Journal of Controlled Release 205 (2015) 155–161

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Multivalent display of pendant pro-apoptotic peptides increases cytotoxic activity

David S.H. Chu [a], Michael J. Bocek [a], Julie Shi [a], Anh Ta [a], Chayanon Ngambenjawong [a], Robert C. Rostomily [b], Suzie H. Pun [a,*]

[a] Department of Bioengineering and Molecular Engineering and Sciences Institute, University of Washington, Seattle, WA 98195, USA
[b] Department of Neurological Surgery, University of Washington, Seattle, WA 98195, USA

ARTICLE INFO

Article history:
Received 1 October 2014
Received in revised form 11 January 2015
Accepted 13 January 2015
Available online 14 January 2015

Keywords:
RAFT polymerization
Peptide delivery
Multivalency
Apoptotic peptides

ABSTRACT

Several cationic antimicrobial peptides have been investigated as potential anti-cancer drugs due to their demonstrated selective toxicity towards cancer cells relative to normal cells. For example, intracellular delivery of KLA, a pro-apoptotic peptide, results in toxicity against a variety of cancer cell lines; however, the relatively low activity and small size lead to rapid renal excretion when applied in vivo, limiting its therapeutic potential. In this work, apoptotic peptide–polymer hybrid materials were developed to increase apoptotic peptide activity via multivalent display. Multivalent peptide materials were prepared with comb-like structure by RAFT copolymerization of peptide macromonomers with *N*-(2-hydroxypropyl) methacrylamide (HPMA). Polymers displayed a GKRK peptide sequence for targeting p32, a protein often overexpressed on the surface of cancer cells, either fused with or as a comonomer to a KLA macromonomer. In three tested cancer cell lines, apoptotic polymers were significantly more cytotoxic than free peptides as evidenced by an order of magnitude decrease in IC_{50} values for the polymers compared to free peptide. The uptake efficiency and intracellular trafficking of one polymer construct was determined by radiolabeling and subcellular fractionation. Despite their more potent cytotoxic profile, polymeric KLA constructs have poor cellular uptake efficiency (<1%). A significant fraction (20%) of internalized constructs localize with intact mitochondrial fractions. In an effort to increase cellular uptake, polymer amines were converted to guanidines by reaction with *O*-methylisourea. Guanidinylated polymers disrupted function of isolated mitochondria more than their lysine-based analogs, but overall toxicity was decreased, likely due to inefficient mitochondrial trafficking. Thus, while multivalent KLA polymers are more potent than KLA peptides, these materials can be substantially improved by designing next generation materials with improved cellular internalization and mitochondrial targeting efficiency.

1. Introduction

One class of potential anti-cancer agents draws inspiration from cationic antimicrobial peptides (CAP), natural host defense mechanisms widely conserved in diverse species [1,2]. These peptides eliminate a wide range of bacteria, fungi, viruses, and protozoa [3,4] by disrupting negatively-charged membranes through electrostatic interactions, leading to pore formation, cellular depolarization, and cell death [5]. Minimal bacterial resistance was developed against antimicrobial amphiphilic polymers over several hundred cellular divisions compared to rapid development of antibiotic resistance, suggesting that drug strategies based on membrane-disruption are less susceptible to drug resistance [6]. Most CAPs have low cytotoxicity towards healthy eukaryotic cells, whose cellular membranes contain high levels of zwitterionic phosphatidylcholine resulting in minimal CAP interaction. Cancer cells, however, frequently overexpress anionic phospholipids, such as phosphatidylserine and *O*-glycosylated mucins, resulting in net-negative membranes that interact with CAPs [2,7]. Therefore, many CAPs show selective toxicity towards cancer cells relative to normal cells.

