Biomaterials 32 (2011) 5213–5222

Contents lists available at ScienceDirect

Biomaterials

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

Cell penetrating peptide conjugated bioreducible polymer for siRNA delivery

Hye Yeong Nam [a], Jaesung Kim [a], Soojin Kim [b], James W. Yockman [a], Sung Wan Kim [a], David A. Bull [c,*]

[a] Center for Controlled Chemical Delivery (CCCD), Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, UT 84112, USA
[b] Division of Hematology, School of Medicine, University of Utah, Salt Lake City, UT 84132, USA
[c] Division of Cardiothoracic Surgery, School of Medicine, University of Utah Health Sciences Center, Room 3C127, 30 North 1900 East, Salt Lake City, UT 84132, USA

ARTICLE INFO

Article history:
Received 27 January 2011
Accepted 24 March 2011
Available online 17 April 2011

Keywords:
PCM
CPP
Bioreducible polymer
Cardiomyocyte
Apoptosis

ABSTRACT

The primary cardiomyocyte–specific peptide (PCM) and the cell-penetrating peptide (CPP), HIV-Tat (49–57), were incorporated into the polymer, cystamine bisacrylamide-diaminohexane (CBA-DAH), to increase the delivery of RNAi to target cells, specifically cardiomyocytes. Interestingly, the impact of PCM and Tat conjugation on cellular uptake and transfection efficiency was greater in H9C2 rat cardiomyocytes than in NIH 3T3 cells. We examined the potential for siRNA targeting SHP-1 or Fas to inhibit the apoptosis of cardiomyocytes under hypoxic conditions using PCM and Tat-modified poly(CBA-DAH), (PCM-CD-Tat). To evaluate for efficacy in inhibiting apoptosis, either Fas siRNA/polymer or SHP-1 siRNA/polymer were transfected into cardiomyocytes treated under hypoxic and serum-deprived conditions. After incubation under hypoxic conditions, treatment with either the SHP-1 siRNA complex or the Fas siRNA complex resulted in an increase in cell viability and a reduction in LDH-cytotoxicity. The cells transfected with either of the siRNA polyplexes had a lower incidence of apoptosis as demonstrated by Annexin V–FITC/PI staining. Both the SHP-1 siRNA/PCM-CD-Tat complex and the Fas siRNA/PCM-CD-Tat complex warrant further investigation as therapeutic agents to inhibit the apoptosis of cardiomyocytes.
Published by Elsevier Ltd.

1. Introduction

Post-transcriptional gene silencing (PTGS) through RNA interference (RNAi) has been proposed as a potent biological strategy for the treatment of gene-related disease [1–3]. RNA interference is mediated by double-stranded RNA (dsRNA), 21–23 nucleotides in length, which binds to and triggers the degradation of target messenger RNA (mRNA) through RNA-induced silencing complex (RISC) in a sequence-specific manner [4,5]. Although RNA interference is a very promising technology, there are significant barriers to its development as a clinically viable therapy including: (i) the need for high levels of siRNA to achieve efficacy; (ii) the generation of an innate immune response; (iii) the inherent instability of the siRNA; and (iv) limited efficacy with systemic delivery. Delivery of siRNA with polymeric carriers such as polyethylenimine (PEI) and bioreducible disulfide polymers has been proposed as a means to overcome the inherent instability and poor and/or selective cellular uptake of siRNA. These polymeric carriers, however, still face major barriers with regard to transfection efficiency and cell-specific targeting for siRNA delivery.

In our previous studies, we have reported the development of novel cardiomyocyte-targeting bioreducible polymers for siRNA delivery to the myocardium [1,6]. In these studies, our primary cardiomyocyte-targeting peptide (PCM) conjugated bioreducible polymer targeted siRNA delivery to cardiomyocytes with higher transfection efficiency. To further enhance the transfection efficiency of siRNA targeted to cardiomyocytes, we designed and synthesized a new bioreducible polymer conjugated with both a PCM peptide and a Tat peptide. The Tat sequence (C-^{47}YGRKKRRQRRR^{57}), a cell-penetrating peptide (CPP) which facilitates the cellular uptake of HIV-1 [7–10], was introduced to the PCM-poly(CBA-DAH) (PCM-CD) to enhance the cellular uptake of siRNA. CPPs have been successfully employed as a non-viral approach for the delivery of genetic material [11–13]. In the present study, our newly modified bioreducible polymer was evaluated for specific binding to cardiomyocytes and enhanced cellular penetration into cardiomyocytes by using the cellular uptake profile and intracellular trafficking in H9C2 cells, comparing with NIH 3T3 cells.

