell line); breast cancer, MDA-MB-231; melanoma, A375; colorectal cancer, HCT116 and HT29. All cell lines were purchased from Institute of Material medical, Chinese Academy of Medical Sciences and Peking Union Medical College (Beijing, China), were cultured in DMEM containing 10% heat-inactivated FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. The cells were culture in a humidified atmosphere of 5% CO_2 in air at 37 °C.

2.1.2. Animals

Female Balb/c nude mice (8 weeks, ~20 g) were purchased from Vital River Laboratory Animal Center (Beijing, China). All the animals were kept in standard housing conditions with free access to standard food and water. All care and handling of animals was performed under the guideline approved by the ethic committee of Peking University Health Science Center Animal Care and Use. Subcutaneous tumors on the right flank of mice were initiated by the injection of 3.8×10^6 viable U87MG cells in a volume of 0.2 mL. The tumor growth was measured using a caliper, and tumor volume was calculated using the formula volume = $length \times width^2/2$. Tumors were allowed to reach about a volume of 100–200 mm^3 for the following *in vivo* experiments.

2.2. Methods

2.2.1. Synthesis and characteristics of RGD-PEG-Suc-PD0325901 conjugate (W22) and its analogues (W20 and W23)

Conjugates W20, W22 and W23 were synthesized using the previous method which we reported with a minor modification