Additionally, intracellular delivery of these CAPs can induce mitochondrial dysfunction [8]. Mitochondrial membranes resemble bacterial membranes and are disrupted upon exposure to CAPs, inducing cellular apoptosis through the release of cytochrome c [8]. The peptide sequence $(KLAKLAK)_2$, or "KLA", has been shown to permeabilize mitochondrial membranes in a local peptide concentration-dependent manner [4,8]. KLA has therefore been investigated as a pro-apoptotic agent in fusion peptide [8–11], polymer conjugate [12], and nanoparticle conjugate [13] form. These materials have been studied in several cancer cell lines and animal models both *in vitro* and *in vivo*, showing

* Corresponding author at: 3720 15th Ave NE, Foege N530P, Box 355061, Seattle, WA 98195, United States.
E-mail address: spun@uw.edu (S.H. Pun).

http://dx.doi.org/10.1016/j.jconrel.2015.01.013
0168-3659/

promising cancer cell killing [10,12,13]. However, the requirement for high intracellular concentrations poses a significant barrier to clinical translation.

Multivalent polymeric display can significantly increase the activity of functional peptides and drugs. Dendrimeric display of folate, for example, has been shown to increase binding avidity up to 5 orders of magnitude [14]. Likewise, multivalent display of apoptotic peptides increased activity by over an order of magnitude [12,15]. Multivalent strategies to increase peptide bioactivity can allow for rational design and optimization of materials for cancer applications.

In this work, peptide copolymers were synthesized via reverse addition-fragmentation chain transfer (RAFT) polymerization of *N*-(2-hydroxypropyl) methacrylamide (HPMA) with methacrylamido-functionalized peptide macromonomers and evaluated in several cancer lines. Two peptide sequences were used, the KLA apoptotic sequence and a GKRK targeting ligand for p32, a mitochondrial protein frequently overexpressed on the surface of tumor cells [16], isolated from phage display. Two peptide-HPMA copolymers with differing display of the peptides were evaluated: (i) pHGcK, a copolymer of GKRK, KLA, and HPMA, and (ii) pHGfK, a copolymer of GKRK-KLA fusion peptide and HPMA. These polymers were evaluated for *in vitro* cellular toxicity, plasma membrane disruption, intracellular trafficking, and inhibition of mitochondrial respiration.

2. Materials and methods

2.1. Materials

N-(2-hydroxypropyl)methacrylamide (HPMA) was purchased from Polysciences (Warrington, PA). The initiator VA-044 was purchased from Wako Chemicals (Richmond, VA). Fmoc-protected amino acids and HBTU were purchased from AAPPTec (Louisville, KY), N-succinimidyl methacrylate from TCI America (Portland, Oregon), and Rink Amide Resin from EMD Biosciences (Darmstad, Germany). All other materials were reagent grade or better and were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

2.2. Material synthesis

2.2.1. Peptide monomers

Three peptides were synthesized using (D) and (L) amino acids and 6-aminohexanoic acid (Ahx): Ahx(D)[KLAKLAK]$_2$ (composed of only (D) amino acids); AhxGKRK(D)[KLAKLAK]$_2$ (composed of (L) amino acid uptake sequence GKRK with (D)-amino acid KLA); and AhxGKRK (composed of only (L) amino acids). Peptides were synthesized on solid support with Rink amide linker following standard Fmoc chemistry on an automated PS3 peptide synthesizer (Protein Technologies, Phoenix, AZ). Prior to peptide cleavage from the resin, the amino termini of the peptides were deprotected and coupled with N-succinimidyl methacrylate. These functionalized peptide monomers are respectively called MaAhxKLA, MaAhxGKRK-KLA, and MaAhxGKRK. Synthesized peptides were cleaved from resin by treatment of solid support with a solution of TFA/H_2O/triisopropylsilane (TIPS)/1,3-dimethoxybenzene (90:2.5:2.5:5, v/v/v/v) for 2.5 h under gentle mixing. Cleaved peptide monomers were precipitated in cold ether, dissolved in methanol and re-precipitated twice in cold ether. Each peptide monomer was purified to >95% purity using RP-HPLC and analyzed by MALDI-TOF MS.

