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Dual-Receptor Targeted Strategy in Nanoparticle Design Achieves Tumor Cell Selectivity Through Cooperativity

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ABSTRACT

Targeted liposomal nanoparticles are commonly used drug delivery vehicles for targeting cancer cells that overexpress a particular cell surface receptor. However, typical target receptors are also expressed at variable levels in healthy tissue, leading to non-selective targeting and systemic toxicity. Here, we demonstrated that the selectivity of peptide-targeted liposomes for their target cells can be significantly enhanced by employing a dual-receptor targeted approach to simultaneously target multiple tumor cell surface receptors. Dual-receptor targeted approach can be tuned to create cooperativity in binding only for the cancer cells, therefore leaving the healthy cells and tissue unharmed. We evaluated this strategy in a multiple myeloma disease model where the liposomes were functionalized with two distinct peptide antagonists to target VLA-4 and LPAM-1, two receptors with increasing relevance in multiple myeloma. By employing a multifaceted strategy to synthesize dual-receptor targeted liposomes with high purity, reproducibility, and precisely controlled stoichiometry of functionalities, we identified optimal design parameters for enhanced selectivity *via* systematic analysis. Through control of the liposomal formulation and valency of each targeting peptide, we identified that the optimal dual-receptor targeted liposome consisted of a peptide density of 0.75% VLA4pep and 1% LPAM1pep, resulting in an 8-fold and 12-fold increased cellular uptake over VLA-4 and LPAM-1 single targeted liposomes respectively. This formulation resulted in a cooperative ratio of 4.3 and enhanced uptake for myeloma cells that simultaneously express both VLA-4 and LPAM-1 receptors, but displayed no increase in uptake for cells that express only one or neither of the receptors, resulting in a 28-fold selectivity of the dual-targeted liposomes for cells displaying both targeted receptors over cells displaying neither receptor. These results demonstrated that through refined design and well-characterized nanoparticle formulations, dual-receptor targeted liposomes have the potential to improve cancer therapy by providing enhanced selectivity over conventional single-receptor targeted approaches.

Keywords

nanoparticle, ligand-targeted liposome, dual-receptor targeted, multiple myeloma, selectivity, VLA-4, LPAM-1

INTRODUCTION

Nanoparticle-based drug delivery systems are being broadly developed to be used in the treatment of various diseases especially cancer.[1-7] PEGylated liposomes in particular have been shown in many studies to demonstrate extended circulation half-life and preferentially home to the tumor site *via* the enhanced permeability and retention (EPR) effect both in the experimental research and in the clinic.[8-11] To increase the efficacy of anticancer drugs, an additional layer of sophistication is typically incorporated in the nanoparticle design by using an active targeting approach.[12,13] This is achieved by conjugating targeting ligands such as antibodies, antibody fragments, peptides, or small molecules to the nanoparticle surfaces to selectively target tumor specific cell surface receptors.[14-17] Active targeting of liposomes can significantly increase the amount of drug delivered to the target cell relative to free drug or passively targeted liposomes.

Despite the advantages active targeting can provide, to date, this approach has not consistently demonstrated successful outcomes for clinical applications, in part due to differences in targeted receptors, types of targeting ligands, drug properties, and cancer models employed.[18-20] Moreover, irreproducibility in batch synthesis and complications associated with large scale production of the nanoparticle systems, especially targeted versions add further complexity to an already untrivial problem.[21,22] For targeted systems that employ a very high affinity ligand, the target receptor must only be present on the tumor cells. Nevertheless, the typical receptors that are being targeted on tumor cells are also expressed on healthy cells even if at a lower expression rate.[16,23] As a result, high affinity systems can give rise to off-site targeting and consequently, non-selective toxicity.[24,25] In more sophisticated targeting systems, selectivity can be achieved by the cooperativity effect of specific multivalent binding interactions between the drug carriers and the targeted tumor cells. In such systems, the particles

multivalently present targeting ligands that inherently have weak-moderate affinity (K_d) for the target receptors; therefore, the particles only have avidity for those cells that enable simultaneous multivalent interactions leading to highly selective particles.[26-29] It is important to emphasize this point and the distinction between systems where nanoparticles simultaneously target multiple receptors on a cell surface to use multivalency to simply achieve enhanced avidity versus our systems that is using simultaneous targeting to achieve selectivity between a healthy cell and a diseased one. This is accomplished by identifying two or more specific receptors that are known to be slightly overexpressed on the target cells, while none of the receptors have significant enough overexpression that distinguish them from healthy cells and accommodate a single targeting system to achieve selectivity.

In multiple myeloma, expression of Very Late Antigen-4 (VLA-4, also known as $\alpha_4\beta_1$ integrin) and Leukocyte Peyer's Patch Adhesion Molecule-1 (LPAM-1, also known as $\alpha_4\beta_7$ integrin) is often correlated with poor survival outcomes through the advent of drug resistance, making them attractive therapeutic targets.[30,31] However, these integrins are ubiquitously expressed on healthy tissue, which limits the therapeutic efficacy of strategies that prioritize targeting these receptors one at a time.[32] Therefore, rather than a targeted approach that focuses on a single-receptor, a dual-receptor targeted approach provides improved cell targeting and selectivity.[33,34] Successful demonstrations of dual-receptor targeted nanoparticles have been largely observed with receptor-ligand pairs that exhibit high binding affinity ($K_d \approx$ low nM), such as the folic acid-folate receptor and antibody-antigen interactions.[33,34] Alternatively, with a broad range of affinities, the use of peptides as the targeting ligands in a dual-receptor targeted strategy may further enhance selectivity through multiple low to moderate affinity interactions.[21,35]

Peptides are a class of commonly used integrin ligands, and several cyclic peptide sequences have been reported in literature as VLA-4 and LPAM-1 antagonists. Cyclic peptides are advantageous targeting ligands owing to their ease of synthetic preparation and

modification, lower antigenicity, decreased opsonization, and increased enzymatic degradation resistance *in vivo*.[22,36,37] Importantly, liposomal nanoparticles provide excellent scaffolds for the multivalent presentation of peptide ligands, enabling the precise optimization of loading densities of select peptides to modulate binding avidity to achieve selectivity.[38,39]

In this study, we established that the targeting selectivity of peptide-targeted liposomes can be dramatically enhanced for their target cells by using a dual-receptor targeted approach to simultaneously target two modestly overexpressed cell surface receptors by using weak-moderate affinity ligands. Specifically, we demonstrated efficient and selective targeting of multiple myeloma cells overexpressing both VLA-4 and LPAM-1 receptors by synthesizing dual-receptor targeted liposomes with antagonist peptides (Figure 1). The liposomes were prepared by employing a multifaceted synthetic strategy we previously established in our laboratories, where both of the targeting peptides were synthesized and purified as lipid conjugates prior to incorporation into liposomes. Conjugation of peptides to lipids prior to particle formation eliminates the inconsistencies that are typically associated with synthesis of targeted nanoparticle systems where conjugation is performed after the nanoparticles have already been synthesized. Furthermore, this synthetic strategy allows for precise control over the stoichiometry of targeting ligands on the nanoparticles, since the purified liposome components are used at the desired ratios to synthesize nanoparticles with high reproducibility and purity with precisely controlled stoichiometry of each targeting peptide.[21,22,40] Dual-receptor targeted liposomes were evaluated with multiple different blood cancer cell lines expressing variable levels of each receptor. In our dual targeting strategy, selectivity can be introduced into the system by optimizing the valency such that a single targeting ligand is insufficient for effective targeting. Through systematic evaluation of the peptide density ratios on liposome surface through various batches, the dual-receptor targeted liposomes provided cooperatively enhanced cellular uptake by cells overexpressing both target receptors and minimal uptake by cells that do not simultaneously express both receptors. Here, we show that through refined liposome

design, systematic optimization of peptide stoichiometry, and precise formulation control, dual-receptor targeted liposomes can achieve selectivity in targeting cancers, while still improving cellular uptake over traditional single-receptor targeted approaches.

EXPERIMENTAL SECTION

Materials

N-Fmoc-amino acids, NovaPEG Rink amide resin, Wang resin, 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and bovine serum albumin (BSA) from EMD Millipore (Billerica, MA); Fmoc-$(EG)_n$-OH modification reagents from Quanta Biodesign (Powell, OH); Fmoc-PEG2000-OH from JenKem Technology (Allen, TX); palmitic acid, cholesterol (CHOL), *N,N*-diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), triisopropylsilane (TIS), ethane dithiol (EDT) acetonitrile (ACN), 2-Propanol (IPA), *N,N*-dimethylformamide (DMF), dichloromethane (DCM), and piperidine from Sigma-Aldrich (St. Louis, MO); fluorescein 5-Isothiocyanate (FITC) from Toronto Reseach Chemicals (Toronto, Canada); 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), hydrogenated soy phosphatidylcholine (HSPC), methoxy PEG2000-DSPE (PEG2000-DPSE), and fluorescein PE from Avanti Polar Lipids, Inc (Alabaster, AL).

Synthesis of peptides and peptide-lipid conjugates

Ligands were synthesized using Fmoc chemistry on a solid support using Rink amide or Wang resin. Residues were activated with HBTU and DIEA in DMF for 3 minutes and coupling efficiency was monitored using Kaiser test. The Fmoc protected residues were de-protected with three applications of 20% piperidine in DMF for 3 minutes each time. The molecules were cleaved from the solid support using 94/2.5/2.5/1 TFA/H_2O/EDT/TIS mixture twice for 30 minutes each time. We purified the molecules using RP-HPLC on an Agilent (Santa Clara, CA) 1200 series system with a semi-preparative Zorbax C18 column or Zorbax C3 column with

either acetonitrile or isopropanol gradients in the mobile phase. We monitored the column eluent with a diode array detector allowing a spectrum from 200 to 400 nm to be analyzed. The purified product was characterized using a Bruker Autoflex III Smartbeam Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometer (MALDI-TOF-MS, Billerica, MA). Peptide cyclization through disulfide bond formulation was performed in DMF with DIEA under stirring overnight. Synthesis of the peptide-lipid conjugates resulted in yields of approximately 30% for the VLA-4 conjugate and 20% for the LPAM-1 conjugate. All synthesized products were confirmed by analytical HPLC injection to be ≥98% pure.

