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Identification of a Novel Lysosomal Trafficking Peptide using Phage Display Biopanning Coupled with Endocytic Selection Pressure

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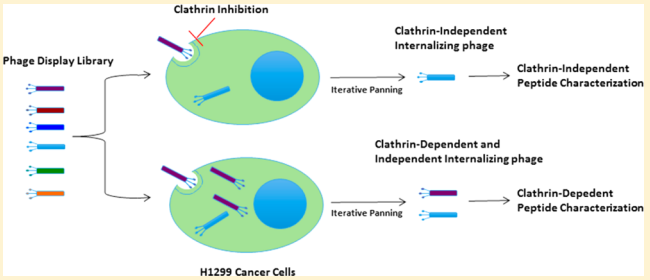
Supporting Information

ABSTRACT: Methods to select ligands that accumulate specifically in cancer cells and traffic through a defined endocytic pathway may facilitate rapid pairing of ligands with linkers suitable for drug conjugate therapies. We performed phage display biopanning on cancer cells that are treated with selective inhibitors of a given mechanism of endocytosis. Using chlorpromazine to inhibit clathrin-mediated endocytosis in H1299 nonsmall cell lung cancer cells, we identified two clones, ATEPRKQYATPRVFWTDAPG (15.1) and a novel peptide LQWRRDDNVHNFGVWARYRL (H1299.3). The peptides segregate by mechanism of endocytosis and subsequent location of subcellular accumulation. The H1299.3 peptide primarily utilizes clathrin-mediated endocytosis and colocalizes with Lamp1, a lysosomal marker. Conversely, the 15.1 peptide is clathrin-independent and localizes to a perinuclear region. Thus, this novel phage display scheme allows for selection of peptides that selectively internalize into cells via a known mechanism of endocytosis. These types of selections may allow for better matching of linker with targeting ligand by selecting ligands that internalize and traffic to known subcellular locations.



INTRODUCTION

Direct drug conjugates generally consist of three distinct parts: the targeting ligand, the linker region, and the drug.[1] The idea, simply put, is to conjugate a highly toxic and nonspecific drug to a cell-specific targeting ligand using a chemical linker region. This process has the potential to alter the pharmacodynamics and kinetics of the original drug with the goal to enhance therapeutic efficacy while minimizing adverse event associated with the treatment.[2] In terms of cancer therapies, the goal is to enhance accumulation or internalization of the conjugate within the tumor while minimizing toxic, nonspecific interactions with healthy cells and clearance organs.[1,3]

One of the major hurdles in developing efficacious direct drug conjugates is releasing active drug from the targeting agent. Most drug conjugates are inactive, existing as pro-drugs. Thus, the conjugate must be designed to be stable while traveling in the blood system but able to release active drug within the tumor or directly within cancer cells. Typically this is performed using physiological properties associated with either the tumor environment or endocytic pathways.[2] Endocytic pathways are particularly advantageous as the drug is released intracellularly to directly exert cytotoxic effects and is not free to circulate through the bloodstream in an active form.[4,5]

Many endocytic release strategies focus on internalization via the canonical clathrin-mediated endocytic pathway that includes internalization into coated pits that mature into endosomes, and eventually the ligand accumulates in low pH lysosomes.[6] This well-defined pathway has multiple physical properties which can be used for releasing the drug including decreasing pH as the ligand progresses from endosomal to lysosomal compartments as well as lysosomal specific proteases, such as the cathespins.[2,3] However, recent advances from studying endocytosis have highlighted that this pathway is one of many endocytic pathways that cells utilize to internalize extracellular ligands including raft-mediated, Arf6, Clic Geek, and macropinocytosis, to mention a few.[7,8] Many of these internalization pathways do not share the same trafficking route as the canonical clathrin-mediated pathway and can result in delivery of the cargo through different physiological environments and alternative subcellular locations. As such, release of free drug from the conjugate may be suboptimal. For example, certain raft-mediated endocytic mechanisms do not proceed through low pH endosomes, thus impairing release by pH sensitive linkers.[9−11] However, this type of trafficking pattern may be useful for delivering pH sensitive cargo such as nucleic acids or protein toxins.[12,13] Thus, knowledge of endocytic trafficking patterns has the potential to allow for better pairing of both linkers and cargo with a given targeting ligand.