To investigate whether our cardiomyocyte-targeted siRNA carrier improved siRNA delivery to cardiomyocytes, we used siRNA targeting SHP-1 or *Fas*. We have previously shown that our Fas siRNA delivery system inhibits cardiomyocyte apoptosis [1,6]. Along with TNF-α and Fas, the Src homology domain 2 (SH2) containing

* Corresponding author. Tel.: +1 801 581 5811; fax: +1 801 585 3936.
E-mail address: david.bull@hsc.utah.edu (D.A. Bull).

0142-9612/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.biomaterials.2011.03.058

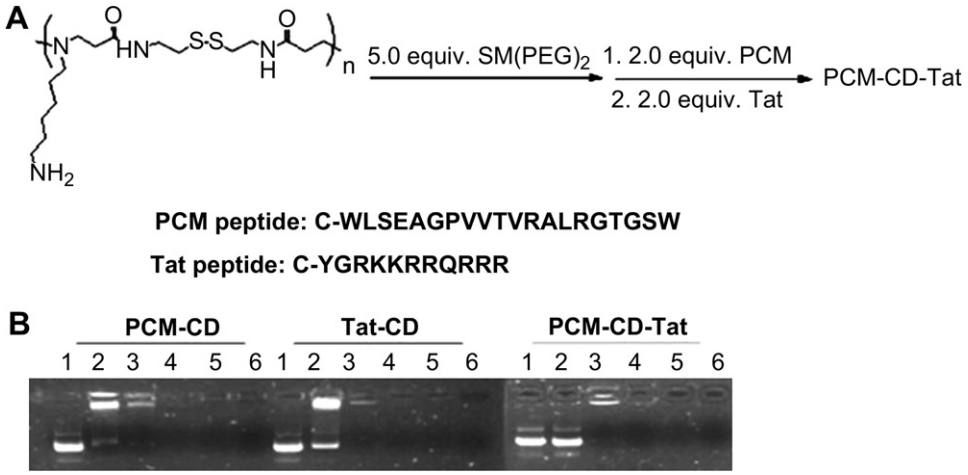

Fig. 1. (A) Schematic diagram of PCM and Tat-conjugated poly(CBA-DAH) (PCM-CD-Tat). (B) Agarose gel electrophoresis of polymers complexed with plasmid DNA at the various weight ratios of polymer/pDNA = 0, 1, 5, 10, 20 and 40 (lanes 1, 2, 3, 4, 5 and 6, respectively).

tyrosine phosphatase-1 (SHP-1) plays an important role in apoptosis and a negative regulatory role in the phosphorylation of Akt [14–18]. Akt activation is a pro-survival signal which inhibits cardiomyocyte apoptosis through extracellular signal–regulated kinase (ERK)-1/2 [19]. Therefore, silencing of SHP-1 or Fas expression with siRNA delivery should reduce apoptosis under *in vitro* or *in vivo* conditions of ischemia. In the present study, we evaluated the potential of siRNA targeting SHP-1 or Fas to inhibit the apoptosis of cardiomyocytes under hypoxic and serum-deprived conditions *in vitro*, using our newly synthesized PCM-CD-Tat for siRNA delivery to the myocardium. Gene silencing and inhibition of apoptosis under hypoxic conditions were examined by FACS analysis and real-time RT-PCR. The innate immune response to the siRNA was measured by the interferon-α response in peripheral blood mononuclear cells (PBMCs).

2. Materials and methods

2.1. Materials

N,N′-Cystaminebisacrylamide (CBA) was purchased from PolySciences, Inc. (Warrington, PA). *tert*-Butyl-*N*-(6-aminohexyl) carbamate (*N*-Boc-1,6-diaminohexane, *N*-Boc-DAH), trifluoroacetic acid (TFA), triisobutylsilane (TIS), N,N-diisopropylethylamine (DIPEA) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO). Fetal bovine serum (FBS), Dulbecco's phosphate buffered saline (DPBS), Roswell Park Memorial Institute 1640 medium (RPMI 1640), and Dulbecco's modified Eagle's medium (DMEM) were supplied from Invitrogen (Carlsbad, CA).