Liposome preparation

Liposomes were prepared by dry film hydration. Peptide-lipid conjugates were individually synthesized and purified as described above. All components in chloroform stocks were combined at the desired ratios, briefly mixed, dried to form a thin film using nitrogen gas, and then placed under vacuum overnight to remove residual solvent. The lipid films were hydrated at 65 °C in PBS pH 7.4, gently agitated, and extruded at 65 °C through a 0.1 μm polycarbonate filter. Single-receptor targeted liposomes adhered to the following formula (95-x):10:5:x HSPC:CHOL:PEG2000-DSPE:peptide where x was varied between 0-5 to control the peptide density. Dual-receptor targeted liposomes were formulated as (95-y-z):10:5:y:z HSPC:CHOL:PEG2000:VLA4pep:LPAM1pep, where y and z represent the peptide valency for VLA4pep and LPAM1pep, respectively. Control liposomes were always formulated as 95:10:5 HSPC:CHOL:PEG2000. Fluorescein PE (0.25%) was added as a fluorescent marker for cellular uptake quantification.

Characterization of liposomes

Particle size was measured using DLS analysis *via* the *90Plus* Nanoparticle Size Analyzer (Brookhaven Instruments Corp., Long Island, NY), using 658 nm light observed at a fixed angle of 90˚ at 20˚C. Zeta potential was measured using the *ZetaPlus* zeta potential analyzer (Brookhaven Instruments Corp.).

Cell culture

NCI-H929, MM.1S, U266, IM9, RPMI-8226, Jurkat, MOLT-4, and Raji were obtained from American Type Culture Collection (Rockville, MD). RPMI-Dox40 were kindly provided by Dr. William Dalton (H. Lee Moffitt Cancer Center, Tampa, FL). All lines were cultured in RPMI 1640 media (Cellgro, Manassas, VA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine (Gibco, Carlsbad, CA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). NCI-H929 cells were supplemented with an additional 10% FBS and 55 µM 2-mercaptoethanol. MM.1S and U266 cells were supplemented with an additional 10% FBS.

Receptor expression analysis and cell-based peptide binding assays

For integrin expression assays, cells were incubated with anti-CD49d (phycoerythrin), anti-CD29 (fluorescein), or anti-integrin β_7 (fluorescein) antibodies (BD Biosciences, San Jose, CA) in binding buffer (1.5% BSA in PBS pH 7.4) on ice for 1 h, washed twice, and analyzed on a Guava easyCyte 8HT flow cytometer (Millipore). Isotype matched antibodies were used as negative controls. For cell-based peptide binding assays, cells were incubated with increasing concentrations of fluorescein-conjugated peptides for 1 h on ice. Samples were washed twice and analyzed on Guava easyCyte 8HT flow cytometer.

***In vitro* nanoparticle uptake assays**

1 x 10^5 cells/well were plated 24 h prior to each experiment in a 24 well dish. Nanoparticles were added at 100 μM phospholipid concentration and incubated for 3 h at 37 °C. Fluorescein PE (0.25% total phospholipid) was added as a fluorescent marker to each nanoparticle formulation. After incubation, cells were washed 3 times with PBS and analyzed *via* flow cytometry. For competition experiments, peptides at 1 mM concentration were incubated with the cells for 30 min prior to addition of the liposomes.

Confocal microscopy

1 x 10^5 cells/well were plated 24 hours prior to each experiment in a 24 well dish. Nanoparticles were labeled with fluorescein PE, added at 100 μM phospholipid concentration, and incubated for 3 h at 37 °C. After incubation, the cells were washed 3 times with PBS and incubated with 50 nM Lysotracker Red (Molecular Probes, Carlsbad, CA) for 30 min at 37 °C to allow internalization. Cells were washed 3 times, spun onto glass slides, fixed in PFA, stained with 2 μg/mL Hoechst dye (bisBenzimide H 33342 trihydrochloride, Sigma) for 15 min, and washed 3 times. Cover slides were mounted using Prolong Gold Antifade Reagent (Molecular Probes). Cells were visualized by Nikon A1R confocal microscope with a 40x oil lens (Nikon Instruments, Melville, NY). Image acquisition was performed by Nikon Elements Ar software (Nikon).

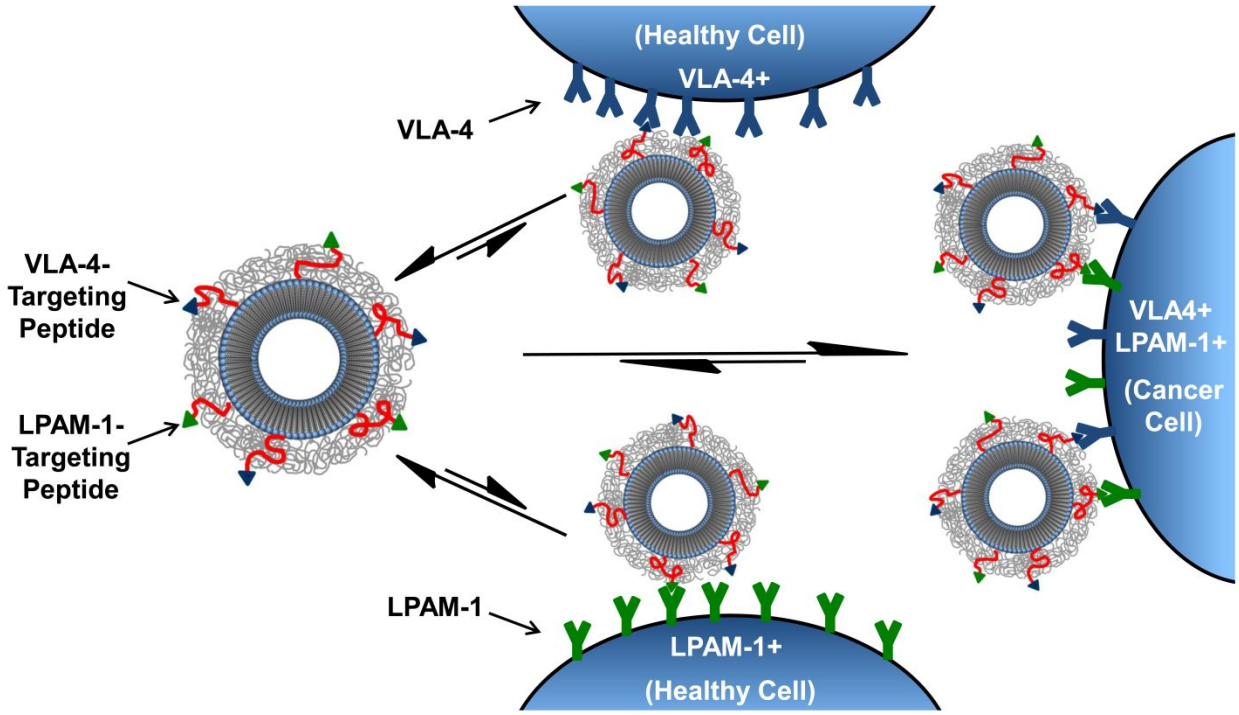


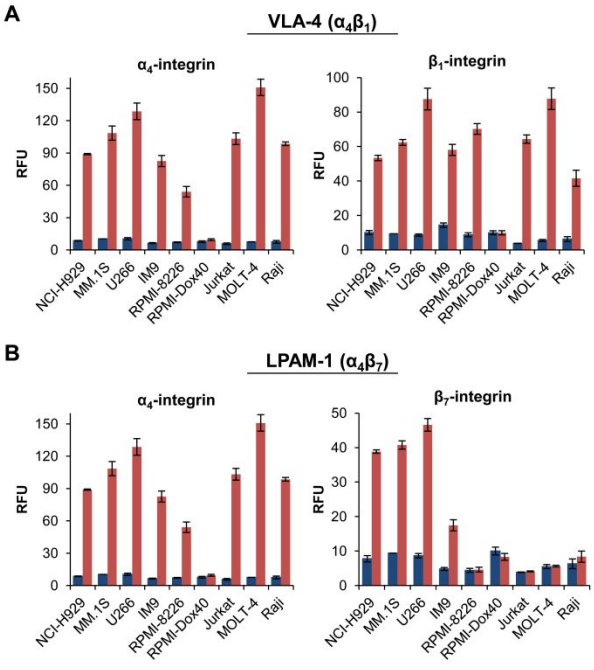
Figure 1. Schematic of dual-receptor targeted liposomes selectively targeting cancer cells expressing both VLA-4 and LPAM-1 target receptors. Dual-receptors targeted liposomes functionalized with VLA-4 and LPAM-1 antagonist peptides are designed for efficient and selective targeting of multiple myeloma cells overexpressing both VLA-4 and LPAM-1 receptors. Through systematic control of peptide density, the dual-receptor targeted liposomes cooperatively enhance cellular uptake by cells with both target receptors and show minimal uptake by cells that do not simultaneously express both receptors.

RESULTS AND DISCUSSION

Evaluation of VLA-4 and LPAM-1 expression in blood cancer cell lines

First we evaluated the VLA-4 and LPAM-1 expression in various blood cancer cell lines including multiple myeloma (NCI-H929, MM.1S, U266, IM9, RPMI-8226, RPMI-Dox40), leukemia (Jurkat, MOLT-4), and lymphoma (Raji) cell lines *via* flow cytometry by using fluorescently labeled integrin specific antibodies (Figure 2). VLA-4 positive (V+) and LPAM-1-positive (L+) cell lines included NCI-H929 (V+/L+), MM.1S (V+/L+), U266 (V+/L+), and IM9

(V+/L+). VLA-4-positive (V+) and LPAM-1-negative (L-) cell lines included RPMI-8226 (V+/L-), Jurkat (V+/L-), MOLT-4 (V+/L-), and Raji (V+/L-); the VLA-4 negative (V-) and LPAM-1 negative (L-) cell line was RPMI-Dox40 (V-/L-). These cell lines provided us with effective tools to study a dual-receptor targeted approach.



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Cell line	VLA-4 ($\alpha_4\beta_1$)	LPAM-1 ($\alpha_4\beta_7$)
NCI-H929	+	+
MM.1S	+	+
U266	+	+
IM9	+	+
RPMI-8226	+	-
Jurkat	+	-
MOLT-4	+	-
Raji	+	-
RPMI-Dox40	-	-

Figure 2. VLA-4 and LPAM-1 expression in blood cancer cell lines. Multiple myeloma (NCI-H929 (V+/L+), MM.1S (V+/L+), U266 (V+/L+), IM9 (V+/L+), RPMI-8226 (V+/L-), RPMI-Dox40 (V-/L-)), leukemia (Jurkat (V+/L-), MOLT-4 (V+/L-)), and lymphoma (Raji (V+/L-)) cancer cell lines were assayed for VLA-4 and LPAM-1 expression by using flow cytometry. (A) VLA-4 expression was determined by α_4 (left) and β_1 (right) integrin expression. (B) LPAM-1 expression was determined by α_4 (left) and β_7 (right) integrin expression. Red columns are primary

antibodies and blue columns are isotype controls. All experiments were repeated in triplicates and data represents means (±s.d.) (C) Summary of VLA-4 and LPAM-1 expression.