This study sought to develop a method that identifies cell-targeting ligands that are specifically internalized by cancer cells and utilize a defined endocytic pathway using phage display biopanning. Current phage biopanning methods focus on

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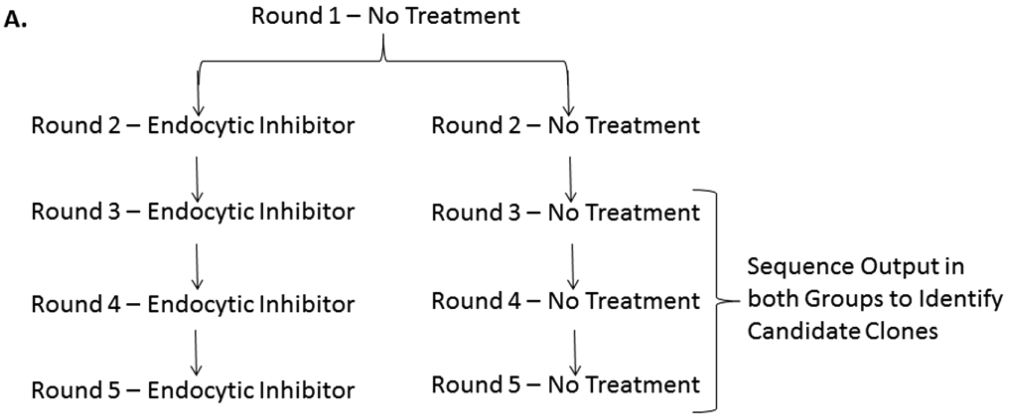
A.



B.

20mer Peptide Sequences from 5 rounds of phage display biopaning on H1299 cells (split after first round)		
Chlorpromazine (125 uM)	Non-Treated Cells	Selectivity*
ATEPRKQYATPRVFWTDAPG (HCC15.1)	ATEPRKQYATPRVFWTDAPG (HCC15.1)	18
	LQWRRDDNVHNFGVWARYRL (H1299.3)	11

*Selectivity = (Input Phage I/O)/(Control Phage I/O)

Figure 1. Phage display biopanning selection to identify cancer specific peptides that accumulate via known mechanism of endocytosis. (A) In this scheme phage display biopanning techniques are used to isolate peptides that are specific for cancer cells as well as internalize via a known mechanism of endocytosis. Round 1 of panning is performed according to standard protocols. The output of round 1 is then split into two groups. One group is treated with a specific inhibitor of endocytosis while the second group remains untreated. Panning proceeds in parallel between both groups. Phage clones are sequenced in both groups starting in round 3 to identify potential hits between groups. (B) Results from H1299 biopanning experiment using chlorpromazine as endocytic inhibitor. One clone, HCC15.1 is present in both the treated and nontreated groups, while H1299.3 is present only in the nontreated group. HCC15.1 and H1299.3 selectively internalize into H1299 cells 18- and 11-fold greater than control phage, respectively.

identifying peptides that bind to and/or mediate cellular uptake. Little attention has been given to identifying peptides that have the added level of specificity for delivery to a subcellular location. Understanding the mechanism of endocytosis may allow for better pairing of a targeting ligand with a linker that is cleavable based on the anticipated subcellular trafficking which is associated with a given mechanism of endocytosis.[6,7]

