Targeted delivery of a novel palmitylated D-peptide for antglioblastoma molecular therapy

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Abstract

Effective glioblastoma treatment with low toxicity is one of the most difficult challenges in cancer therapy. The interaction between tumor suppressor protein p53 and its negative regulator murine double minute 2 (MDM2) provides a promising target for specific therapy because an important subtype of glioblastoma harbors wild-type p53 and overexpressed MDM2. Several D-peptides have been previously reported to effectively antagonize MDM2 for binding to p53 with high affinity and unsurpassed specificity. However, poor cell penetration and lack of efficient delivery method hampered the therapeutic applicability of the most potent D-peptide, D-PMIβ. In this study, a novel lipophilic derivate of D-PMIβ (pDP) was developed. Liposome was chosen as a carrier for pDP, and cyclic pentapeptide c(RGDyK) was used as a targeting moiety for the treatment of glioblastoma. D-PMIβ was N-terminally modified with palmitic acid and the resultant c(RGDyK) decorated liposomes (RGD-liposomal pDP) showed almost 100% encapsulation efficiency and 10% loading efficiency. The abilities of palmitylated D-peptide to antagonize MDM2 and reactivate p53 specifically were confirmed by the western blot assay. The IC50 ratio of RGD-liposomal pDP in treating human umbilical vascular endothelial normal cells vs. U87 tumor cells was 10 times higher than that of RGD-liposomal doxorubicin. After intravenous administration, the median survival time of intracranial U87 glioblastoma-bearing nude mice treated with RGD-liposomal pDP (29 days) was significant longer than that of mice treated with blank RGD-liposome (23 days) (p<0.001). These results indicated that palmitylated D-peptide inhibitor of p53-MDM2 combined with RGD modified liposomes provided a potential molecular therapy for glioblastoma.

Keywords: Glioblastoma, p53, D-peptide, lipophilic peptide, cyclic-RGD decorated liposomes

Introduction

Glioblastoma is recognized as the most lethal and malignant form of primary brain tumors in adults, which has poor prognosis and is difficult to treat (Veeravagu et al., 2008; Magrini et al., 2006; Li et al., 2002). Most of the currently used treatment strategies show poor efficacy, and their collective lack of specificity toward tumor cells causes severe side effects and failed therapy. New therapeutic paradigms based on novel molecular targets and mechanisms need to be developed. To this end, targeting the pleiotrophin (PTN) and its receptor anaplastic lymphoma receptor tyrosine kinase represents an attractive antglioblastoma strategy since PTN is overexpressed in various types of glioblastoma but rarely found in normal tissue (Grzelinski et al., 2009). The interaction between the tumor suppressor protein p53 and its negative regulator murine double minute 2 (MDM2) provides another promising target for antglioblastoma therapy (Toledo and Wahl, 2006; Kirsch and Kastan, 1998).

p53 functions primarily as a transcription factor that transactivates gene expression in response to cellular stress signals, resulting in cell cycle arrest, senescence, or apoptosis (Vogelstein et al., 2000). p53-deficient mice are developmentally normal but susceptible to spontaneous tumors (Donehower et al., 1992). Thus, p53 plays a crucial role in the prevention of tumor development. Not surprisingly, in 50% of human cancers, p53 is defective due to somatic mutations or deletions (Kirsch and Kastan, 1998). Although the remaining half harbors wild-type p53, the p53 pathway is partially abrogated by
amplified and/or overexpressed MDM2. MDM2 is a p53-specific, E3 ubiquitin ligase and the most important negative regulator of the tumor suppressor protein (Toledo and Wahl, 2006; Honda et al., 1997). MDM2 controls p53 stability (protein levels) by targeting p53 for ubiquitin-mediated proteasomal degradation. In addition, MDM2 binds with high affinity to the N-terminal transactivation domain of p53, directly blocking its ability to regulate responsive genes expression. It has been validated in vitro and in vivo that inhibitors of the p53-MDM2 interaction can reactivate the p53 signaling pathway and kill tumor cells in a p53-dependent manner (Vassilev et al., 2004; Shangary et al., 2008; Bernal et al., 2007; Zhang et al., 2009). Since MDM2 is over-expressed in primary glioblastoma without concomitant p53 mutation, inhibitors of the p53-DM2 interaction may be clinically beneficial in treating glioblastoma (Magrini et al., 2006).