Identification of VLA-4- or LPAM-1-antagonist peptides that bind to VLA-4 and/or LPAM-1 overexpressing blood cancer cells with specificity

Due to the increasingly critical roles of VLA-4 and LPAM-1 in cancers, several antagonistic peptides for these receptors have been identified. Among these, the cyclic peptide sequences, YCDPC (VLA4pep; Figure 3A) and CRSDTLCGE (LPAM1pep; Figure 3B) have previously been identified as a potential VLA-4 antagonist and a LPAM-1 antagonist, respectively, in adhesion inhibitory assays.[41,42] However, neither of these peptides has been evaluated for their specific binding to VLA-4 and LPAM-1 expressing blood cancer cells.

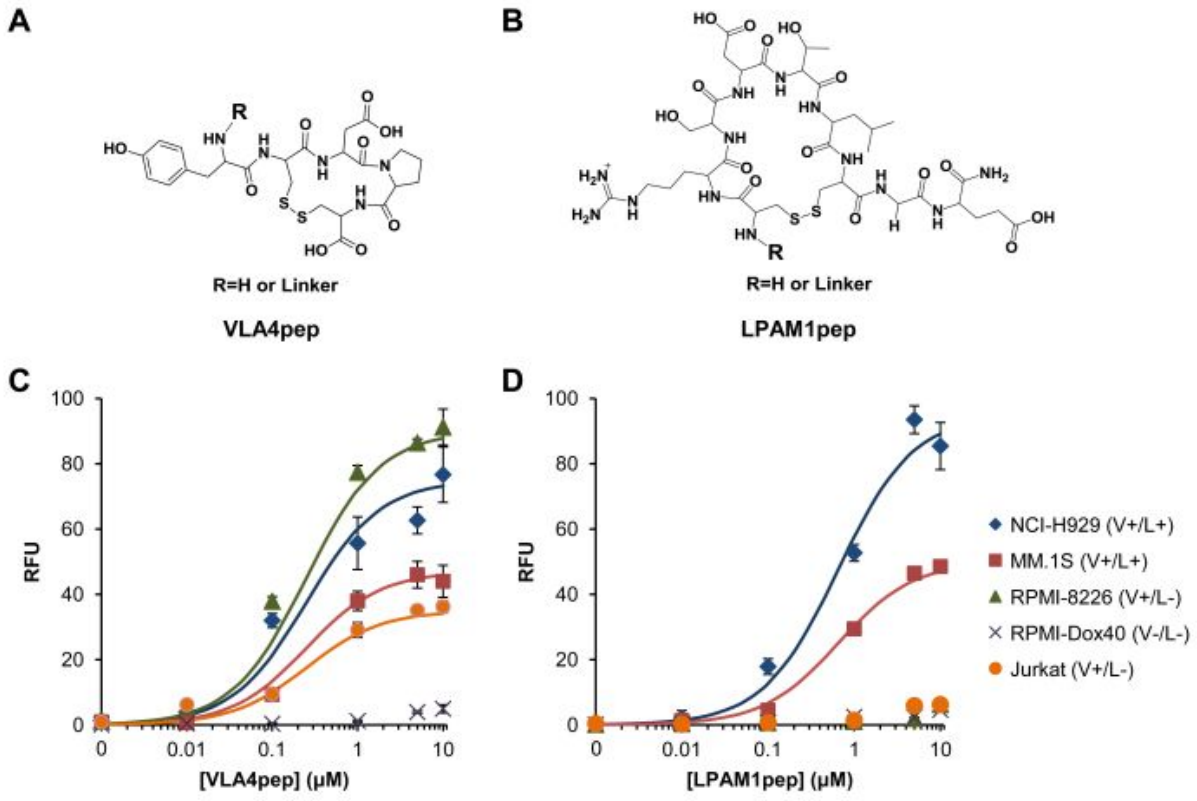


Figure 3. Identification of VLA-4 and LPAM-1-antagonist peptides. (A) Structure of VLA-4-antagonist peptide (VLA4pep). (B) Structure of LPAM-1-antgonist peptide (LPAM1pep). (C) Cellular binding assays using fluorescein labeled VLA4pep. (D) Cellular binding assays using fluorescein labeled LPAM1pep. In all experiments, NCI-H929 (V+/L+), MM.1S (V+/L+), RPMI-8226 (V+/L-), RPMI-Dox40 (V-/L-), and Jurkat (V+/L-) cell lines were incubated with the indicated peptides on ice and cellular binding was detected by flow cytometry. Negligible binding with scrambled peptides was observed which was subtracted from each data point. All experiments were repeated in triplicates and data represents means (±s.d.)

To validate specific binding of VLA4pep to VLA-4 expressing cell lines, NCI-H929 (V+/L+), MM.1S (V+/L+), RPMI-8226 (V+/L-), and Jurkat (V+/L-) cell lines were incubated on ice with fluorescein labeled VLA4pep and peptide binding to cells was analyzed by flow cytometry (Figure 3C). Control experiments performed with a fluorescein labeled scrambled peptide showed only minimal background binding, which was subtracted from each data point. VLA4pep bound to NCI-H929 (V+/L+), MM.1S (V+/L+), RPMI-8226 (V+/L-), and Jurkat (V+/L-) cell lines with an apparent K_d of 250 nM. Additional control experiments done with RPMI-Dox40 (V-/L-) showed negligible binding due to lack of VLA-4 receptor expression, as expected. Similar experiments were also performed with fluorescein labeled LPAM1pep with LPAM-1 expressing cell lines NCI-H929 (V+/L+) and MM.1S (V+/L+) (Figure 2D). LPAM1pep bound to NCI-H929 (V+/L+) and MM.1S (V+/L+) with an apparent K_d of 650 nM. Negligible binding was observed to RPMI-8226 (V+/L-), RPMI-Dox40 (V-/L-), and Jurkat (V+/L-) cell lines that do not express LPAM-1, as expected. These results demonstrated that both peptides bound to their respective target receptors with high specificity as VLA4pep bound to only VLA-4 expressing cell lines and LPAM1pep bound to only LPAM-1 expressing cell lines.

To confirm lack of cross reactivity between VLA4pep and LPAM1pep, we performed competitive binding experiments on ice with fluorescein labeled VLA4pep in the presence of

excess, unlabeled LPAM1pep by using NCI-H929 (V+/L+) and MM.1S (V+/L+) cells. The results showed that unlabeled LPAM1pep did not inhibit the binding of fluorescein labeled VLA4pep to the cells (Supporting Information, Figure S1A). A similar experiment was also performed with fluorescein labeled LPAM1pep in the presence of excess, unlabeled VLA4pep. Similarly, unlabeled VLA4pep did not inhibit the binding of fluorescein labeled LPAM1pep to the cells (Supporting Information, Figure S1B). These results confirmed lack of cross reactivity between VLA4pep and LPAM1pep when binding to the cell surface and the high specificity of VLA4pep and LPAM1pep for their respective target receptors. Additionally, to confirm independent binding events of VLA4pep and LPAM1pep, we simultaneously incubated the NCI-H929 (V+/L+) and MM.1S (V+/L+) cells with fluorescein labeled VLA4pep and LPAM1pep on ice to examine cellular binding. Our results showed that the binding of each peptide was additive as the observed fluorescence signal increased to the approximate sum of the fluorescence signal obtained from the binding of each peptide, demonstrating that both peptides could bind simultaneously to the cell surface (Supporting Information, Figure S1C).

Design and preparation of peptide-targeted liposomes

To prepare peptide-targeted liposomes with high purity and reproducibility, we synthesized, purified, and characterized the peptide-lipid conjugates prior to liposome preparation as we have recently described.[21,40] This method of incorporating the peptide-ligands in the liposomes allows for precisely defined stoichiometry of surface functionalities, elimination of coupling yield variability, and elimination of decreased binding activity due to chemical side reactions. In our design, the peptide-lipid conjugates consisted of i) VLA4pep or LPAM1pep, ii) an EG2 spacer, iii) a short oligolysine chain with 3 lysine residues (Lys_{linker}) to improve peptide solubility and availability, iv) a PEG2000 linker to present the targeting peptide above the PEG cloud, and v) two hydrophobic fatty acid chains for association with the lipid bilayer of the liposomes (Figure 4A). By using these design elements in an earlier study, we demonstrated

significantly improved cellular uptake compared to traditional targeting strategies that lacked the EG2 spacer and Lys_{linker} to enhance peptide hydrophilicity.[40] Synthetic procedures were carried out with solid phase peptide synthesis protocols using Fmoc chemistry. Completed products were cleaved from the resin, purified *via* RP-HPLC, and characterized with MALDI-TOF. Peptide cyclization through disulfide bond formation was performed in DMF with DIEA at room temperature while stirring overnight. Full synthetic scheme and mass spectrometry data of the synthesized peptide-lipid conjugates are provided (Supporting Information, Table ST1, Figure S2, and Figure S3).