2.2.2. Polymers

Four polymers were synthesized: HPMA-*co*-(MaAhxGKRK-KLA) (pHGfK), HPMA-*co*-MaAhxKLA-*co*-MaAhxGKRK (pHGcK), and two HPMA-*co*-MaAhxGKRK copolymers (pHG35k, pHG64k). pHGfK, pHGcK, and pHG35k were synthesized with a monomer to chain transfer agent ratio of 142 and pHG64k with a ratio of 226; all polymers had 10% peptide mole feed. Monomers were dissolved in 9:1 acetate buffer (1 M, pH 5.1):ethanol (v/v) such that the final monomer concentration of the solution was 0.7 M. The RAFT chain transfer agent (CTA) used was ethyl cyanovaleric trithiocarbonate (ECT, molecular weight 263.4 g/mol) and the initiator (I) used was VA-044. The molar ratios of total monomer:CTA:I at the start of polymerization were 142:1:0.1 and 226:1:0.1, respectively. The reaction solutions were transferred to round bottom flasks, capped with a rubber septum, purged with argon for 10 min, and then submerged in a 44 °C oil bath to initiate polymerization. The polymerization was allowed to proceed for 24 h. The flasks were removed from the oil bath and polymers dialyzed against distilled H_2O to removed unreacted monomers and buffer salts. The dialyzed polymers were lyophilized dry.

2.2.3. Guanidinylation of peptide and polymers

15 mg of the pHGcK and pHGfK copolymers and 12 mg of AcAhxKLA were dissolved in 1 mL of half-saturated $NaHCO_3$. 60 mg of o-methylisourea was dissolved in 1 mL of half-saturated $NaHCO_3$ and added to each solution. Guanidinylation reaction was allowed to proceed at room temperature under stirring for 3 days. After 3 days, polymer reactions were dialyzed again distilled H_2O to purify. Guanidinylated peptide was purified by RP-HPLC and analyzed by MALDI-TOF MS.

2.2.4. Size exclusion chromatography

Molecular weight analysis was carried out by size exclusion chromatography. The copolymers were dissolved at 2 mg/mL in running buffer (150 mM acetate buffer, pH 4.4) for analysis by size exclusion chromatography-multiangle laser light scattering (SEC-MALLS). Analysis was carried out on an OHpak SB-804 HQ column (Shodex, New York, NY) in line with a miniDAWN TREOS multiangle light scattering detector (Wyatt, Santa Barbara, CA) and an OptiLab rEX refractive index detector (Wyatt). Absolute molecular weight averages (M_n, M_w) were calculated using ASTRA software (Wyatt).

2.2.5. Amino acid analysis

The polymer composition was determined through modified amino acid analysis following the method of Bidlingmeyer and coworkers [17]. Briefly, hydrolyzed polymer samples were run on a ZORBAX Eclipse Plus C18 (Agilent Technologies, Santa Clara, CA) HPLC column with pre-column derivatization using o-phthalaldehyde/β-mercaptopropionic acid to label hydrolyzed amino acids and 1-amino-2-propanol (hydrolyzed HPMA). Calibration curves were generated using serial dilutions of (L)-lysine, (L)-arginine, and reagent grade 1-amino-2-propanol.

2.2.6. ^{3}H-pHGfK radiolabeling

pHGfK was ^{3}H-labeled using ^{3}H-acetic anhydride. 5 mg of polymer was dissolved in 500 μL of 5% triethylamine in N,N-dimethylformamide. 2.5 μL of H^3-acetic anhydride was added and reaction allowed to proceed under mixing for 2 h. Polymer was precipitated in ice-cold ether, dissolved in methanol and re-precipitated twice in ice-cold ether.

2.3. Polymer cytotoxicity

The cytotoxicity of the polymers was evaluated *in vitro* using the MTS assay. GL261 (murine glioma), SNB-19 (human glioblastoma), and HeLa (human cervical cancer) cells were plated overnight in 96-well plates at a density of 3000, 1500, and 2500 cells per well per 0.1 mL growth media, respectively. Polymers of various concentrations were prepared in phosphate buffered saline (PBS) and then diluted 10-fold in complete growth media. The cells were rinsed once with PBS and incubated with 100 μL of the polymer solution for 48 h at 37 °C, 5% CO_2. At 48 h, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium (MTS) (Promega, Madison, WI) was added to each well. Cells were then incubated for 3 h and absorbance measured at 1.5 h and 3 h at 490 nm using a plate reader (Tecan Safire^2, Männerdorf, Switzerland). IC_{50} values