More recently, we have obtained several biostable D-peptide inhibitors of the p53-MDM2 interaction by mirror-image phage display technique (Schumacher et al., 1996; Liu et al., 2010a), which can bind to the p53-binding cavity of MDM2 at affinities of submicromolar levels, on par or even better than Nutlin-3—a small molecule antagonist of MDM2 (Vassilev et al., 2004). However, these D-peptides failed to show p53-dependent antitumor effect due to their poor lipid solubility and inability to traverse the cell membrane. Moreover, the limited solubility in water of the most potent D-peptide, D-PMIβ, makes it difficult to achieve high encapsulation efficiency by nanocarriers. It has been reported that peptide derivates modified with lipophilic moieties achieved high peptide encapsulation within liposomes (Liang et al., 2005; Babu et al., 1995), thus providing a potential breakthrough for efficient D-peptide loading and delivery.

The integrin αvβ3 is a receptor ubiquitously expressed on the surface of malignant glioma cells. It has been validated that cyclic RGD peptides (ligands of integrin receptors) can facilitate the glioblastoma-specific delivery of therapeutic and diagnostic reagents (Chen et al., 2004a; Hsu et al., 2006; Zhan et al., 2010).

Based on the combined information above, the aim of this study was to develop a novel palmitylated D-PMIβ derivate (pDP) and its targeted delivery system for the molecular treatment of glioblastoma (Figure 1). N-terminally modified D-PMIβ (pDP) was synthesized using standard Boc-solid phase peptide synthesis method. Briefly, after chain assembly of D-PMIβ (sequence: DTDADWDYDADNDFDEDKDLDLDR), N-Boc groups on resins were deprotected by trifluoroacetic acid. Then, palmatic acid N-hydroxysuccinimide ester was conjugated with peptide chains for 30 min at a molar ratio of 5:1 under the catalysis of N,N-diisopropylethylamine. Cyclic pentapeptide c(RGDyK) was synthesized via Fmoc-solid phase peptide synthesis strategy as previously reported (Zhan et al., 2010).

Synthesis of palmitylated D-peptide and cyclic pentapeptide c(RGDpYK)

N-terminally palmitylated D-PMIβ derivate was synthesized using standard Boc-solid phase peptide synthesis method. Briefly, after chain assembly of D-PMIβ (sequence: bφφφφwφφφφnφφφφpφφφφkφφφφlφφφφlφφφφr), N-Boc groups on resins were deprotected by trifluoroacetic acid. Then, palmatic acid N-hydroxysuccinimide ester was conjugated with peptide chains for 30 min at a molar ratio of 5:1 under the catalysis of N,N-diisopropylethylamine.

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All the crude peptides mentioned above were purified by preparative RP-HPLC (Hitachi, Japan) before further usage. Homogeneity of the final products was confirmed by ESI-MS.

Preparation and characterization of liposomes

Preparation of liposomes

Cyclic-RGD-PEG 3400-DSP was synthesized by coupling thioacetyl c(RGDyK) with MAL-PEG 3400-DSP as

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**Materials and methods**

**Materials**

Phosphatidylethanolamine distearoyl methoxy(polyethylene glycol 2000 conjugate (mPEG2000-DSP) and palmitic acid N-hydroxysuccinimide ester were obtained from Sigma (USA). Hydrogenated soybean phosphatidylcholine (HPC) was purchased from Lipoid GmbH (Germany). Cholesterol was purchased from Shanghai Chemical Reagent Company (China). Maleimide-derivatized PEG-DSP (Mal-PEG3400-DSP) was purchased from Layson Bio, Inc (USA). All Boc-amino acids and Fmoc-amino acids were purchased from Peptides International (Japan) and Bachem (USA). All other reagents and chemicals were of analytical grade.