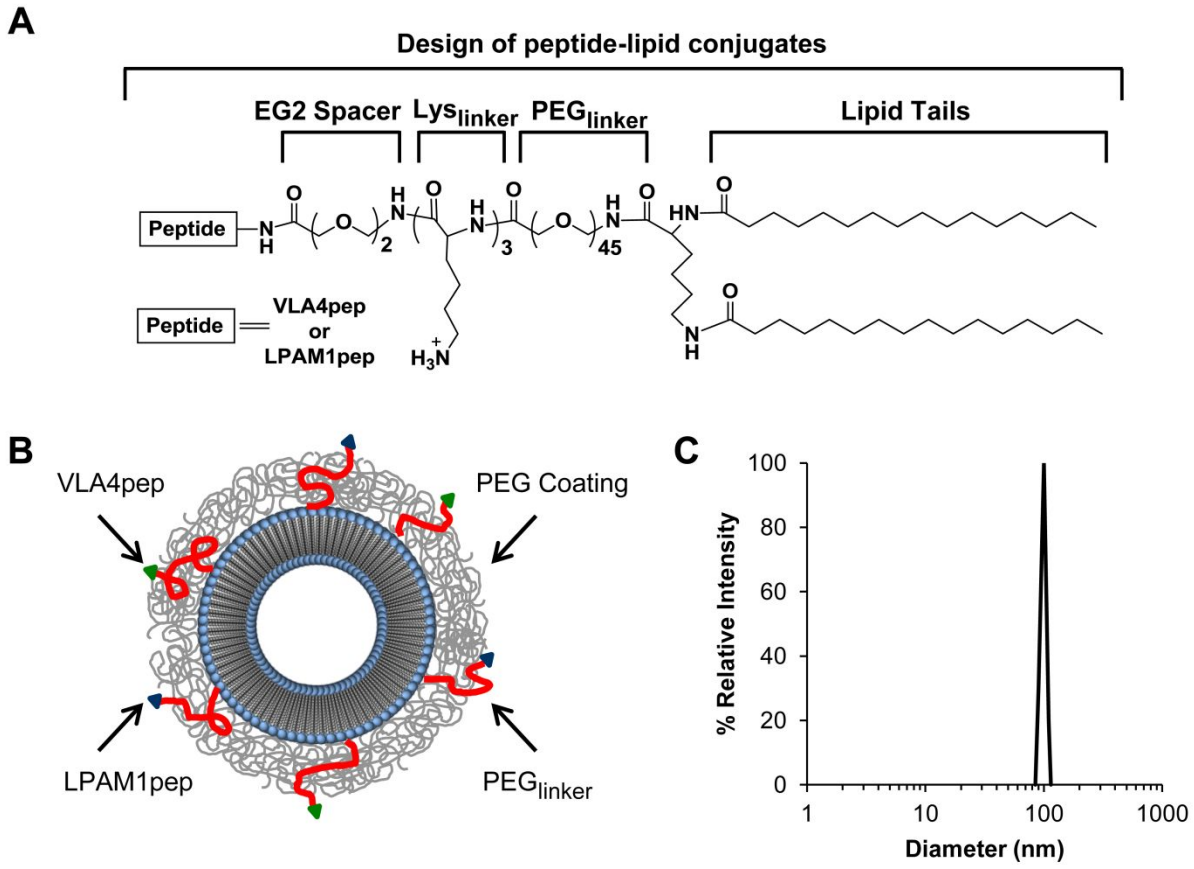


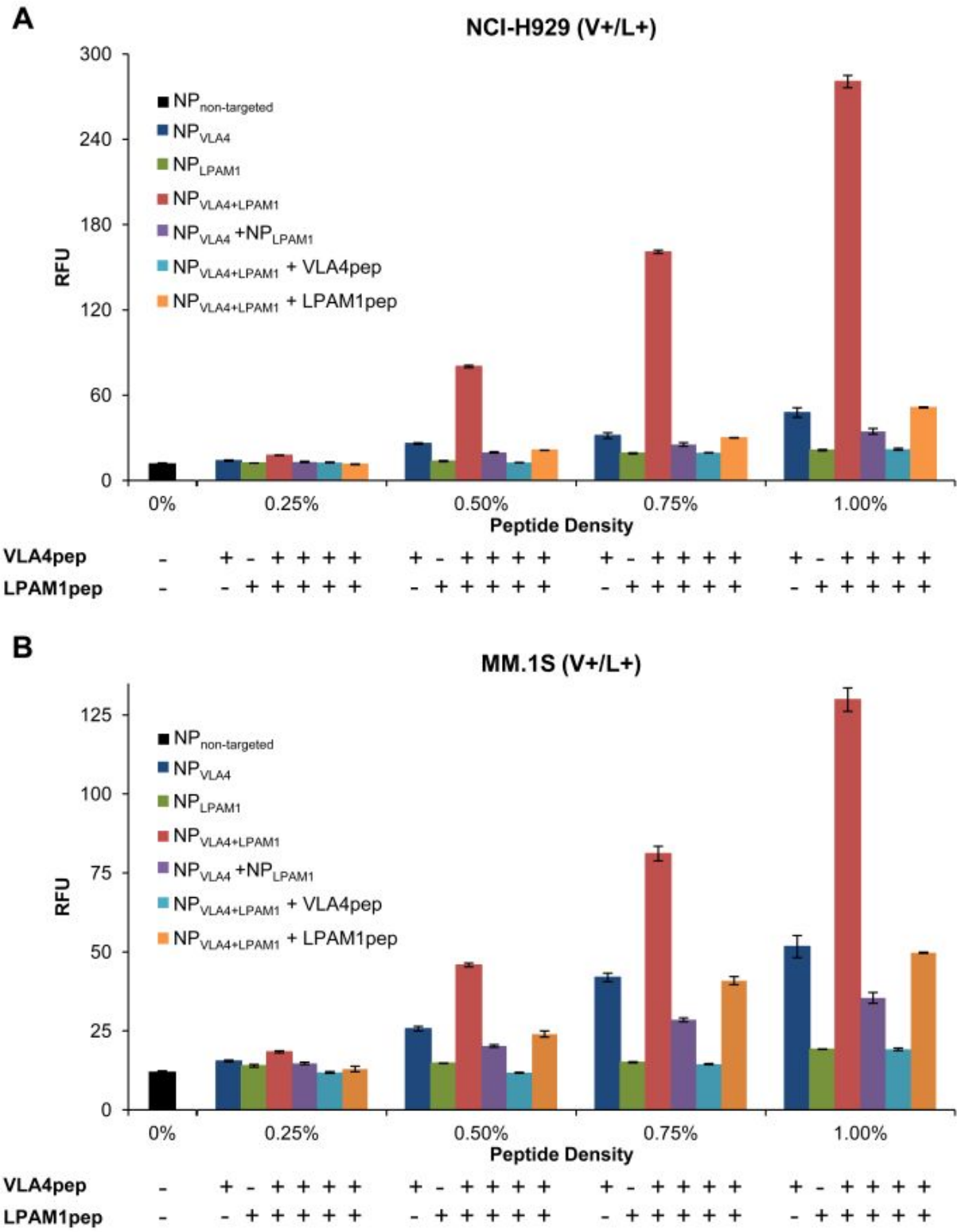
Figure 4. Design and characterization of peptide-conjugated liposomes. (A) Structure of peptide-lipid conjugates. Design includes the targeting peptide, EG2 spacer, lysine linker, PEG linker, and lipid tails. (B) Schematic of the dual peptide-targeted liposomes. (C) Dynamic light scattering analysis of liposomes.

Liposomes were prepared using purified peptide-lipid conjugates, PEG2000-DSPE, HSPC, and cholesterol, and sized *via* extrusion through a polycarbonate membrane to yield nanoparticles with 100 nm diameter as determined by dynamic light scattering (DLS) (Figure 4B-C). During preparation, the liposomal components were mixed at specific stoichiometries, precisely controlling the density of functional ligands on each particle to maintain reproducibility in nanoparticle production. Dual VLA-4- and LPAM-1-targeted liposomal nanoparticles ($NP_{VLA4+LPAM1}$) were formulated as (95-y-z):10:5:y:z HSPC:CHOL:PEG2000:VLA4pep:LPAM1pep, where y and z represent the peptide valency for VLA4pep and LPAM1pep, respectively. As a control, non-targeted liposomes ($NP_{non\text{-}targeted}$) were formulated without any peptide as 95:10:5 HSPC:CHOL:PEG2000. Additionally, single-receptor targeted liposomes with VLA4pep (NP_{VLA4}) and LPAM1pep (NP_{LPAM1}) were also prepared as (95-x):10:5:x HSPC:CHOL:PEG2000:peptide as controls. For each formulation, the mean diameter of the particles remained constant (Supporting Information, Table ST2 for particle size and zeta potential analysis of select nanoparticle formulations).

Dual-receptor targeted liposomes demonstrate enhanced cooperative cellular uptake for myeloma cells that express both VLA-4 and LPAM-1 receptors

In order to quantify the enhancement in cellular uptake by the dual-receptor targeted approach, we prepared $NP_{VLA4+LPAM1}$ with equimolar ratios of VLA4pep and LPAM1pep and evaluated cellular uptake using the NCI-H929 (V+/L+) and MM.1S (V+/L+) myeloma cell lines which express both VLA-4 and LPAM-1 receptors simultaneously. Dual-receptor targeted liposomes were formulated as (95-y-z):10:5:y:z HSPC:CHOL:PEG2000:VLA4pep:LPAM1pep, where y and z represent the peptide valency for VLA4pep and LPAM1pep, respectively. For the NCI-H929 (V+/L+) cells, we observed a dramatic enhancement in cellular uptake obtained by a dual-receptor targeted approach. Using $NP_{VLA4+LPAM1}$ at peptide densities of 0.5%, 0.75%, and 1.0% resulted in ~8, ~13, and ~23-fold enhancement in cellular uptake over $NP_{non\text{-}targeted}$,

respectively (Figure 5A). On the other hand, NP_{VLA4} showed only ~2, ~2.5, and ~4-fold enhancements at 0.5%, 0.75%, and 1.0% peptide densities, respectively, and NP_{LPAM1} showed only ~1.5 fold enhancement at 1.0% peptide density with negligible uptake at lower LPAM1pep densities. This demonstrated that the dual-receptor targeted liposomes cooperatively (more than additive effects) enhanced cellular uptake for cells simultaneously expressing both target receptors. As a control experiment, cellular uptake obtained by $NP_{VLA4+LPAM1}$ was also compared to coadministration of single-receptor targeted liposomes, NP_{VLA4}+NP_{LPAM1}. NP_{VLA4}+NP_{LPAM1} showed only ~1.5, ~2, and ~3-fold uptake enhancements at 0.5%, 0.75%, and 1.0% density of each peptide, significantly less than $NP_{VLA4+LPAM1}$ which resulted in ~8, ~13, and ~23-fold cellular uptake enhancements at the same peptide densities, respectively. These results showed that cooperative cellular uptake can only be obtained by presenting both peptides on the same liposome ($NP_{VLA4+LPAM1}$) and not by coadministration of single-receptor targeted liposomes (NP_{VLA4}+NP_{LPAM1}). Additionally, to confirm the specificity of $NP_{VLA4+LPAM1}$ for the target receptors VLA-4 and LPAM-1, competition experiments were performed in the presence of excess soluble free peptides. When NCI-H929 (V+/L+) cells were incubated with $NP_{VLA4+LPAM1}$ in the presence of excess VLA4pep ($NP_{VLA4+LPAM1}$+VLA4pep), cellular uptake significantly decreased, returning to the levels observed for NP_{VLA4}, as expected. Similar results were observed using excess LPAM1pep as the competitor peptide ($NP_{VLA4+LPAM1}$ + LPAM1pep). These demonstrated that $NP_{VLA4+LPAM1}$ specifically targeted the VLA-4 and LPAM-1 receptors and that both receptors must be available to increase cellular uptake cooperatively.