This proof of concept study utilizes phage display biopanning coupled with specific inhibitors of endocytosis to identify peptides that both selectively accumulate in cancer cells and are bias toward the noninhibited mechanism of endocytosis.[14] H1299 nonsmall cell lung cancer (NSCLC) cells were treated with chlorpromazine to inhibit clathrin-mediated endocytosis,[14] then subjected to side-by-side phage display biopanning on cells in the presence and absence of the inhibitor allowing us to identify phage clones that are group specific and clones that are enriched in both conditions. Using this method, we identified two potential targeting peptides. The first peptide (ATEPRKQYATPRVFWTDAPG, named 15.1) appeared in both the chlorpromazine treated and nontreated group. Our group previously identified this peptide from a panning on HCC15 NSCLC cells and demonstrated that this peptide accumulates in a peri-nuclear subcellular location in H1299 cells.[15] This study demonstrates that HCC15.1 primarily internalizes via a cholesterol dependent mechanism of endocytosis. The second peptide is a novel peptide here termed H1299.3 (LQWRRDDNVHNFGVWARYRL). It was present only in the non-chlorpromazine treated group, and appears to internalize primarily through a clathrin-dependent mechanism of endocytosis. Further, the peptide demonstrates selective accumulation in cancers cells and colocalizes with Lamp-1, a marker for lysosomes, indicating that H1299.3 may utilize the canonical clathrin-mediated endocytic pathway to selectively accumulate in cancer cells. Thus, we present a proof of concept phage display method that may allow for identification of cancer specific peptides that internalize via a known mechanism of endocytosis. This method may facilitate rapid discovery of efficacious direct drug conjugates by allowing informed selection of linkers and cargo based on endocytic trafficking.

RESULTS

Novel Panning Scheme. We developed the phage display scheme outlined in Figure 1 to select peptides that both accumulate specifically in a given cell type and internalize via a known mechanism of endocytosis. Phage display biopanning in Round 1 is performed using established protocols with cell binding and internalization as the selection criteria. The amplified phage recovered from Round 1 is then split into two groups. One group is treated with a specific inhibitor of endocytosis and one group remains untreated. The remaining rounds of panning are then carried out in parallel with each group, sequencing selected clones at each round. Clones enriched in one or both groups are then further characterized as targeting ligands for drug conjugates.

Paramount to this protocol is utilizing inhibitors that are specific to a mechanism of endocytosis. Chlorpromazine at 125 μM specifically inhibited 63% of clathrin-mediated endocytosis in H1299 cells as determined by reduction of transferrin uptake. At this concentration minimal cytotoxicity and no effect on raft-mediated endocytosis was observed (Supporting Information Figure S1, data not shown). Unfortunately, consistent inhibition of raft-mediated endocytosis in H1299 cells using pharmacological inhibitors proved difficult in our hands. Therefore, it was necessary to compare between the

