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Gold nanoparticle-DNA aptamer conjugate-assisted delivery of antimicrobial peptide effectively eliminates intracellular *Salmonella enterica* serovar Typhimurium

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ABSTRACT

Antimicrobial peptides (AMPs) are a promising new class of antibacterial compounds. However, their applications in the treatment of intracellular pathogenic bacteria have been limited by their *in vivo* instability and low penetrating ability into mammalian cells. Here, we report that gold nanoparticles conjugated with DNA aptamer (AuNP-Apt) efficiently delivered AMPs into mammalian living systems with enhanced stability of the AMPs. C-terminally hexahistidine-tagged A3-APO (A3-APO^{His}) AMPs were loaded onto AuNPs conjugated with His-tag DNA aptamer (AuNP-Apt^{His}) by simple mixing and were delivered into *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium)-infected HeLa cells, resulting in the increased viability of host cells due to the elimination of intracellular *S.* Typhimurium cells. Furthermore, the intravenous injection of AuNP-Apt^{His} loaded with A3-APO^{His} into *S. Typhimurium*-infected mice resulted in a complete inhibition of *S. Typhimurium* colonization in the mice organs, leading to 100% survival of the mice. Therefore, AuNP-Apt^{His} can serve as an innovative platform for AMP therapeutics to treat intracellular bacterial infections in mammals.

1. Introduction

The rising incidence of nosocomial infections and the spreading of multidrug-resistant bacteria represent a global health threat and a burden to healthcare systems. Notably, intracellular pathogenic bacteria are often hard to eliminate, because the persistence of bacteria in host cells considerably decreases the therapeutic efficacy of antibacterial compounds due to the poor uptake of the compounds into infected host cells [1–4]. Combined with the overall rapid rise in the occurrence of bacterial resistance, the need for novel treatment strategies is evident, and this can be pursued by developing new classes of antibacterial compounds and efficient intracellular drug delivery systems.

Among many intracellular pathogenic bacteria, *Salmonella* infection is a major public health concern because it is a primary enteric pathogenic disease affecting both humans and animals [5,6]. The *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) is the best characterized in this genus. This pathogen is able to colonize the intestinal tract and modulate epithelial tight junction integrity sufficiently to allow the physical movement of polymorphonuclear leukocytes across the intestinal monolayer and to penetrate the gut epithelium to ultimately gain access to systemic sites [7–9]. Although antibiotics are used for the treatment of invasive *Salmonella* infections, the multidrug resistance (MDR) of this bacterium has been a major concern [10].

Many new classes of antibacterial compounds have been developed to treat MDR bacteria. Among them, antimicrobial peptides (AMPs) are considered promising drug candidates due to their broad ranges of activity and the decreased resistance developed by the target cells [11,12]. AMPs are biologically active

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molecules produced by a wide variety of organisms as an essential component of their innate immune response [13]. The mechanisms of action of AMPs are thought to involve the binding of the AMPs to lipopolysaccharide and lipoteichoic acid, with subsequent membrane disruption through pore formation or other processes [14]. In addition, bacterial membranes are composed of phosphatidylglycerol and phosphatidylethanolamine, and thus are negatively charged. The positively charged amino acid residues of AMPs are thought to mediate electrostatic interactions with microorganisms via the exposed negative charges on their surfaces. This may alter the secondary structure of the AMPs, facilitating their antimicrobial effects [15].

However, applications of AMPs in clinical settings have been hampered by the lack of an effective delivery system [16]. AMPs cannot be administered orally, as they are quickly degraded before reaching the site of infection. The systemic delivery of AMPs is also challenging, because the host immune system rapidly identifies and targets AMPs for clearance. For these reasons, higher AMP doses are required to achieve a therapeutic efficacy, which would be cost-prohibitive and, more importantly, cause severe side effects.

During the last decade, investigators have applied liposomes [17,18], chitosan [19], poly (lactide-co-glycolic acid) (PLGA)-nanoparticles [20], and lactic acid bacteria [21] for the delivery of AMPs. However, these molecules have limitations due to their complexity for loading AMPs and their intrinsic cytotoxicity. Therefore, in this study, to overcome the limitations of conventional delivery systems for AMPs, we employed a gold nanoparticle-DNA aptamer (AuNP-Apt) conjugate-based delivery system, which has been proven for the easy and efficient delivery of recombinant proteins into mammalian living systems without cytotoxicity [22]. In this study, we investigated whether this system can be used for the delivery of AMPs into mammalian living systems. We demonstrated that our AuNP-Apt system protectively and efficiently delivered C-terminally hexahistidine-tagged A3-APO, which has potent *in vitro* and *in vivo* activities by disrupting the bacterial membrane against Gram-negative bacteria [23–25] into *S. Typhimurium*-infected cells and mice, and the delivered A3-APO effectively eliminated intracellular *S. Typhimurium* cells, leading to a markedly enhanced survival rate of the infected host cells and mice.