A
NCI-H929 (V+/L+)
RFU
300
240
180
120
60
0
NP non-targeted
NP VLA4
NP LPAM1
NP VLA4+LPAM1
NP VLA4 +NP LPAM1
NP VLA4+LPAM1 + VLA4pep
NP VLA4+LPAM1 + LPAM1pep
0%
0.25%
0.50%
0.75%
1.00%
Peptide Density
VLA4pep - + - + + + + + - + + + + + - + + + + + - + + + + +
LPAM1pep - - + + + + + - + + + + + - + + + + + - + + + + +
B
MM.1S (V+/L+)
RFU
125
100
75
50
25
0
NP non-targeted
NP VLA4
NP LPAM1
NP VLA4+LPAM1
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NP VLA4+LPAM1 + VLA4pep
NP VLA4+LPAM1 + LPAM1pep
0%
0.25%
0.50%
0.75%
1.00%
Peptide Density
VLA4pep - + - + + + + + - + + + + + - + + + + + - + + + + +
LPAM1pep - - + + + + + - + + + + + - + + + + + - + + + + +

Figure 5. Dual-receptor targeted liposomes cooperatively enhance cellular uptake for cell lines expressing both receptors. The cellular update of fluorescein labeled, dual-receptor targeted liposomes ($NP_{VLA4+LPAM1}$) by (A) NCI-H929 (V+/L+) and (B) MM.1S (V+/L+) cell lines was determined by flow cytometry. $NP_{VLA4+LPAM1}$ were prepared using equimolar ratios of VLA4pep and LPAM1pep at the indicated peptide densities. As control experiments, non-targeted ($NP_{non\text{-}targeted}$), VLA4-targeted (NP_{VLA4}), LPAM-1-targeted (NP_{LPAM1}) liposomes and a 1:1 mixture of single-receptor targeted liposomes (NP_{VLA4}+NP_{LPAM1}) were also tested. Competition experiments were also performed using $NP_{VLA4+LPAM1}$ in the presence of excess VLA4pep or LPAM1pep to determine specificity of uptake. All experiments were repeated in triplicates and data represents means (±s.d.)

These results observed with the NCI-H929 cell line were also confirmed with the MM.1S (V+/L+) cell line which also expresses both VLA-4 and LPAM-1 receptors simultaneously. For the MM.1S (V+/L+) cell line, $NP_{VLA4+LPAM1}$ at peptide densities of 0.5%, 0.75%, and 1.0% resulted in ~4, ~7, and ~11-fold cellular uptake enhancements over $NP_{non\text{-}targeted}$ (Figure 5B). This was again a significant cooperative enhancement over both NP_{VLA4} with ~2, ~3.5, and ~4-fold enhancements at 0.5%, 0.75%, and 1.0% VLA4pep densities, respectively, and NP_{LPAM1} which only demonstrated ~1.5-fold uptake enhancement at 1.0% LPAM1pep density with negligible uptake at lower LPAM1pep density. Additionally, NP_{VLA4}+NP_{LPAM1} showed only ~1.5, ~2, and ~3-fold uptake enhancements at 0.5%, 0.75%, and 1.0% peptide density, further confirming that the improved cellular uptake achieved by dual-receptor targeted liposomes was cooperative and achievable only by having both peptides present on the same liposome scaffold, rather than mixtures of single-receptor targeted liposomes. Collectively, through systematic optimization and precise surface control, these results demonstrated that dual-receptor targeted liposomes can provide cooperative uptake with significantly more cellular accumulation compared to conventional single-receptor targeted and non-targeted liposomes.

Dual-receptor targeted liposomes do not cooperatively enhance cellular uptake in cells that express only one or none of the VLA-4 or LPAM-1 receptors

To determine if the dual-receptor targeted approach required both VLA-4 and LPAM-1 receptor expression on the cell surface for cooperatively enhanced cellular uptake, we examined the uptake efficiency of $NP_{VLA4+LPAM1}$ with cell lines that only express one or none of the target receptors. For this, the RPMI-8226 (V+/L-) and Jurkat (V+/L-) cell lines that lack LPAM-1 expression and also the RPMI-Dox40 (V-/L-) cell line that lacks both VLA-4 and LPAM-1 expression were used. $NP_{VLA4+LPAM1}$ was prepared using equimolar ratios of VLA4pep and LPAM1pep and cellular uptake was evaluated by flow cytometry. We did not observe any uptake enhancement with $NP_{VLA4+LPAM1}$ over NP_{VLA4} or NP_{LPAM1} when the RPMI-8226 (V+/L-) cell line was used at any of the peptide densities tested (Figure 6A). For example, at 0.5% peptide density, $NP_{VLA4+LPAM1}$ showed ~3.5-fold uptake enhancement over $NP_{non\text{-}targeted}$ similar to NP_{VLA4} (~3.5-fold), confirming that both receptors must be expressed on the cell surface to achieve cooperatively enhanced cellular uptake by a dual-receptor targeted approach. NP_{LPAM1} showed negligible cellular uptake, consistent with the high specificity of these formulations for their intended target receptor as RPMI-8226 (V+/L-) lacks LPAM-1 expression. Control experiments performed in the presence of excess free VLA4pep ($NP_{VLA4+LPAM1}$+VLA4pep) showed that cellular uptake returned to the values of $NP_{non\text{-}targeted}$, further demonstrating the high specificity of $NP_{VLA+LPAM1}$ for the target receptors.

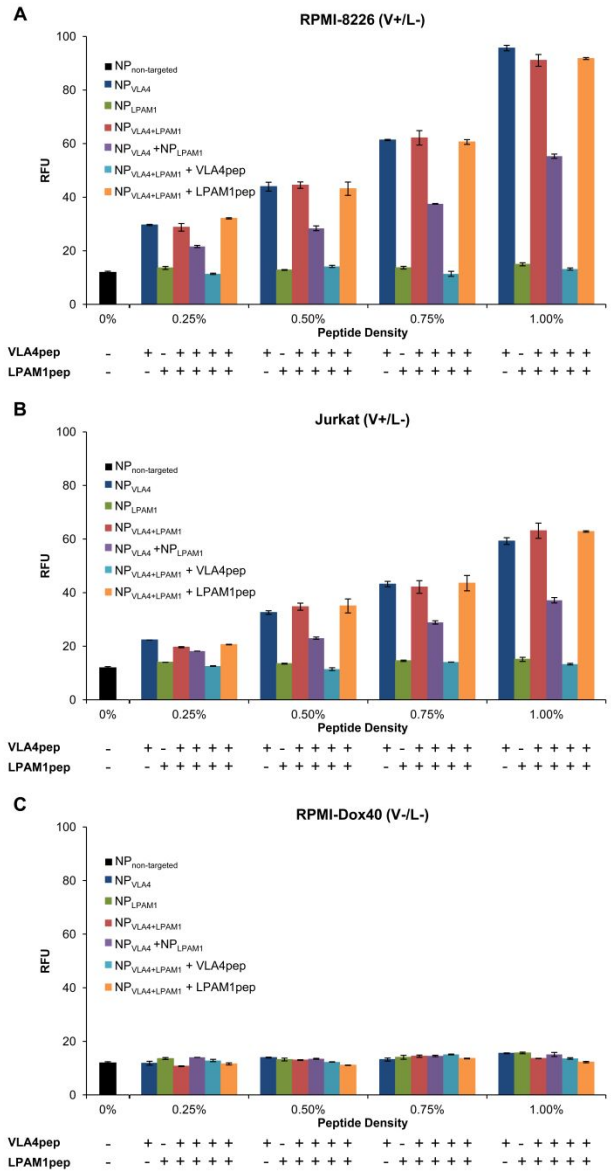

Figure 6. Dual-receptor targeted liposomes do not enhance uptake with cell lines that do not express both receptors simultaneously. The cellular update of fluorescein labeled, dual-receptor targeted liposomes ($NP_{VLA4+LPAM1}$) by (A) RPMI-8226 (V+/L-), (B) Jurkat (V+/L-) and (C) RPMI-Dox40 (V-/L-) cell lines was determined by flow cytometry. $NP_{VLA4+LPAM1}$ were prepared using equimolar ratios of VLA4pep and LPAM1pep at the indicated peptide densities. As control experiments, non-targeted ($NP_{non-targeted}$), VLA4-targeted (NP_{VLA4}), LPAM-1-targeted (NP_{LPAM1}) liposomes, and a 1:1 mixture of single-receptor targeted liposomes (NP_{VLA4}+NP_{LPAM1}) were used. Competition experiments were also performed using $NP_{VLA4+LPAM1}$ in the presence of

excess VLA4pep or LPAM1pep to determine specificity of uptake. All experiments were repeated in triplicates and data represents means (±s.d.)

Similar results were also observed for the Jurkat (V+/L-) cell line (Figure 6B). Both $NP_{VLA4+LPAM1}$ and NP_{VLA4} showed the same enhancement of uptake over $NP_{non\text{-}targeted}$ at all evaluated peptide densities due to lack of LPAM-1 expression. Additional experiments were also performed with the RPMI-Dox40 (V-/L-) cell line that does not express either the VLA-4 or LPAM-1 receptor. As expected, there was no enhancement of uptake by any of the targeted formulations ($NP_{VLA4+LPAM1}$, NP_{VLA4}, and NP_{LPAM1}) over $NP_{non\text{-}targeted}$ due to lack of target receptor expression (Figure 6C). These results demonstrated that dual-receptor targeted liposomes do not provide cooperatively enhanced cellular uptake in cells that express only one or none of the VLA-4 or LPAM-1 receptor.

Dual-receptor targeted liposomes with variable peptide stoichiometry elucidate the significance of liposome design on enhanced cellular uptake

To further evaluate how the relative stoichiometry of VLA4pep and LPAM1pep incorporated into dual-receptor targeted liposomes influences cellular uptake, we prepared $NP_{VLA4+LPAM1}$ by holding the density of one targeting peptide constant at either 0.25%, 0.5%, 0.75%, or 1.0% and varying the other peptide density from 0.25% to 1.75%. For this study, the NCI-H929 (V+/L+) cell line that expresses both VLA-4 and LPAM-1 was used to evaluate the cellular uptake of the liposomes by flow cytometry. To analyze the cooperative effects of simultaneously targeting two receptors, a cooperative ratio of each combination of peptide densities was also calculated. This ratio was equal to the uptake of the dual-receptor targeted liposome divided by the sum of the uptakes of the two individually-targeted liposomes of matching peptide densities. If this ratio was greater than one, the peptide ratios were determined to have a cooperative effect on liposome uptake. Initially, we examined the effect of

fixed VLA4pep density at 0.25% (Figure 7A, 8A), 0.5% (Figure 7B, 8B), 0.75% (Figure 7C, 8C), or 1.0% (Figure 7D, 8D) when LPAM1pep density was varied from 0.25 to 1.75% on cellular uptake. At 0.25% VLA4pep density, both cellular uptake and the relative cooperative effects increased and reached a plateau at ~1.25% LPAM1pep density, as $NP_{VLA4+LPAM1}$ showed a cooperative ratio of 2.5 (Figure 7A, 8A). Beyond this point, the cooperative effects began to decrease with increasing LPAM1pep density and cellular uptake reached a plateau, presumably due to saturation of the receptors and receptor trafficking mechanisms. Similar results were observed at higher densities of VLA4pep (Figure 7B-D, 8B-D). For example, when VLA4pep density was kept constant at 0.5% while varying LPAM1pep density, maximum cooperativity occurred at ~1.0% LPAM1pep density with a cooperative ratio of 3.5 (Figure 7B, 8B). These trends were also observed for formulations with fixed VLA4pep densities of 0.75% (Figure 7C, 8C) and 1.0% (Figure 7D, 8D). For these densities of VLA4pep, the maximum cooperative ratio occurred when using 1.0% LPAM1pep. Systematic stoichiometric control of the peptide density in dual-receptor targeted liposomes enabled us to identify that as long as VLA4pep density was 0.5% or greater, the incorporation of 1.0% LPAM1pep in the formulation was ideal for maximum cooperativity, beyond which cellular uptake reaches a plateau and cooperativity decreases.