**Human glioblastoma cancer cell lines**

U87 (wild-type p53), U251 (mutant p53), and human umbilical vascular endothelial cells (HUVEC; wild-type p53), obtained from Shanghai Cell Bank of Chinese Academy of Sciences, were routinely grown in Dulbecco's Modified Eagle Medium medium supplemented with 10% fetal bovine serum, 100 μU/mL penicillin, and 100 μg/mL streptomycin under an atmosphere of 5% CO2/air at 37°C.

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**Preparation and characterization of liposomes**

**Preparation of liposomes**

Cyclic-RGD-PEG 3400-DSP was synthesized by coupling thioacetyl c(RGDyK) with MAL-PEG 3400-DSP as
previously reported (Liu et al., 2010c). The successful conjugation of c(RGDFK) with MAL-PEG$_{3400}$-DSPE was confirmed via 1H-NMR.

RGD-liposomal pDP was prepared by the rotation evaporation method. In brief, hydrogenated soy phosphatidylcholine, cholesterol, mPEG$_{2000}$-DSPE, and c(RGDFK)-PEG$_{3400}$-DSPE were dissolved in 2 mL of chloroform at a molar ratio of 55:45:2:1 (total amount of liposomal membrane materials was about 36 mg), then mixed with 4 mg of palmitylated D-PMIβ peptide (pDP) dissolved in 2 mL of methanol. After organic solvents were volatized entirely to form a thin film at 60°C for 2 h. The obtained suspension was repeatedly extruded through polycarbonate membranes with 200, 100, and 80 nm pore sizes successively, using a mini-extruder (Avanti Polar Lipid, AL). For in vitro and in vivo bioactivity assays, RGD-liposomal pDP were purified through a Sephadex CL-4B column equilibrated with saline buffer (pH 7.4). The concentration of encapsulated peptide within liposome was detected by a C4 column-based RP-HPLC method (Slimani et al., 2006).

Blank c(RGDFK)-PEG-liposome and pDP-loaded PEG-liposome (liposomal pDP without RGD decoration) were performed in parallel and used as negative controls. c(RGDFK)-liposomal doxorubicin, as the positive control liposome for in vivo assay, was prepared by the ammonium sulfate gradient method (Pan et al., 2008).

Characterization of liposomes

pDP encapsulation and loading efficiency To determine the encapsulation efficiency of pDP in the RGD-liposomal pDP, the obtained liposomes before chromatographically purification were divided into two parts equally. One part was purified using the Sephadex CL-4B column. The eluent was collected and the encapsulated pDP concentration was measured. The other part of liposomes was diluted into same volume of the eluent above. Then, the total amount of pDP concentration was detected. The encapsulation efficiency of liposomes was directly calculated as the amount of the encapsulated pDP divided by the total amount of pDP. The pDP loading efficiency of liposomes was calculated accordingly.

The particle size of liposomes was determined using dynamic light scattering (Nicomp 380 DLS, USA).

Characterization of conjugated cyclic-RGD pentapeptide on the surface of liposome The immune-gold staining technique was used to confirm the covalent conjugation of cyclic-RGD pentapeptide with liposomes (Hu et al., 2009). The blank c(RGDFK)-PEG-liposomes were used here. Firstly, 50 μg of recombinant human integrin αvβ3 was incubated with NHS-biotin (10 times excessive amount) in 100 μL PBS buffer (pH 7.4) for 30 min. After ultrafiltration, the biotinylated protein obtained was resuspended in 75 μL reaction buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 150 mM NaCl, 1 mM MgCl$_2$, and 1 mM MnCl$_2$, pH 7.4) (Liu et al., 2010c). Then 1 mg of the blank c(RGDFK)-PEG-liposome was incubated with 25 μL biotinylated protein αvβ3 solution above for 4 h in 200 μL reaction buffer. The integrin-labeled liposome was separated from unbound biotinylated protein through the Sephadex CL-4B column, followed by incubation with streptavidin-gold conjugate for 1 h at a volume ratio of 50:1 (protein:streptavidin-gold). After repurified using the Sephadex CL-4B column, the liposome eluent was stained with a phosphotungstic acid solution (2%, w/v) and examined by transmission electron microscopy. The blank c(RGDFK)-PEG-liposome without incubation with biotinylated integrin and the PEG-liposome were used as negative controls.