2. Materials and methods

2.1. Peptide synthesis and purification

The procedure for peptide synthesis has been previously described [26]. To obtain N-terminal fluorescently labeled peptides, the resin-bound peptides were treated with 20% (v/v) piperidine in dimethylformamide to remove the protective Fmoc group from the N-terminal amino acid residue. The peptides were then cleaved from the corresponding resins, precipitated with ether, and extracted. The resultant crude peptides were purified by reversed-phase preparative high-performance liquid chromatography (HPLC) on a Jupiter C_{18} column (250 × 21.2 mm, 15 μm, 300 Å) using a 0–60% (v/v) acetonitrile gradient in water also containing 0.05% (v/v) trifluoroacetic acid. The purity of the extracted peptides (more than 95%, v/v) was then confirmed using analytical reversed-phase HPLC on a Jupiter proteo C_{18} column (250 × 4.6 mm, 90 Å, 4 μm). The molecular mass of the peptides was confirmed using a matrix-assisted laser desorption ionization mass spectrometer (MALDI II; Kratos Analytical, Inc., Spring Valley, NY, USA).

2.2. Synthesis of AuNP-Apt conjugates

Standard citrate-reduced AuNPs (15 nm in diameter) were purchased from BBI Life Science (UK). His-tag DNA aptamers (5′-GCTATGGGTGGTCTGGTTGGGATTGGCCCCGGGAGCTGGC-A10-Thiol-3′) were purchased from Bioneer (Korea). DNA aptamers were conjugated to AuNP according to previously described procedures [27].

2.3. Preparation of the AuNP-Apt-peptide complex

Aptamer-conjugated AuNPs were pre-incubated at 80 °C for 5 min to prevent the formation of secondary structures. AuNP-Apt^{His} (1 nM) and purified His-tagged peptides were incubated at room temperature in 1 × AMP binding buffer (200 mM Tris-Cl, pH 8.8, 200 mM NaOH, and 1 mM $MgCl_2$), which was equilibrated in 1 × phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4), for 10 min. The pH of each solution was adjusted with hydrogen chloride.

2.4. Binding capacity assay

Binding capacities between AMPs and AuNP-Apt^{His} conjugates were measured by previously described procedures [22].

2.5. Antibacterial assay

Salmonella enterica serovar Typhimurium ATCC14028 cells were cultured at 37 °C in Luria-Bertani (LB) medium, and the minimum inhibitory concentration (MIC) of each peptide was determined in microdilution assays. In brief, two-fold serial dilutions covering a range from 8 to 64 μM for each peptide were added to duplicate media containing cultures of bacteria (5×10^5 colony forming unit (CFU)/ml) at their mid-logarithmic phase of growth. The samples were then incubated for 18–24 h at 37 °C. After incubation, MICs were identified as the lowest peptide concentrations based on the OD_{600} measurements of the cultures.

2.6. Mammalian cell culture

HeLa (human cervical carcinoma) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Welgene, Korea) containing 10% (v/v) fetal bovine serum (Welgene) and 1% (v/v) penicillin–streptomycin (Welgene) at 37 °C with 5% (v/v) CO_2 in humidified air.

2.7. Cytotoxicity assay

HeLa (2×10^4/well) cells were plated and cultured for 18–24 h in 96-well culture dishes. The cells were further incubated with peptides in the culture media for an additional 24 h, and then viability was measured by a CellTiter-Glo® Luminescent Cell Viability Assay kit.

2.8. Live and dead cell assay

To analyze the live and dead cells, *S. Typhimurium* ATCC14028 was incubated with the peptides and monitored using fluorescence microscopy (LX71, Olympus, Tokyo, Japan). A3-APO and A3-APO^{His} peptides were added to the cells using the same procedure as used for the antimicrobial assay. The peptides were added at their respective MICs to 500 μl of a cell suspension (1×10^7 CFU/ml). After 20 min of incubation, the cells were pelleted by centrifugation at 8000 × *g* for 5 min. The cells on the slides were examined under a microscope (IX71, Olympus) [28].