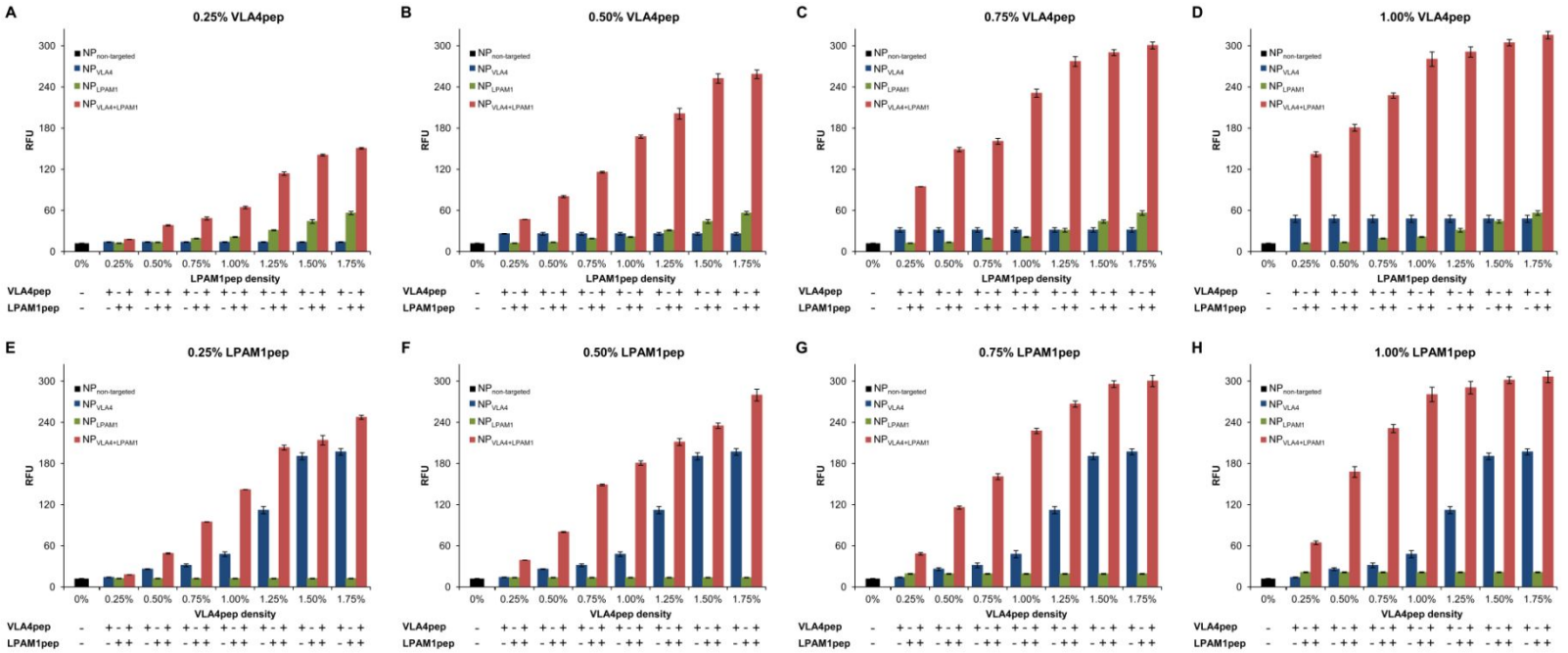

Figure 7. The effect of peptide stoichiometry on the cellular uptake of dual-receptor targeted liposomes. The cellular uptake of fluorescein labeled, dual-receptor targeted liposomes $NP_{VLA4+LPAM1}$ with variable peptide stoichiometry and densities were evaluated for the NCI-H929 (V+/L+) cell line. Peptide stoichiometries included fixed VLA4pep at (A) 0.25%, (B) 0.5%, (C) 0.75%, and (D) 1.0% while varying LPAM1pep between 0.25%-1.75% as well as fixed LPAM1pep at (E) 0.25%, (F) 0.5%, (G) 0.75%, and (H) 1.0% while varying VLA4pep between 0.25%-1.75%. Control experiments included non-targeted ($NP_{non\text{-}targeted}$), VLA4-targeted (NP_{VLA4}), and LPAM-1-targeted (NP_{LPAM1}) liposomes. Uptake was determined by flow cytometry. All experiments were repeated in triplicates and data represents means (±s.d.)

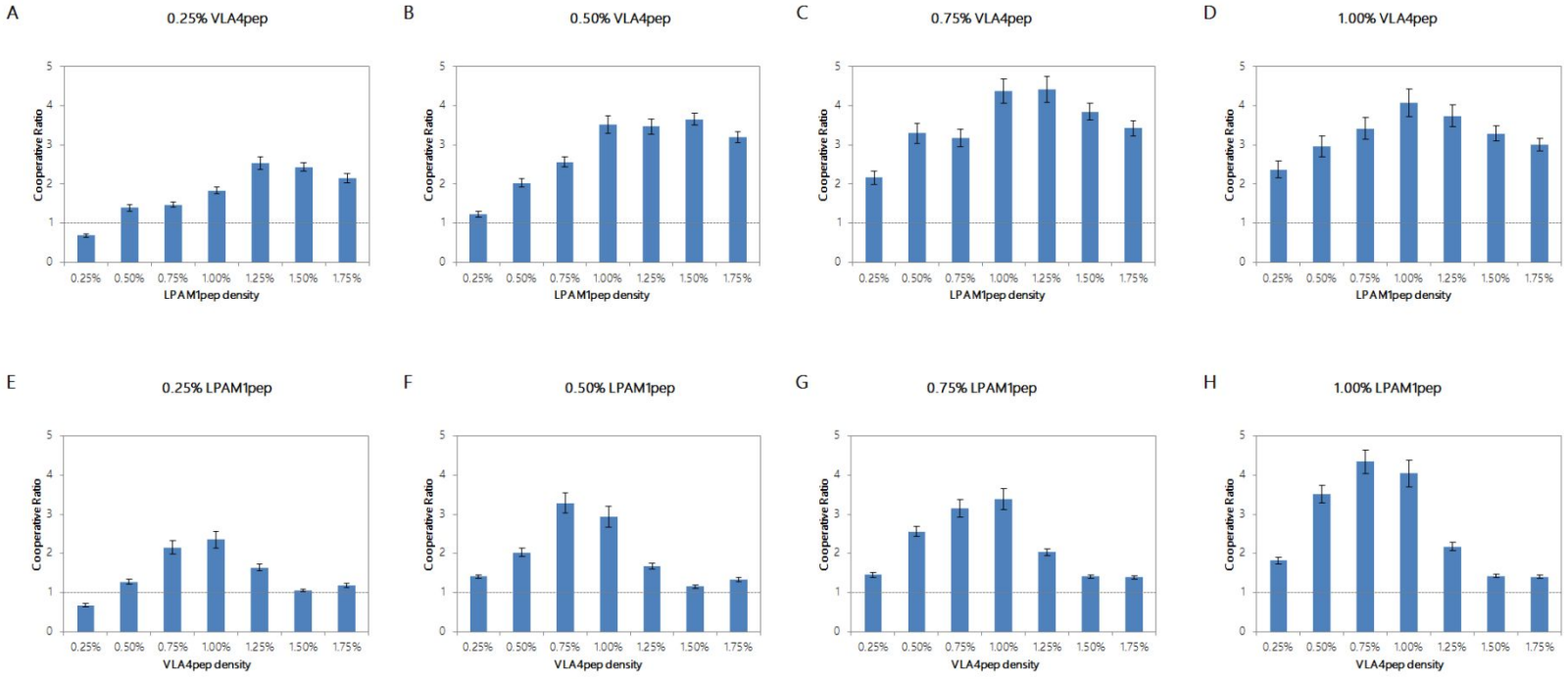

Figure 8. Cooperative ratio for the uptake of dual-receptor targeted liposomes. Visualization of the cooperative ratio for the uptake presented in Figure 7. Peptide stoichiometries included fixed VLA4pep at (A) 0.25%, (B) 0.5%, (C) 0.75%, and (D) 1.0% while varying LPAM1pep between 0.25%-1.75% as well as fixed LPAM1pep at (E) 0.25%, (F) 0.5%, (G) 0.75%, and (H) 1.0% while varying VLA4pep between 0.25%-1.75%. Control experiments included non-targeted ($NP_{non\text{-}targeted}$), VLA4-targeted (NP_{VLA4}), and LPAM-1-targeted (NP_{LPAM1})

liposomes. Uptake was determined by flow cytometry. All experiments were repeated in triplicates and data represents means (±s.d.)