siRNA Fas siRNA and GFP siRNA were synthesized and supplied by Dharmacon (Lafayette, CO) and predesigned SHP-1 siRNA was obtained by Ambion (Austin, TX, USA). The siRNA sequences used in this study were the following: rat Fas siRNA, (sense) 5′–ACACGGACAGGAAACACTA–3′, (anti-sense) 5′–UAGUGUUUCCUGUCCGUGU–3′ and green fluorescent protein (GFP) siRNA, (sense) 5′–GCACGACTTCTTCAAGTCC–3′, (anti-sense) 5′–GGACUUGAAGUCGUGC–3′. The sequences of predesigned rat SHP-1 siRNA candidates are shown in Fig. 1. For the cellular uptake study and intracellular trafficking assay, the anti-sense strand of the siRNA was labeled with Cy3 dye at the 3′-terminal end.

2.2. Synthesis of the PCM-poly(CBA-DAH)-Tat

Poly(CBA-DAH), CD was synthesized according to procedures published previous by our group [20–23]. Then, conjugation of the Tat and PCM peptides to poly (CBA-DAH) was performed using hetero-bifunctional cross linker NHS-PEG $_2$-Maleimide, SM(PEG)$_2$ (Pierce, Rockford, IL). Poly(CBA-DAH) was activated with 5 M equivalents of SM (PEG)$_2$ per 10 DAH groups in anhydrous DMF for 4 h at room temperature and purified with a desalting column. Cysteine-terminated PCM (2 eq.) was added to the activated polymer and the reaction mixture was stirred for 24 h at room temperature. Tat-SH (2 eq.) was added and the reaction was allowed to continue an additional 24 h. The crude mixture was purified by extensive dialysis against ultra pure water. For the synthesis of Tat-poly(CBA-DAH) and PCM- poly(-CBA-DAH), 2 M equivalents of SM (PEG)$_2$ per 10 DAH groups and PCM-SH (2 eq.) and Tat-SH (2 eq.) were used. The conjugation of PCM and Tat peptides was confirmed by ^{1}H NMR (400 MHz, D_2O).

2.3. Polyplex formation and characterization

The pCMV-Luci plasmid was condensed with the polymers in HEPES Buffered Saline (HBS, 10 mM HEPES, 1 mM NaCl, pH 7.4) at various weight ratios and incubated at room temperature for 30 min. Modified polymers were combined with unmodified CD polymers (1:1) to produce complex formation for further experiments. Each polyplex was loaded onto an agarose gel (1.0%, w/v) containing a SYBR gel staining solution and was electrophoresed at 100 V for 30 min in TAE (10 mM Tris/HCl, 1% (v/v) acetic acid, 1 mM EDTA) buffer. The retardation band of the polyplexes was imaged with a UV illuminator using a Gel Documentation System (Bio-Rad, Hercules, CA). The average sizes and Zeta-potential measurements of polyplexes were examined using Nano ZS (ZEN3600, Malvern Instruments) with a He–Ne ion laser (633 nm). 0.06 mL of polyplex solutions (0.5 μg of pDNA) were prepared in Hepes buffered saline (10 mM Hepes, 1 mM NaCl, pH 7.4) at various weight ratios ranging from 1 to 40. After 30 min incubation, polyplex solutions were diluted to final volume of 0.6 mL before measurement. The results were obtained as the hydrodynamic diameters and polydispersity index for size and mean values ± SEM for zeta-potential.

Table 1
Size and zeta-Potential of Polyplexes.

Weight ratio	PCM-CD		Tat-CD		PCM-CD-Tat	
	Size (nm)[a]	Zeta-potential	Size (nm)	Zeta-potential	Size (nm)	Zeta-potential
1	218.4/0.277	−9.96 ± 9.54	170.6/0.138	−3.17 ± 12	138.2/0.213	−11.9 ± 9.36
5	309.9/0.413	11.2 ± 5.48	204.8/0.250	13.5 ± 5.13	189.4/0.280	17.4 ± 5.36
10	204.6/0.323	17.9 ± 7.29	144.6/0.287	22.2 ± 6.02	126.7/0.284	25.1 ± 5.89
20	181.46/0.352	18.5 ± 4.42	115.4/0.246	23.2 ± 6.54	197.3/0.241	24.6 ± 5.98
40	190.5/0.463	21.5 ± 6.87	96.81/0.404	25.2 ± 7.31	168.9/0.258	24.0 ± 5.85

[a] The sizes were expressed as the cumulated hydrodynamic diameter/polydispersity of the polyplexs.