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Delivery of doxorubicin to glioblastoma multiforme *in vitro* using solid lipid nanoparticles with surface aprotinin and melanotransferrin antibody for enhanced chemotherapy

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ABSTRACT

Solid lipid nanoparticles (SLNs) conjugated with aprotinin (Apr) and melanotransferrin antibody (Anti-MTf) were used to carry doxorubicin (Dox) across the blood–brain barrier (BBB) for glioblastoma multiforme (GBM) chemotherapy. Dox-entrapped SLNs with grafted Apr and Anti-MTf (Apr-Anti-MTf-Dox-SLNs) were applied to a cellular monolayer comprising human brain-microvascular endothelial cells (HBMECs) with a regulation of human astrocyte (HAs) and to a proliferated colony of U87MG cells. Based on the average particle diameter, zeta potential, entrapping efficiency of Dox, and grafting efficiency of Apr and Anti-MTf, we found that 1,2-dipalmitoyl-sn-glycero-3-phosphocholine of 40% (w/w) in lipids was appropriate for fabricating Apr-Anti-MTf-Dox-SLNs. In addition, Apr-Anti-MTf-Dox-SLNs could prevent Dox from fast dissolution and did not induce a serious cytotoxicity to HBMECs and HAs when compared with free Dox. Moreover, the treatment with Apr-Anti-MTf-Dox-SLNs enhanced the ability of Dox to infuse the BBB and to inhibit the growth of GBM. The current Apr-Anti-MTf-Dox-SLNs can be a promising pharmacotherapeutic preparation to penetrate the BBB for malignant brain tumor management.

Abbreviations

Anti-MTf	melanotransferrin antibody
Anti-MTf-Dox-SLN	doxorubicin-entrapped solid lipid nanoparticle with grafted melanotransferrin antibody
Apr	aprotinin
Apr-Anti-MTf-Dox-SLN	doxorubicin-entrapped solid lipid nanoparticle with grafted aprotinin and melanotransferrin antibody
Apr-Dox-SLN	doxorubicin-entrapped solid lipid nanoparticle with grafted aprotinin
BBB	blood–brain barrier
Dox	doxorubicin
Dox-SLN	doxorubicin-entrapped solid lipid nanoparticle
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
GBM	glioblastoma multiforme
HA	human astrocyte
HBMEC	human brain-microvascular endothelial cell
HBMEC/HA	monolayer of HBMECs regulated by HAs
LDLR	low-density lipoprotein receptor
LRP	low-density lipoprotein receptor-related protein
PI	propidium iodide
SLN	solid lipid nanoparticle

1. Introduction

Glioblastoma multiforme (GBM) is a deadly brain neoplasm of the highest lethal grade with a median survival time within 1.5 years of post-diagnosis [1]. The GBM lethality is derived primarily from its aggressive characteristics and frequent recurrences [2]. To prevent the GBM recurrences after resection, a pharmacotherapy for virtually targeting the residual cells is excessively required [3,4]. In addition, during the inhibition to GBM propagation in the brain, a delivery of antitumor preparation across the blood–brain barrier (BBB), a complicated tissue site between the circulation system and the central nervous system, becomes an inevitable challenge [5].

Aprotinin (Apr), a proteolytic enzyme with a peptide single chain of 58 residues and molecular weight of 6511.51 Da, can specifically bind to low-density lipoprotein receptor (LDLR)-related

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Nomenclature

C_{Apr}	aprotinin concentration for grafting (μg/ml)
C_{Dox}	doxorubicin concentration for entrapment in Dox-SLNs (mg/ml)
D	average diameter of Apr-Anti-MTf-Dox-SLNs (nm)
EE_{Dox}	entrapping efficiency of doxorubicin (%)
$GE_{Anti\text{-}MTf}$	grafting efficiency of melanotransferrin antibody (%)
GE_{Apr}	grafting efficiency of aprotinin (%)
P_{DPPC}	DPPC weight percentage in solid lipid nanoparticles (%)
$P_{viability,HA}$	viability of human astrocytes treated with Apr-Anti-MTf-Dox-SLNs (%)
$P_{viability,HBMEC}$	viability of human brain-microvascular endothelial cells treated with Apr-Anti-MTf-Dox-SLNs (%)
$P_{viability,U87MG}$	viability of U87MG cells treated with Apr-Anti-MTf-Dox-SLNs (%)
$P_{release,Dox}$	cumulative release percentage of doxorubicin from Apr-Anti-MTf-Dox-SLNs (%)
$P_{BBB,Dox}$	blood–brain barrier permeability coefficient for doxorubicin (cm/s)
$P_{BBB,PI}$	blood–brain barrier permeability coefficient for propidium iodide (cm/s)
t	time for doxorubicin release (h)
TEER	*transendothelial* electrical resistance ($\Omega \times cm^2$)
ζ	zeta potential of Apr-Anti-MTf-Dox-SLN (mV)

protein (LRP) with a high affinity [6,7]. LRP is normally expressed by human brain-microvascular endothelial cells (HBMECs), the most important cellular component in the BBB [8,9]. It has been observed that the BBB permeability for Apr was about 1.55 pmol/cm^2, which was much higher than that for transferrin (about 0.25 pmol/cm^2) after transport for 90 min [10]. Moreover, membrane-bound melanotransferrin (human p97) is an antigen expressed by HBMECs and appears in the brain tissue [11,12]. It has been found that melanotransferrin antibody (Anti-MTf; anti-p97) was capable to tightly conjugate melanotransferrin [13]. Intriguingly, U87MG cells, which were the typical cells isolated from the brain of a GBM patient, also expressed LDLR, LRP, and melanotransferrin [14–16]. Thus, a combination of Apr and Anti-MTf in drug vector to carry chemotherapeutic agents can be a feasible strategy to target the BBB and GBM via the inherent immunity.