Next, we examined the effect of fixed LPAM1pep density at 0.25% (Figure 7E, 8E), 0.5% (Figure 7F, 8F), 0.75% (Figure 7G, 8G), or 1.0% (Figure 7H, 8H) and variable VLA4pep density from 0.25 to 1.75% on cellular uptake. At 0.25% LPAM1pep density, cooperative cellular uptake reached a maximum at 0.75-1.0% VLA4pep, as $NP_{VLA4+LPAM1}$ showed a cooperative ratio of 2.2 (Figure 7E, 8E). Interestingly, at higher densities of VLA4pep, although cellular uptake increased, the cooperative effects decreased to negligible values. For example, at 0.25% LPAM1pep and 1.5% VLA4pep density, $NP_{VLA4+LPAM1}$ showed a cooperative ratio of only 1.05. This indicates that $NP_{VLA4+LPAM1}$ lost selectivity for cell lines expressing both target receptors above VLA4pep density of 1.5% as the single-receptor targeted NP_{VLA4} was just as efficient at promoting cellular uptake as $NP_{VLA4+LPAM1}$. Similar results were also observed when LPAM1pep density was increased to 0.5% while varying VLA4pep density from 0.25 to 1.75%. Maximum cooperativity occurred at 0.5% LPAM1pep and 0.75% VLA4pep as $NP_{VLA4+LPAM1}$ showed a cooperative ratio of 3.3 (Figure 7F, 8F). Again, despite increased cellular uptake, the cooperative ratio decreased to minimal values upon increasing VLA4pep to 1.5% with $NP_{VLA4+LPAM1}$ showing only a negligible cooperative ratio of 1.15. These trends were also observed for formulations with fixed LPAM1pep densities of 0.75% (Figure 7G, 8G) and 1.0% (Figure 7H, 8H). Importantly, for all LPAM1pep densities examined, the maximum cooperative ratio occurred when using 0.75% VLA4pep. Combined with the findings reported above (Figures 7A-D, 8A-D), our results demonstrated that the maximum cooperative cellular uptake occurred when the dual targeted liposomes were prepared with 0.75% VLA4pep and 1.0% LPAM1pep, as this combination displayed the highest cooperative ratio of all tested at 4.3. These findings showed that the maximum cooperative ratio and cellular uptake is highly dependent on the

liposome formulation, the targeted receptors, and densities of each of the targeting peptides. In order for dual-receptor targeted liposomes to promote efficient and cooperative uptake, systematic optimization of the density of each peptide on the liposome surface is required to provide enhancements over single-receptor targeted formulations.

Dual-receptor targeted liposomes demonstrate selectivity in targeting of myeloma cells that simultaneously express both VLA-4 and LPAM-1 receptors

Selective delivery of liposomal vehicles to their intended target cells is essential to reduce systematic toxicity and improve efficacy. In order to determine if the engineered dual-receptor targeted liposomes were selective only for cells that express both VLA-4 and LPAM-1 receptors simultaneously, we analyzed the cellular uptake of $NP_{VLA4+LPAM1}$ a cell line expressing both receptors (NCI-H929 (V+/L+)) and compared it to the uptake observed with cell lines expressing only one receptor (Jurkat (V+/L-)) or no target receptors (RPMI-Dox40 (V-/L-)). A dual-receptor targeted liposome, $NP_{VLA4+LPAM1}$, was prepared using 0.75% VLA4pep and 1.0% LPAM1pep, which was determined as the optimal densities to provide maximum cooperative uptake (Figure 9). Single-receptor targeted NP_{VLA4} or NP_{LPAM1}, as well as non-targeted liposomes, $NP_{non\text{-}targeted,}$ were used as controls. A significant enhancement in cellular uptake was observed using $NP_{VLA4+LPAM1}$ (~23 fold over $NP_{non\text{-}targeted}$) for the NCI-H929 (V+/L+) cell line, while only minimal uptake of $NP_{VLA4+LPAM1}$ was observed with the Jurkat (V+/L-) (~5-fold over $NP_{non\text{-}targeted}$), and no enhancement of uptake was observed with the RPMI-Dox40 (V-/L-) cell line. This experiment demonstrated that $NP_{VLA4+LPAM1}$ was selectively taken up by the NCI-H929 (V+/L+) cells that express both VLA-4 and LPAM-1 simultaneously, but not with the Jurkat (V+/L-) or RPMI-Dox40 (V-/L-) that expressed only one or none of the receptors. In all cell lines, the single-receptor targeted nanoparticles showed only 1.5-5 fold enhancements over $NP_{non\text{-}targeted}$ which shows that by incorporating both peptides into a single liposomes, we enhance the avidity of the $NP_{VLA4+LPAM1}$ preferentially and selectively to target cells expressing both target receptors.

Collectively, these results demonstrated that by accounting for the properties of each receptor-pair, the dual-receptor targeted liposomes can be effectively designed and optimized to cooperatively enhance cellular uptake while providing selectivity to improve the efficiency of cell targeting over conventional, single-receptor targeted strategies.

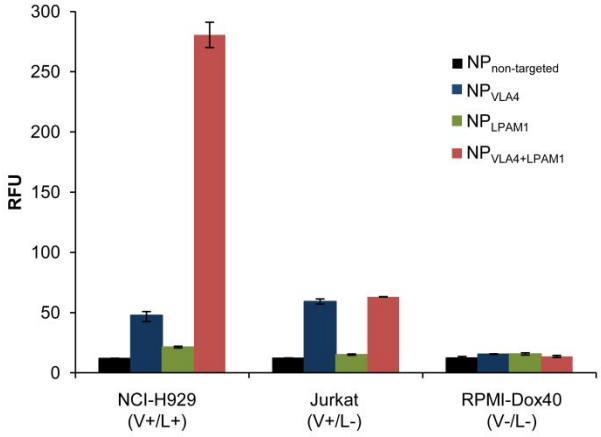

Figure 9. Dual-receptor targeted liposomes are selective only for cells that express both VLA-4 and LPAM-1 receptors. The cellular uptake of fluorescein labeled, dual-receptor targeted liposomes $NP_{VLA4+LPAM1}$ was evaluated for cell lines expressing both receptors (NCI-H929(V+/L+)), one receptor (Jurkat (V+/L-)), and no receptors (RPMI-Dox40(V-/L-)). Control experiments included non-targeted ($NP_{non\text{-}targeted}$), VLA4-targeted (NP_{VLA4}), and LPAM-1-targeted (NP_{LPAM1}) liposomes. Uptake was determined by flow cytometry. All experiments were repeated in triplicates and data represents means (±s.d.).

Validation of cellular uptake results with confocal microscopy

While flow cytometry is a powerful tool to quantify association of nanoparticles with cells, it does not distinguish between cellular binding and cellular internalization. To confirm cellular uptake and internalization of the liposomes, we performed confocal microscopy experiments with fluorescein labeled $NP_{VLA4+LPAM1}$. $NP_{non\text{-}targeted}$ and cell only controls were also included. Intracellular acidic vesicles (endosomes/lysosomes) were labeled with LysoTracker Red and the

colocalization of the nanoparticles in intracellular vesicles was examined. For both the NCI-H929 (V+/L+) and MM.1S (V+/L+) cells, we observed significant internalization into lysosomes with $NP_{VLA4+LPAM1}$, validating efficient internalization (Supporting Information, Figure S4).

CONCLUSIONS

Nanotechnology has the potential to make significant advances in the treatment and diagnosis of cancer to improve patient outcome in the clinic.[43] However, despite the advantages active targeting can provide, this approach has not consistently demonstrated success in preclinical evaluations. This is predominantly due to the fact that the target receptor is rarely overexpressed exclusively on tumor cells, rather is also expressed at variable levels in healthy tissue, leading to non-selective targeting and systematic toxicity. To address the issue of selectivity in peptide-targeted liposomes, in this study we applied nanotechnology and multivalency to develop a dual-receptor targeted liposomal nanoparticle approach, which significantly enhanced the selective targeting of multiple myeloma cells expressing both VLA-4 and LPAM-1 receptors simultaneously. Through systematic optimization and precise formulation control, the dual-receptor targeted liposomes significantly enhanced selectivity while at the same time cooperatively improving cellular uptake, providing a strategy to improve active targeting of tumor cells over conventional, single-receptor targeted strategies.

In our approach, to prepare the peptide-targeted liposomes, we used a multifaceted synthetic strategy we have recently developed, where the peptide-lipid conjugates are synthesized, purified, and characterized prior to liposome preparation. This method yielded nanoparticles with high purity and reproducibility, providing several advantages over conventional methods that involve ligand post insertion or ligand conjugation to preformed liposomes. Among the advantages provided by our multifaceted synthetic strategy were precisely defined control over the valency of surface functionalities, elimination of coupling yield variability, and elimination of decreased binding activity due to chemical side reactions.

Combined, these provided a method to consistently prepare well-defined liposomes with minimal batch-to-batch variability. In addition, our design included a short Lys_{linker} adjacent to the targeting peptide, which we have shown to significantly improve cellular uptake of peptide-targeted liposomes.[40] Consequently, the purity and precision of the liposomes prepared by our synthetic strategy enabled us to systematically evaluate and refine the design of the dual-receptor targeted nanoparticles, which would not have been possible with conventional nanoparticle preparation methods.

Our findings demonstrated that, with careful design, dual-receptor targeted liposomes have the potential to significantly improve the efficacy of active-targeted approaches, while enhancing selectivity for the tumor cells and thereby reducing possible systemic toxicity. In our experiments, through systematic optimization of the liposomal formulation and valency of each targeting peptide, the dual-receptor targeted liposomes cooperatively increased cellular uptake and enhanced selectivity for multiple myeloma cells that simultaneously express both VLA-4 and LPAM-1 receptors. Importantly, the dual-receptor targeted liposomes demonstrated negligible uptake by cell lines expressing only one or none of the VLA-4 or LPAM-1 receptors which is essential for discriminating between desired target cells and healthy tissue. The cellular uptake and cooperativity achieved by a dual-receptor targeted approach was found to be highly dependent on the peptide valency, requiring precise control of peptide density and methodical evaluation for each receptor to obtain the most effective formulation. We predict that this high dependence on peptide density is due to the density and distribution of the targeted receptors on the surface of the cell. The cooperativity shown is thus a combination of the individual monovalent binding affinities and the number of binding interactions that the liposome is able to achieve. The addition of a second targeting peptide allows binding to approximately twice as many target receptors on the cells, increasing uptake more than additively due to cross-avidity between the two targeting complexes. Combined, our results demonstrated that through the optimization of key parameters such as peptide valency and stoichiometry for each targeted

receptor, dual-receptor targeted liposomes can be effectively designed to reduce non-selective targeting and enhance cellular uptake only for target cells expressing both receptors.

The dual-receptor targeted approach described in this study is particularly important for diseases for which despite all efforts, ideal cell-surface receptors for targeting have not been identified. Ideal targets for cancer therapy are expressed homogeneously and at high levels by the tumor cell population and have negligible or low expression in healthy tissues.[16,23] For diseases such as multiple myeloma, however, the currently identified target receptors such as VLA-4, LPAM-1, CD38, CD138, and CS-1 are also expressed at variable levels in healthy tissue. For example, VLA-4 is also expressed in lymphocytes, monocytes, and hematopoietic stem cells,[44] and LPAM-1 is also expressed on leukocytes and hematopoietic progenitor cells,[45,46] limiting the therapeutic efficacy of singly targeting LPAM-1 or VLA-4 alone. This issue, however, could be overcame by the herein described dual-receptor targeted approach where we have shown that multiple myeloma cells expressing both target receptors simultaneously can be selectively targeted through refined design and systematic optimization. Thus, to discriminate between malignant and healthy cells, dual-receptor targeted liposomes have the potential to enhance the selectivity and targeting efficiency for diseases such as multiple myeloma for improved patient outcome.