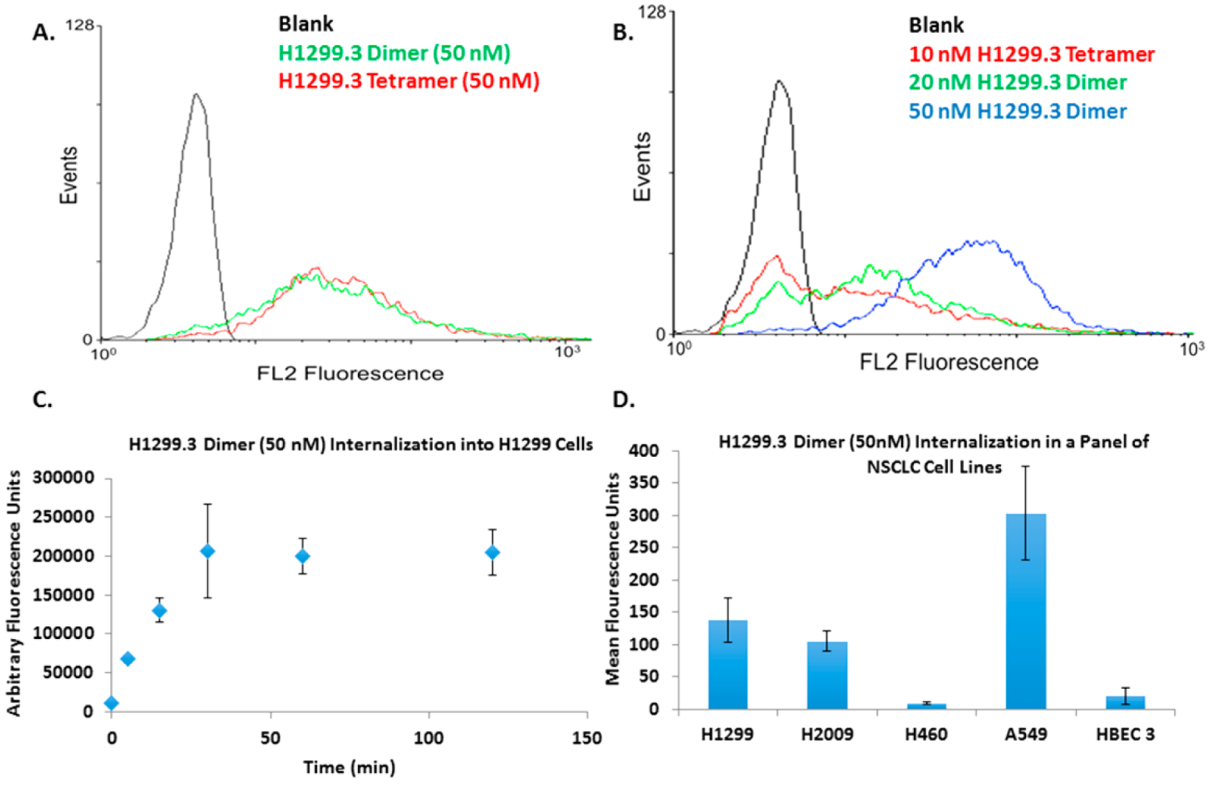


Figure 2. H1299.3 peptide mediates cellular internalization. (A) Representative histogram generated from treating H1299 cells with 50 nM of dimeric and tetrameric forms H1299.3 conjugated to PE (green and red traces, respectively). The black trace is H1299 cells not treated with peptide. (B) H1299.3 dimeric peptide titration demonstrates dose dependent internalization. (C) Time course of H1299.3 dimeric peptide accumulation in H1299 cells. (D) Internalization of H1299.3 in a panel of NSCLC cell lines. Internalization values ranged from 8.6 ± 1.9 to 303.3 ±72.2 mean A.F.U. Importantly, H1299.3 demonstrated minimal accumulation in HBEC3 cells, a model of normal lung epithelial cells, compared to all NSCLC cell excluding H460 ($p < 0.01$).

chlorpromazine-treated and nontreated cells to identify clones that internalize via clathrin-mediated endocytosis.

After verifying inhibition of clathrin-mediated endocytosis, biopanning was performed on H1299 cells according to the scheme outlined in Figure 1 using chlorpromazine as the endocytic inhibitor. Starting at Round 3, 6−12 phage clones per group were sequenced (Supporting Information Figure S2). We identified a novel phage clone LQWRRDDNVHNFGVWARYRL, termed H1299.3 by observing repeat sequences in the nontreated group (Figure 1b, Supporting Information Figure S2). H1299.3 phage clone was observed twice in round 3 and again in round 4. This clone was not observed the chlorpromazine treated cells. Further, the peptide was not listed in existing peptide databases, and blast searches indicated the peptide did not align to a known protein or contain punitive domains/motifs.

However, at later rounds of biopanning both the chlorpromazine and nontreated groups converged on the phage clone ATEPRKQYATPRVFWTDAPG previously identified in our lab as HCC15.1 (6 for 6 clones in round 5 for both groups). This phage clone was selected on the HCC15 NSCLC cell line and has affinity for H1299 cells as reported.[15] As such, it is not surprising that this clone was isolated again. No other repeat sequences were observed in this panning experiment, nor were consensus sequences observed among phage clones (Supporting Information Figure S2).

Both phage clones accumulate in H1299 cells above the background internalization of the library. The selectivity values of each clone were calculated by measured the difference in accumulation between the selected phage and a control, random phage, in H1299 cells using the following formula [(Phage Clone[Output Titer/Input Titer])/(Control Phage-[Output Titer/Input Titer])]. HCC15.1 and H1299.3 accumulated 18- and 11-fold above control phage in H1299 cells (Figure 1). Thus, both HCC15.1 and H1299.3 phage clones accumulate in H1299 cells and the binding is mediated by the displayed peptide. However, HCC15.1 was the convergent clone probably due to both slightly higher internalization as well as unintended selection pressures which are inherent to all phage panning protocols.[16] Early sequencing, starting in round 3, allowed for partial mitigation of amplification bias and other unintended selection pressure by allowing the observation of enriched clones that accumulate in H1299 cells that might be lost in later rounds. It is important to note that these clones were isolated without a negative selection step. Thus, highly selective clone can be identified using this technique without the use of a negative selection step.