In vitro bioactivity assays of RGD-liposomal pDP against tumor cells

Western blot assay According to the model of p53 regulated by MDM2, the treatment of tumor cells (p53 wild type) with p53-MDM2 inhibitors should result in: 1) stabilization and accumulation of p53; 2) activation of MDM2 expression; and 3) activation of other p53-regulated genes and the p53 pathway (e.g. p21) (Vassilev et al., 2004). Thus, the western blotting technique was used to semiquantitatively determine three key factors (i.e. p53, MDM2, and p21) that representing the p53 pathway, by, was necessary to demonstrate the molecular mechanism of tumor growth inhibition by the liposomal D-peptide. Two human glioblastoma cells U87 (p53 wild type) and U251 (p53 mutant type) were used here for comparison due to their different p53 status and both expression of integrin αvβ3 with positive responding to RGD-assisted diagnosis and therapy (Naganuma et al., 2004; Chen et al., 2004; Wang et al., 2009). The expressions of p53, MDM2, p21, and β-actin in U87 and U251 tumor cells after exposure to RGD-liposomal pDP for 12 h were analyzed by western blot assay. Cells were collected and lysed in protein lysis buffer. Protein lysates were resolved in 10% SDS-PAGE followed by transfer to nitrocellulose polyvinylidene difluoride membranes (Amersham Pharmacia). Membranes were probed with primary antibodies including p53, MDM2 (Bioworld Technology), p21 (Santa Cruz), and β-actin (control for protein load, Sigma), respectively. Appropriate secondary antibodies conjugated with horseradish peroxidase (Bio-Rad) and enhanced chemiluminescence reagents (Amersham Pharmacia) were used for detection and visualization.

In vitro cell viability assays

The antitumor activities of the RGD-liposomal pDP, the RGD-liposomal doxorubicin, the pDP-loaded PEG-liposome, and Nutin-3 against human glioblastoma U87 cells were evaluated by a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay performed in triplicate. The D-peptides (D-PMIβ) and pDP were used as controls. U87 cells were seeded in 96-well plates (3000 cells/well) and allowed to grow for 24 h before being treated with the liposomes. Then, cells were incubated with various concentrations of liposomes and controls in supplement-free culture medium for 72 h.
The optical density measurement was performed on a multiwell spectrophotometer reader (Bio-Rad, USA) at a test wavelength of 595 nm and a reference wavelength of 650 nm. The cell viability and IC50 value were calculated from these data.

The cytotoxicity of these formulations against normal cells was also performed by the MTT assay as described above. The human umbilical vein endothelial HUVEC cells were used as normal cell model. The comprehensive therapeutic effect was evaluated by the therapeutic index calculated as a ratio of IC50 values against HUVEC normal cells vs. U87 tumor cells.

**Antiglioblastoma study of RGD-liposomal pDP**

The antiglioblastoma efficacy of the RGD-liposomal pDP was studied in intracranial U87 glioblastoma bearing nude mice model. All the animal experiments were carried out in accordance with guidelines evaluated and approved by the ethics committee of Fudan University. The male Balb/c nude mice, 6–8 weeks old, were anesthetized and injected slowly with U87 cells (8 × 10^5 cells suspended in 5 μL PBS) into the right striatum (1.8 mm lateral, 0.6 mm anterior to the bregma, and 3 mm of depth). The surgical procedures were performed using a stereotactic fixation device with mouse adaptor.

The intracranial tumor-bearing nude mice were randomly divided into four groups (n = 8) and administered via tail vein with varies of formulations. The four groups of mice were treated in parallel at day 8, 12, 16, and 20 postinoculation with the blank RGD-liposome, pDP-loaded PEG-liposome (liposomal pDP without RGD), RGD-liposomal doxorubicin (5 mg/kg each time), and RGD-liposomal pDP (10 mg/kg each time) respectively. The survival time was recorded.

