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Multivalent interactions between streptavidin-based pretargeting fusion proteins and cell receptors impede efficient internalization of biotinylated nanoparticles

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ABSTRACT

Pretargeting represents a promising strategy to enhance delivery of nanoparticles. The strategy involves first introducing bispecific antibodies or fusion proteins (BFP) that can bind specific epitopes on target cells with one arm, and use the other arm to capture subsequently administered effector molecules, such as radionuclides or drug-loaded nanoparticles. Nevertheless, it remains unclear whether BFP that bind slowly- or non-internalizing epitopes on target cells can facilitate efficient intracellular delivery. Here, we investigated the cellular uptake of biotin-functionalized nanoparticles with streptavidin-scFv against TAG-72, a membrane protein on Jurkat T-cell leukemia cells. Unlike conventional active-targeted nanoparticles, we found that pretargeting resulted in preferential retention of ~100 nm nanoparticles at the plasma membrane rather than internalization into cells. We found no improvement in nanoparticle internalization by simply reducing nanoparticle concentration or surface biotin density. Interestingly, by adding both the BFP and a monoclonal antibody against TAG-72, we observed a twofold improvement in internalization of pretargeted nanoparticles. Our work illustrates that the cellular fate of pretargeted nanoparticles can be controlled by carefully tuning the interactions between pretargeting molecules and nanoparticles on the cell surface.

Statement of Significance

Pretargeting is a multi-step strategy that utilizes bispecific proteins that recognize both cellular epitopes and subsequently administered therapeutic molecules. This approach has been extensively studied for radiotherapy of blood cancers; however, pretargeting remains largely underexplored for nanoparticle targeting, including whether pretargeting can facilitate efficient *intracellular delivery*. Here, we found that high density of targeting proteins on the cell surface can effectively limit internalization of pretargeted nanoparticles. Our work underscores the need to carefully assess specific cell-pretargeting molecule pairs for applications requiring intracellular delivery, and the key design requirements for such bispecific pretargeting molecules.

1. Introduction

The primary goal of targeted drug delivery is to maximize the dose of therapeutic molecules in target tissues while minimizing exposure and toxicity in non-target tissues. A commonly exploited strategy, often referred to as "active targeting," involves conjugating ligands onto the surface of nanoparticle drug carriers. The expectation is that these nanocarriers can circulate in the bloodstream for sufficient duration such that they can extravasate, encounter, and bind to specific receptors on the surface of target cells [1,2]. Unfortunately, actively targeted nanoparticles often possess relatively poor circulation kinetics as a result of rapid clearance by the mononuclear phagocytic system (MPS), presumably because the presence of ligands on the particle surface

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compromises the otherwise stealth characteristics of polymeric coatings on these nanoparticles [1,3–5]. This in turn limits the fraction of nanoparticles that can reach and extravasate at target cells/tissues [1]. Inadequate nanoparticle targeting is further exacerbated by the fact that many diseases are comprised of heterogeneous populations of cells that cannot be effectively targeted by a single ligand [4]. These potential shortcomings of active targeting have led many investigators to explore alternative strategies to deliver nanoparticles to target cells/tissues, including ultrasound [6], magnetic or electric fields [7–9], and cell-based delivery systems [10,11].

A well-established strategy for enhancing delivery of effector molecules to target cells is "pretargeting," a multi-step approach that takes advantage of molecules that bind both cellular epitopes and effector molecules. Specifically, bispecific antibody or fusion protein (BFP) molecules (henceforth abbreviated BFP) are administered first, with the expectation that they will circulate, extravasate, and accumulate on the surface of target cells, or otherwise be quickly eliminated from systemic circulation. The effector molecules are subsequently administered and captured by cell-bound BFP. This approach has been extensively studied for the treatment of hematological malignancies in pretargeted radioimmunotherapy (PRIT) [12]. Multiple preclinical studies have shown that PRIT can lead to greater tumor-to-background ratios and reduced radioactivity in healthy organs compared to radiolabeled monoclonal antibodies used in conventional radioimmunotherapy, thereby improving imaging contrast and tumor suppression [12–14]. Additionally, several studies have reported promising results for pretargeting in diagnostic applications like PET [15–18] and optical imaging [19,20]. Despite substantial promise of PRIT for treatment of different hematological malignancies, pretargeting remains largely underexplored for use in improving targeting of nanoparticles.