Free Peptides Mediate Cellular Internalization. Previous studies by our group demonstrated that multimeric presentation of peptides on lysine cores is an effective method for retaining specificity and affinity of peptides selected from pIII displayed libraries.[17] We therefore synthesized dimeric and tetrameric H1299.3 peptide with a PEG_{10} spacer on each peptide monomer between a biotinylated mono- or trilysine core, respectively. The peptides are conjugated to streptavidin-Phycoerythrin (SA-PE) and incubated with cells of interest. Surface bound extracellular peptide was removed using an acid wash prior to flow cytometry analysis allowing for measurement

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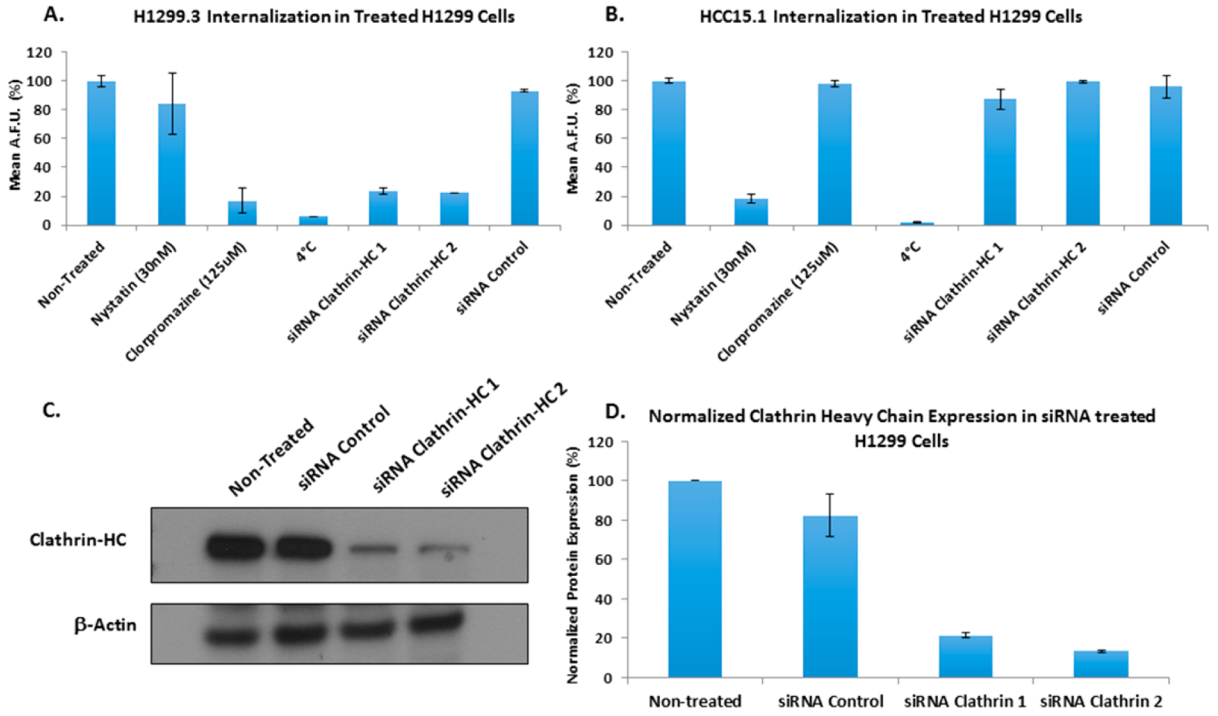