Solid lipid nanoparticles (SLNs) are appropriate colloidal carrier systems for delivering pharmaceuticals to the brain [17]. In a study on the chemoresistance of doxorubicin (Dox), Dox-entrapped SLNs (Dox-SLNs) could significantly induce the apoptosis of MCF-7 and its adriamycin-resistant variant cells [18]. It has also been found that SLNs enhanced the brain uptake of Dox after an intravenous injection to conscious rats [19]. In a study on the antiproliferative effect on malignant HT-29 cells, the 50% inhibitory concentration of Dox-SLNs was 81.87 ± 4.11 nM, which was smaller than that of a conventional Dox formulation (126.57 ± 0.72 nM) [20].

The aim of this study was to investigate the efficacy of Dox-SLNs with grafted Apr and Anti-MTf (Apr-Anti-MTf-Dox-SLNs) in the BBB permeation and anti-GBM proliferation. Since a targeting therapy of anticancer drug for the brain tumors is an urgent need in the clinical practice, a development of Dox carriers with targeting ability can be important. We examined the physicochemical characteristics and biocompatibility of Apr-Anti-MTf-Dox-SLNs, including the particle size, surface charge, Dox entrapment and dissolution, Apr and Anti-MTf grafting, and viability of HBMECs and human astrocytes (HAs). Moreover, the biomedical efficiency of Apr-Anti-MTf-Dox-SLNs was studied via the transport across a validated BBB model, comprising a monolayer of HBMECs regulated by HAs (HBMEC/HA), and the immunochemical staining of U87MG cells.

2. Materials and methods

2.1. Preparation of Apr-Anti-MTf-Dox-SLNs

1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC; Avanti Polar Lipids, Alabaster, AL), cacao butter (aka cocoa butter; OCG Cacao, Whitinsville, MA), hexadecanoic acid (palmitic acid; Sigma-Aldrich, St. Louis, MO), Dox hydrochloride (D1515, Sigma-Aldrich), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[carboxy(polyethylene glycol)-2000] (DSPE-PEG(2000)-COOH; Avanti Polar Lipids) were dissolved in methanol (J. T. Baker, Phillipsburg, NJ) using a magnetic stirrer at 400 rpm and 25 °C for 30 min. The total concentration of the organic phase solute in methanol was 40 mg/ml, where the Dox concentration was 1 or 2 mg/ml. The weight percentage of DSPE-PEG(2000)-COOH was 2.5% in the lipid phase. The weight percentage of DPPC was 0%, 10%, 20%, 30%, or 40% in the lipid phase. Equal weights of cacao butter and hexadecanoic acid composed the rest of the lipid phase. The organic phase of 10 ml and the aqueous phase of 50 ml containing 0.8% (w/v) cholesteryl hemisuccinate (Sigma–Aldrich), 0.2% (w/v) taurocholate (Sigma–Aldrich), 0.6% (w/v) Tween 80 (Sigma–Aldrich), and 2% (v/v) *n*-butanol (Riedel-de Haen, Seelze, Germany) in ultrapure water (Barnstead, Dubuque, IA) were mixed using a homogenizer (PT 2100, Kinematica AG, Lucerne, Switzerland) at 20,000 rpm and 25 °C for 10 min. Chromogenic SLNs were obtained by adding fluorescein isothiocyanate (FITC; Sigma-Aldrich) of 50 ppm to the aqueous phase during preparation. FITC is a common reagent for fluorescent labeling and was encapsulated primarily in the current Dox-SLNs. In addition, FITC could react with amino, sulfhydryl, imidazoyl, tyrosyl, or carbonyl groups and a conjugation of FITC with primary or secondary amines yielded stable products [21]. Thus, FITC could also conjugate with secondary amine of taurocholate as a fluorescent probe in SLNs. To eliminate methanol, the emulsified liquid with newly formed Dox-SLNs was added to ultrapure water of 140 ml at 500 rpm for 2 h and dialyzed in a permeable membrane (regenerated cellulose of 50 kDa, Spectrum Laboratories, Rancho Dominguez, CA) against ultrapure water of 300 ml in a flask of 500 ml at 120 rpm for 1 h. After removing the dialysate, the remaining fluid containing Dox-SLNs was infiltrated through a filtration paper with pores of 1 μm. The filtrate was centrifuged using a centrifuge (AVANTij-25, Beckman Coulter, Palo Alto, CA) at 159,000 ×*g* and 4 °C for 10 min. The bottom pellet containing Dox-SLNs was suspended in ultrapure water of 1 ml containing 1% (w/v) D-mannitol (Sigma–Aldrich). The suspension was placed in a freezer (Panasonic, Gunma, Japan) at −80 °C for 30 min and dehydrated using a lyophilizer (Eyela, Tokyo, Japan) at 2–4 torr and −80 °C for 24 h. The solid product was preserved in a refrigerator at 4 °C. The content of free Dox in dialysate after dialysis and in supernatant after centrifugation was evaluated using a high performance liquid chromatograph (HPLC; Jasco, Tokyo, Japan) with a reverse phase BDS Hypersil C-18 column (Thermo Hypersil-Keystone, Bellefonte, PA). The mobile phase was an acetonitrile (BDH, Poole, England) gradient from 5% to 45% (v/v), propelled using two high pressure pumps (Jasco) in series at a flow rate of 1 ml/min for 20 min, and analyzed using an ultraviolet (UV)-visible detector (Jasco) at 233 nm. This wavelength was adopted from a study on the dissolution of Dox [22]. The entrapping efficiency of Dox in Dox-SLNs, EE_{Dox} (%), was defined as [(total weight of Dox – weight of free Dox)/(total weight of Dox)] × 100%. The solid Dox-SLNs were suspended in