An implicit requirement for effective pretargeting is that the BFP must remain on the cell surface until nanoparticles can extravaste from the circulation and reach target cells. This implies that the ideal BFP should bind cellular epitopes that are either non-internalizing, or at minimum very slowly internalizing. A natural and obvious concern with pretargeting is whether BFP bound to non-internalizing or slowly internalizing cellular epitopes can facilitate *intracellular* delivery of nanoparticles. This is particularly important because the cellular fate of nanoparticles can directly impact the type of therapeutics that can be effectively delivered via pretargeting. To investigate this question, we sought to evaluate whether BFP based on anti-tumor-associated glycoprotein (TAG)-72 single chain variable fragments (scFvs) conjugated to streptavidin (SA) can facilitate intracellular delivery of biotin-functionalized polymeric nanoparticles to a T-cell leukemia cell line. TAG-72, which is highly overexpressed in several cancers including T-cell leukemia [21,22], exhibits limited internalization and shedding [23], making it an ideal cellular target for the pretargeted approach.

2. Materials and methods

2.1. Cell lines and bispecific fusion proteins

Jurkat T-acute lymphoblastic leukemia cell line was obtained from the University of North Carolina at Chapel Hill Tissue Culture Facility, and cultured in RPMI 1640 medium (Gibco by Thermo Fisher, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) and 1% penicillin streptomycin (Gibco) at 37 °C and 5% CO_2. BFP comprised of streptavidin (SA) linked to four scFvs against either CD20 (αCD20-SA) or TAG-72 (αTAG-72-SA) were kind gifts received from Oliver W. Press' group at Fred Hutchinson Cancer Research Cancer (Seattle, WA) [24].

2.2. Nanoparticle synthesis and characterization

Carboxylate-modified green fluorescent polystyrene (PS) beads with mean diameter of 100 nm were purchased from Thermo Fisher Scientific (Grand Island, NY, USA). Methoxy polyethylene glycol (PEG) amine (mPEG-NH_2, MW 3000 g/mol) and biotin PEG amine (biotin-PEG-NH_2, MW 3244 g/mol) were purchased from JenKem Technology (Beijing, China) and Rapp Polymere (Tuebingen, Germany), respectively. mPEG-NH2 and biotin-PEG-NH2 were conjugated to PS particles at varying molar ratios (0–100% biotin) using EDC coupling to produce PS-PEG-biotin beads, as previously described [25]. To indirectly quantify PEG density, the number of residual COOH groups remaining after PEG conjugation was measured using fluorogenic 1-pyrenyldiazomethane, as previously described [25]. Hydrodynamic size and zeta potential of synthesized particles were determined by dynamic light scattering and laser Doppler anemometry, respectively, using a Zetasizer Nano (Malvern, U.K.). Nanoparticle size distribution and concentration were also determined by Nanoparticle Tracking Analysis technology using Malvern NanoSight NS500. For Nanosight characterization, each nanoparticle sample was diluted 1:200,000 in filtered PBS and run in 5 replicates.

2.3. Nanoparticles uptake measured by flow cytometry

Jurkat (TAG-72^+) cells were seeded in 96-well plates at a density of 100,000 cells/well, and treated with no FP, 500 nM control αCD20-SA BFP or 500 nM cell-specific αTAG-72-SA BFP for 4 h at 37 °C. After washing the cells three times with cold PBS to eliminate unbound BFP, cells were incubated with fluorescent PS-particles at a ratio of 10^4 beads/cell for 12 h at 37 °C or 4 °C. Next, cells were washed twice with a cold acid buffer (0.2 M glycine, 0.15 M NaCl, pH 3.0) to further remove surface bound nanoparticles followed by three cold PBS washes. Samples were kept on ice until bead uptake was quantified by flow cytometry analysis (BD FACS Canto). A total of 10,000 cells were counted per sample and the mean fluorescence intensity was measured for each sample.

2.4. Nanoparticle uptake measured by imaging flow cytometry

One million Jurkat cells per well were treated with 500 nM αTAG-72-SA BFP for 4 h at 37 °C. After removing unbound BFP by washing cells three times with ice cold PBS, PS-PEG-biotin (PS-PEGb) 100% nanoparticles (10^3 beads/cell) incubated with cells for 12 h at either 37 °C or 4 °C. Three cold acid washes followed by three cold PBS washes were performed to remove unbound and surface associated nanoparticles (NP). We added 2 μg/ml Hoechst 33342 to live cells for nuclear staining, and resuspended cells in a final volume of 50–70 μl PBS prior to transferring to siliconized tubes. Samples were kept on ice until nanoparticle internalization was evaluated by Amnis ImageStream Flow Cytometer where 10,000 cells per sample were imaged. Amnis IDEAS software (v6.1) was used to quantify the average number of punctate fluorescent spots, corresponding to green NP, per cell.

2.5. Labeled BFP internalization kinetics

We fluorescently labeled 500 nM αTAG-72-SA BFP with 10 μg of fluorescent biotin (Atto488-biotin, Sigma-Aldrich) by mixing and

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