**Data analysis**

Nonlinear regression analysis was performed using GraphPad Prism 5.0 to calculate the Kd values and IC50 values presented as mean±SD. Survival data were presented using Kaplan–Meier plots and were analyzed using a log-rank test. Student’s t-test was applied and p<0.05 was considered statistically significant.

**Results**

**Characterization of prepared liposomes**

As shown in Figure 2, the characteristic peak of maleimide group of MAL-PEG-DSPE was at 6.7 ppm. This MAL peak disappeared in the 1H-NMR spectrum of c(RGDyK)-PEG-3400-DSPE, indicating the successful conjugation of c(RGDyK) with MAL-PEG3400-DSPE.

The surface of the blank RGD-liposome was surrounded by colloidal gold, indicating the presence of cyclic-RGD conjugated to the surface of liposome (Figure 3). No gold particle around the liposome surface was observed in the case of the negative control groups. These results further confirmed the successful preparation of cyclic-RGD-PEG-3400-DSPE.

The pDP encapsulation and loading efficiency of RGD-liposomal pDP were 99.83 ± 4.86% and 9.98 ± 0.40% (n = 6), respectively. After 45 days of storage at 4°C, no significant difference in the particle size of liposomes and no obvious peptide leakage was observed (Table 1).

**In vitro antitumor activity of RGD-liposomal pDP**

In vitro antitumor activity of RGD-liposomal pDP was evaluated in U87 cells. It can be seen in Figure 4 that a potent tumor cell growth inhibition was found in the RGD-liposomal pDP group with a low IC50 value of 550 nM compared to 260 nM of RGD-liposomal doxorubicin and 3.2 μM of Nutlin-3. The liposomal pDP without RGD modification also showed tumor-killing activity with an IC50 value of 1.3 μM. The D-PMIβ did not show any antiglioblastoma activity in vitro as expected and the pDP showed very weak tumor-killing ability, which suggesting a suitable delivery system is crucial to facilitate the p53-dependent antitumor effect of D-peptide therapeutics. As shown in Table 2, the IC50 ratio of RGD-liposomal pDP in treating HUVEC vs. U87 was about 25, which was almost three times and ten times higher than that of Nutlin-3 and RGD-liposomal doxorubicin respectively, indicating a specific tumor-killing activity of RGD-liposomal pDP with high efficiency and low toxicity.

**Molecular mechanism of antitumor effect**

As shown in Figure 5, a dose-dependent increase in the expressions of p53, MDM2, and p21 was observed in U87 tumor cells (p53 wild type) treated with RGD-liposomal pDP. In contrast, only a basal level of p53 was observed in U251 tumor cells (p53 mutant type) and no expressions of MDM2 and p21 were found. These results confirmed the p53-dependent manner of RGD-liposomal pDP in tumor growth inhibition (Vassilev et al., 2004; Shangary et al., 2008).

![Figure 2. 1H-NMR spectra of MAL-PEG1500-DSPE and c(RGDyK)-PEG3400-DSPE at 400MHz.](image)
Antiglioblastoma study of RGD-liposomal D-peptide

Both U87 cells and tumor xenografts had effective response to doxorubicin as reported (Madhankumar et al., 2009; Gupta and Torchilin, 2007), thus the RGD-liposomal doxorubicin was used as a positive control here. The median survival trend and time of each group was illustrated in Figure 6. The average survival time of the blank RGD-liposome group, the liposomal pDP group, treatment group (RGD-liposomal pDP), and the RGD-liposomal doxorubicin group were 23, 26, 29, and 30 days, respectively. RGD-liposomal pDP significantly improved the mean survival time of intracranial glioblastoma bearing nude mice (treatment group vs. empty liposome group, \(p<0.001\); treatment group vs. liposomal pDP group, \(p<0.05\)). And there was no significant difference in the median survival trend for treatment group vs. RGD-liposomal doxorubicin (\(p>0.05\)).
Discussion

Albeit the aggressive growth and relatively incomplete surgical supports made glioblastoma one of the world-wide medical challenges, extensive efforts have been made to explore more effective therapeutic methods over the past decades. More specific and potent therapeutic agents have been designing and screening for efficient tumor-killing and avoidance of severe side effects. Meanwhile, targeted drug delivery systems have also been developing to deliver therapeutic agents across the blood-brain barrier to the site of brain tumor, while sparing the surrounding healthy tissue from damage (Ying et al., 2010).