In conclusion, our study has detailed the development of dual-receptor targeted liposomes for the enhanced selectivity and cooperative cellular uptake of peptide-targeted liposomes, specifically for VLA-4 and LPAM-1 targeting in multiple myeloma. These results demonstrated that when properly designed, a dual-receptor targeted strategy can improve the selectivity and efficiency of active targeting approaches to achieve results otherwise unattainable with traditional strategies targeting only a single receptor. *In vivo* studies are currently ongoing in our lab to assess the therapeutic outcome of the dual-receptor targeted liposomes on selective tumor targeting, uptake by the tumor cell, clearance, and efficacy. The application of this dual-receptor targeted approach may ultimately provide the ability to

customize ligand-targeted liposomes to fit individual patient needs, allowing for a personalized approach in cancer therapy for improved patient outcome.

CONFLICTS OF INTEREST

There are no conflicts to declare.

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REFERENCES

1. M. L. Immordino, F. Dosio and L. Cattel, *International Journal of Nanomedicine*, 2006, **1**, 297-315.
2. P. P. Deshpande, S. Biswas and V. P. Torchilin, *Nanomedicine*, 2013, **8**, 1509-1528.
3. G. T. Noble, J. F. Stefanick, J. D. Ashley, T. Kiziltepe and B. Bilgicer, *Trends in Biotechnology*, 2014, **32**, 32-45.
4. J. D. Ashley, J. F. Stefanick, V. A. Schroeder, M. A. Suckow, T. Kiziltepe and B. Bilgicer, *Journal of Medicinal Chemistry*, 2014, **57**, 5282-5292.
5. H. H. Xiao, J. F. Stefanick, X. Y. Jia, X. B. Jing, T. Kiziltepe, Y. Zhang and B. Bilgicer, *Chemical Communications*, 2013, **49**, 4809-4811.
6. H. H. Xiao, G. T. Noble, J. F. Stefanick, R. G. Qi, T. Kiziltepe, X. B. Jing and B. Bilgicer, *Journal of Controlled Release*, 2014, **173**, 11-17.
7. A. S. Paraskar, S. Soni, K. T. Chin, P. Chaudhuri, K. W. Muto, J. Berkowitz, M. W. Handlogten, N. J. Alves, B. Bilgicer, D. M. Dinulescu, R. A. Mashelkar and S. Sengupta, *Proceedings of the National Academy of Sciences of the United States of America*, 2010, **107**, 12435-12440.

8. Y. Malam, M. Loizidou and A. M. Seifalian, *Trends in Pharmacological Sciences*, 2009, **30**, 592-599.
9. T. M. Allen, W. W. K. Cheng, J. I. Hare and K. M. Laginha, *Anti-cancer Agents in Med. Chem.*, 2006, **6**, 513-523.
10. A. D. Wong, M. Ye, M. B. Ulmschneider and P. C. Searson, *Plos One*, 2015, **10**.
11. Y. Barenholz, *Journal of Controlled Release*, 2012, **160**, 117-134.
12. V. P. Torchilin, *Nature Reviews Drug Discovery*, 2005, **4**, 145-160.
13. T. M. Allen, *Nature Reviews Cancer*, 2002, **2**, 750-763.
14. T. M. Allen and P. R. Cullis, *Advanced Drug Delivery Reviews*, 2013, **65**, 36-48.
15. P. Sapra, P. Tuagi and T. M. Allen, *Curr. Drug Deliv.*, 2005, **2**, 369-381.
16. R. R. Sawant and V. P. Torchilin, *Aaps Journal*, 2012, **14**, 303-315.
17. E. Ruoslahti, *Advanced Materials*, 2012, **24**, 3747-3756.
18. R. Sinha, G. J. Kim, S. M. Nie and D. M. Shin, *Molecular Cancer Therapeutics*, 2006, **5**, 1909-1917.
19. M. Ferrari, *Nature Reviews Cancer*, 2005, **5**, 161-171.
20. O. C. Farokhzad and R. Langer, *Acs Nano*, 2009, **3**, 16-20.
21. J. F. Stefanick, J. D. Ashley, T. Kiziltepe and B. Bilgicer, *Acs Nano*, 2013, **7**, 2935-2947.
22. T. Kiziltepe, J. D. Ashley, J. F. Stefanick, Y. M. Qi, N. J. Alves, M. W. Handlogten, M. A. Suckow, R. M. Navari and B. Bilgicer, *Blood Cancer Journal*, 2012, **2**.
23. P. Sapra and T. M. Allen, *Progress in Lipid Research*, 2003, **42**, 439-462.
24. J. M. Saul, A. Annapragada, J. V. Natarajan and R. V. Bellamkonda, *Journal of Controlled Release*, 2003, **92**, 49-67.
25. K. B. Ghaghada, J. Saul, J. V. Natarajan, R. V. Bellamkonda and A. V. Annapragada, *Journal of Controlled Release*, 2005, **104**, 113-128.
26. M. L. Wolfenden and M. J. Cloninger, *Journal of the American Chemical Society*, 2005, **127**, 12168-12169.
27. C. B. Carlson, P. Mowery, R. M. Owen, E. C. Dykhuizen and L. L. Kiessling, *Acs Chemical Biology*, 2007, **2**, 119-127.
28. M. J. Cloninger, B. Bilgiçer, L. Li, S. L. Mangold, S. T. Phillips and M. L. Wolfenden, in *Supramolecular Chemistry*, John Wiley & Sons, Ltd., pp. 95-115.
29. J. F. Stefanick, T. Kiziltepe, M. W. Handlogten, N. J. Alves and B. Bilgicer, *Journal of Physical Chemistry Letters*, 2012, **3**, 598-602.
30. J. S. Damiano, A. E. Cress, L. A. Hazlehurst, A. A. Shtil and W. S. Dalton, *Blood*, 1999, **93**, 1658-1667.
31. P. Neri, L. Ren, A. K. Azab, M. Brentnall, K. Gratton, A. C. Klimowicz, C. Lin, P. Duggan, P. Tassone, A. Mansoor, D. A. Stewart, L. H. Boise, I. M. Ghobrial and N. J. Bahlis, *Blood*, 2011, **117**, 6202-6213.
32. E. Ruoslahti, *Journal of Clinical Investigation*, 1991, **87**, 1-5.
33. K. Laginha, D. Mumbengegwi and T. Allen, *Biochimica Et Biophysica Acta-Biomembranes*, 2005, **1711**, 25-32.
34. J. M. Saul, A. V. Annapragada and R. V. Bellamkonda, *Journal of Controlled Release*, 2006, **114**, 277-287.
35. R. M. Owen, C. B. Carlson, J. W. Xu, P. Mowery, E. Fasella and L. L. Kiessling, *Chembiochem*, 2007, **8**, 68-82.
36. M. Mammen, S. K. Choi and G. M. Whitesides, *Angewandte Chemie-International Edition*, 1998, **37**, 2755-2794.

37. E. Forssen and M. Willis, *Advanced Drug Delivery Reviews*, 1998, **29**, 249-271.
38. R. M. Schiffelers, G. A. Koning, T. L. M. ten Hagen, M. Fens, A. J. Schraa, A. Janssen, R. J. Kok, G. Molema and G. Storm, *Journal of Controlled Release*, 2003, **91**, 115-122.
39. J. C. Reubi, *Endocrine Reviews*, 2003, **24**, 389-427.
40. J. F. Stefanick, J. D. Ashley and B. Bilgicer, *Acs Nano*, 2013, **7**, 8115-8127.
41. D. Y. Jackson, C. Quan, D. R. Artis, T. Rawson, B. Blackburn, M. Struble, G. Fitzgerald, K. Chan, S. Mullins, J. P. Burnier, W. J. Fairbrother, K. Clark, M. Berisini, H. Chui, M. Renz, S. Jones and S. Fong, *Journal of Medicinal Chemistry*, 1997, **40**, 3359-3368.
42. N. J. P. Dubree, D. R. Artis, G. Castanedo, J. Marsters, D. Sutherlin, L. Caris, K. Clark, S. M. Keating, M. H. Beresini, H. Chiu, S. Fong, H. B. Lowman, N. J. Skelton and D. Y. Jackson, *Journal of Medicinal Chemistry*, 2002, **45**, 3451-3457.
43. M. C. Roco, *Journal of Nanoparticle Research*, 2011, **13**, 427-445.
44. T. A. Springer, *Nature*, 1990, **346**, 425-434.
45. B. Holzmann and I. L. Weissman, *Embo Journal*, 1989, **8**, 1735-1741.
46. Y. Katayama, A. Hidalgo, A. Peired and P. S. Frenette, *Blood*, 2004, **104**, 2020-2026.

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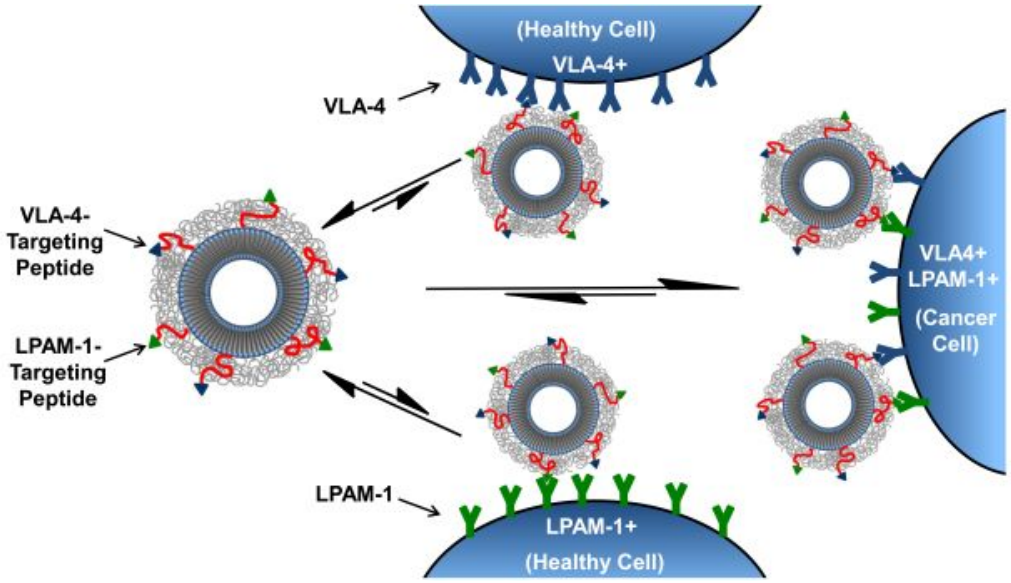

Dual-targeted liposomes achieve increased uptake and selectivity for multiple myeloma cells through cooperative peptide targeting of LPAM-1 and VLA-4.