Figure 3. Peptides H1299.3 and HCC15.1 primarily internalize via different mechanisms. (A) Internalization of H1299.3 in H1299 cells treated with selective inhibitors of endocytosis. Treatment of H1299 cells with inhibitors to clathrin-mediated endocytosis including chlorpromazine and siRNA toward CHC significantly reduced internalization ($p < 0.01$). Membrane fluidity was also a significant factor for internalization ($p < 0.01$). (B) Internalization of HCC15.1 in H1299 cells treated with selective inhibitor of endocytosis. Only treatment with Nystatin and 4 °C resulted in significant reduction in HCC15.1 internalization ($p < 0.01$) indicating the necessity of cholesterol and membrane fluidity for internalization of HCC15.1. (C) Western blot demonstrating clathrin heavy chain (CHC) knockdown using targeted siRNA in H1299 cells. (D) Quantification of CHC knockdown in H1299 cells, normalized for loading, using β-actin as loading control.

of internalized peptide. As shown in Figure 2, the free peptides are able to bind and internalize into H1299 cells, again indicating that this process is peptide mediated and not dependent on the phage particle. A negligible difference in internalization was observed between dimeric and tetrameric forms of H1299.3 by flow cytometry (Figure 2). Thus, increasing valency past the dimeric format does not improve cell binding or internalization. This is particularly fortunate as the tetrameric peptide has limited solubility in aqueous solution. Monomeric peptide was not tested in this study. Uptake of the H1299.3 dimeric peptide is concentration dependent over a 10−50 nM range as predicted for a peptide that internalizes via receptor-mediated endocytosis. Similar to the selectivity data, H1299.3 accumulated less than tetrameric HCC15.1 in H1299 cells (mean afu H1299.3 = 138.3 ± 33.7, and HCC15.1 = 844.9 ± 50.8).

In addition, we also performed a time course to monitor H1299.3 accumulation in H1299 cells (Figure 2c). Dimeric H1299.3 internalized in an exponential fashion, with significant uptake observed as early as 5 min and saturating after 30 min. This is consistent with peptide internalization via receptor mediated endocytosis.

H1299.3 Differentially Accumulates in NSCLC Cell Lines. Internalization of H1299.3 was determined on a panel of NSCLC cell lines (Figure 2d). H1299.3 displays significantly lower internalization in Human Bronchial Epithelial Cells (HBEC3) a model for normal lung epithelium compared to most NSCLC cell lines. Thus, the peptide has a window for selective accumulation into lung cancer cells. In addition, H1299.3 internalized into multiple histological subtypes of NSCLC including Large Cell (H1299 mean afu = 138.3 ± 33.8) and Adenocarcinoma (H2009 mean afu = 104.9 ± 15.5 and A549, mean afu = 303.3 ± 72.2). However, the peptide does not accumulate in all NSCLC cells; no significant uptake is seen in the large cell line, H460 (mean afu = 8.6 ± 1.9). Our previous studies demonstrate that HCC15.1 differentially accumulates in these cell lines.[15] However, H1299.3 and HCC15.1 display differential pattern of accumulation across NSCLC lines. Most striking is HCC15.1 has no affinity for A549 cells. The differential cell-specificity may indicate that these peptides internalize via different mechanisms and/or bind to different cellular receptors.

H1299.3 and HCC15.1 Utilize Different Types of Receptor-Mediated Endocytosis. We sought to determine the primary mechanism of endocytosis of H1299.3 and HCC15.1 peptides in H1299 cells. H1299 cells were preincubated with pharmacological inhibitors before addition of the H1299.3 dimer and internalization of the peptide was quantitated. A significant 83% ($p < 0.01$) reduction in H1299.3 internalization is observed in the presence of chlorpromazine, while treatment with nystatin to block raft mediated endocytosis resulted in only an 18% reduction in internalization (N.S.) compared to nontreated H1299 cells (Figure 3a). Incubation of H1299.3 with H1299 cells at 4 °C also resulted in a significant loss of peptide internalization ($p < 0.01$). Finally, a 76% and 77% reduction in internalization is observed in H1299 cells treated with either of two different siRNA oligos targeting clathrin heavy chain (CHC) ($p < 0.01$). Minimal perturbation is observed in H1299.3 uptake in H1299 cells treated with control siRNA (7% reduction, N.S.). CHC protein knockdown in H1299 cells treated with siRNA oligos was confirmed using Western blot to measure relative CHC protein levels (Figure