Glioblastoma harbors wild-type p53 and elevated levels of MDM2 (Parsons et al., 2008; Prasad et al., 2002). Therefore, antagonists targeting the p53-binding domains of MDM2 that activate the p53 pathway can be promising candidates for the molecular therapy of glioblastoma. Recently, we have reported several D-peptide inhibitors of the p53-MDM2 interactions (Liu et al., 2010a, 2010b). To fulfill the p53-dependent antitumor potentials of these D-peptides, liposomes were chosen as delivery carriers. However, most of D-peptides obtained including the most potent peptide D-PMIβ (Kd value of 30 nM) cannot be successfully loaded into liposome due to the poor lipid solubility and limited water solubility.

The terminal modification strategy has been extensively used in the development of peptide-based therapeutics, which can improve the physico-chemical properties of peptides without loss of biological activities. For example, Nicolau et al. (2002) reported a terminal modification method with fatty acid to increase the lipophilicity of antigen peptide for the construction of liposome-based therapeutic vaccines (Tosi et al., 1995; Muhs et al., 2007). Using this method, the antigen peptide was well loaded and presented by the liposomal carrier with maintained immune activity. In our case, based on the crystal structure of peptide-protein complex, the highly flexible N-terminus of this kind of D-peptide inhibitors were not involved in binding with MDM2 (Figure 7). Therefore, the N-terminal palmitylation was expected to maintain the specific antitumor activity of peptide, which has been confirmed using the western blot assay.

To achieve the antiglioblastoma molecular therapy of D-PMIβ, we developed the novel palmitylated D-PMIβ and its glioblastoma-targeted delivery system. When encapsulated in c(RGDyK) modified liposomes, pDP showed potent p53-dependent growth inhibition activity against glioblastoma in both tumor cell model and nude mice xenograft model. The therapeutic index of the RGD-liposomal pDP was almost 10 times higher than that of the RGD-liposomal doxorubicin, which makes molecular therapy using the RGD-liposomal pDP even more attractive than chemical therapy. To explore its therapeutic applicability, more possible antiglioblastoma mechanisms of this novel D-peptide involved needs to be demonstrated by further work (Vassilev et al., 2004; Secchiero et al., 2007). The combination with other anticancer agents may be another new strategy to further improve the therapeutic efficacy of this type of D-peptide (Kojima et al., 2005; Drakos et al., 2007).

Conclusion

In conclusion, the novel lipophilic D-PMIβ achieved high encapsulation efficiency in c(RGDyK) modified liposomes, which exerted potent antiglioblastoma effect and low toxicity. Therefore, the lipophilic D-peptide inhibitor of p53-MDM2 and its tumor-targeted liposomal formulation represented a promising system for the molecular treatment of glioblastoma.

Figure 7. The crystal structure of complex formed between the D-peptide and 25-109 murine double minute 2 (MDM2). (A) Close-up view of the interface of complex (Liu et al., 2010c; Copyright © 2011 WILEY-VCH Verlag GmbH & Co. KGaA; used by authorized). The three key residues in peptide to bind with MDM2 are colored in red, the rest in yellow. The electrostatic potential at the molecular surface of MDM2 is displayed as negative in red, positive in blue, and apolar in white. The disordered N-terminus of D-peptide is not involved in binding with MDM2. This D-peptide is a parental molecule of D-PMIβ, in which the Asn2 and Leu7 were replaced by Ala2 and Phe7, respectively, to further improve binding affinity. (B) Ribbon-and-stick representation of the complex (D-peptide is colored in yellow and MDM2 in green). The Trp3 (red) is the start site of MDM2-binding domain of this D-peptide, whereas the highly flexible N-terminus is difficult to define.
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Declaration of interest

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References


Targeted delivery of palmitylated D-peptide