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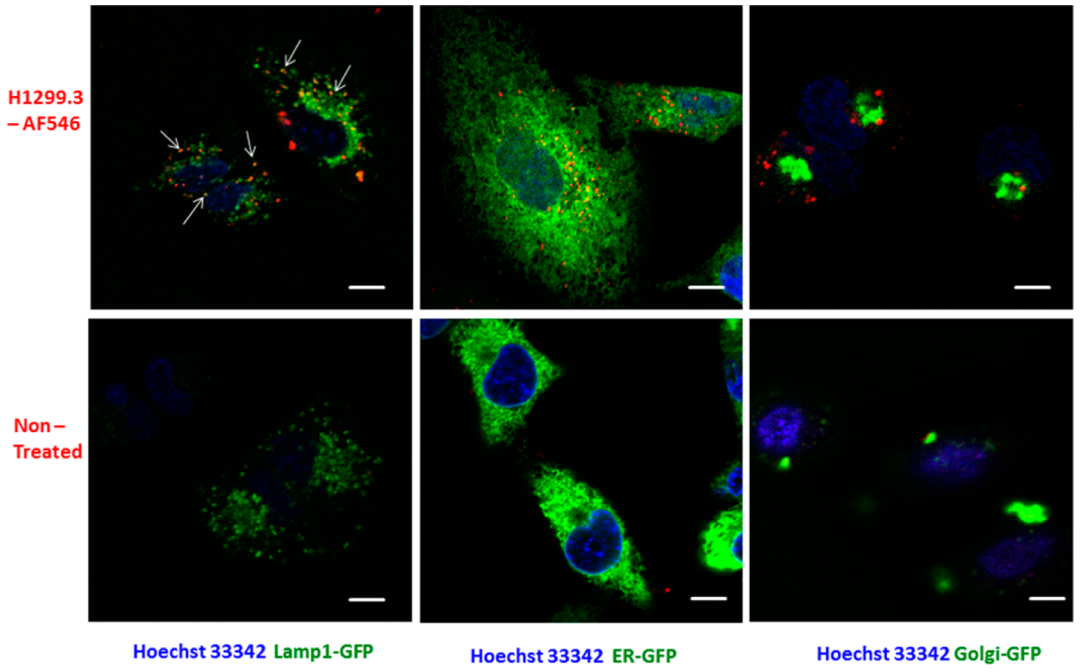

Figure 4. H1299.3 colocalizes with Lamp-1 in A549 cells. A549 cells treated with H1299.3 peptide and an organelle tracking GFP fusion construct. The top three panels are A549 cells treated with 100 nM of H1299.3 dimer conjugated to AF546, while the bottom three panels are negative control A549 cells not treated with peptide but imaged in the red channel. H1299.3 displays a distinct puncti type staining in all treated wells, while no red signal is observed in control groups. A549 cells were treated 24 h prior to imaging with BacMam 2.0 live imaging constructs to stain organelles with GFP. A549 cells were stained from left to right with Lamp 1-GFP as a lysosomal marker, KDEL-GFP as endoplasmic reticulum marker, and *N*-acetylgalactosaminyltransferase 2-GFP as Golgi marker. White arrows indicate costaining between Lamp-1-GFP and H1299.3 peptide. Minimal colocalization is observed with other organelle markers. Scale bar = 10 μm.

3c,d). The 78% and 86% reduction in CHC protein levels in H1299 cells treated with one of two siRNA oligos against CHC, respectively, is consistent with the decrease in peptide uptake. In comparison, treatment with control siRNA oligo resulted in a 17% decrease in CHC expression levels compared to control. In sum, these data support that the H1299.3 peptide undergoes clathrin-mediated endocytosis.

The mechanism of endocytosis of HCC15.1 in H1299 cells was determined by repeating the above assays (Figure 3b). Nystatin treatment significantly decreased HCC15.1 internalization (82%, $p < 0.01$) while chlorpromazine treatment had minimal effect on internalization of HCC15.1 (2% reduction, N.S.). Incubating HCC15.1 with H1299 cells at 4 °C resulted in loss of peptide internalization ($p < 0.01$). Finally, treatment with either of two siRNAs targeting CHC or a control siRNA had minimal effect on HCC15.1 internalization (12%, 1%, and 4% reduction, respectively, N.S.). Unlike the H1299.3 peptide, HCC15.1 does not utilize a clathrin-mediated pathway for internalization but most likely utilizes a raft-dependent mechanism.

H1299.3 Colocalizes with Lamp-1. Live cell, confocal fluorescent microscopy was used to determine subcellular accumulation of these peptides. H1299.3 subcellular localization was determined by conjugating the H1299.3 dimeric peptide directly to AF546 using cysteine maleimide chemistry.[17,18] A549 cells, transfected with GFP-fusion constructs to mark organelles using the Bacmam 2.0 system purchased from Life Technologies,[19,20] were treated with H1299.3-AF546 (Figure 4). A549 cells were used because they internalize the most H1299.3 peptide of the cell lines assayed, thus allowing for the greatest observable signal with the lowest concentration of H1299.3 to avoid false positive data. H1299.3 appears subcellularly as multiple distinguished puncti. The puncti colocalized well with Lamp 1-GFP fusion and localized poorly with both endoplasmic reticulum and Golgi markers. The Mander's coefficient for these groups are 0.608, 0.368, and 0.107 for Lamp 1, ER, and Golgi, respectively, indicating that strongest colocation is with lysosomes. In addition, the matching puntci shape further supports colocalization with lysosomes rather than ER. These data further support the model that the H1299.3 peptide binds to an extracellular receptor and is internalized by a conical clathrin-mediated process which results in accumulation of ligand in a lysosome. By comparison, previous studies demonstrated that HCC15.1 accumulates in a perinuclear location and results in a distinctively different staining pattern than the H1299.3 peptide.[15]

■ DISCUSSION

Phage biopanning has proven to be an effective method for identifying cell-targeting ligands. However, little emphasis has been placed on identifying cell-specific ligands which not only internalize into a specific cell type but can also deliver cargo to a desired subcellular location. Furthermore, only a few studies have actually determined the subcellular localization of cell-targeting peptides after they have been isolated from a combinatorial library.[21,22] This study sought to develop a method that can identify targeting peptides that both accumulate significantly in cancer cells and internalize via a known mechanism of endocytosis. The overall goal is to enhance selection of peptides that traffic to a desirable subcellular localization of ligand accumulation. Recent literature has highlighted the need for matching subcellular localization with drug release strategies for both drug conjugate as well as

nanoparticle platforms.[1−3,23] Phage display techniques are well suited to perform this task, as it is a nonbiased technique to identifying targeting ligands and allows for wide versatility of selection pressures to tune the output ligand to the desired specifications.

In addition, facilitating the ability to match a targeting ligand with a linker understanding primary endocytic mechanism of a targeting ligand may also guide cargo attachment. For example, small molecule drugs may be delivered through the lysosomal pathway; however, trafficking nucleic acid for RNAi therapies or protein toxins through a lysosomal compartment is demonstrated to degrade cargo resulting in suboptimal delivery.[12,13] Thus, utilizing protocols that allow for selection of major endocytic mechanisms and subsequent subcellular localization have the potential to guide selection of both linker and cargo resulting in more rapid creation of effective drug conjugates of multiple subclasses.

This study capitalized on the multitude of endocytic pathways and the differential subcellular accumulation of ligands known to traffic through these endocytic methods. Here we utilized chlorpromazine to specifically inhibit clathrin-mediated endocytosis as a model to demonstrate proof of principle. Our model relies on the specificity of endocytic inhibition as well as consistency of inhibition across panning rounds. As demonstrated in Supporting Information Figure S1, chlorpromazine specifically inhibits clathrin-mediated endocytosis. In our hands we could not specifically and consistently knock down raft-mediated endocytosis using pharmacological inhibitors in H1299 cells. Thus, this study used a comparison between chlorpromazine treated and nontreated cells to identify peptides internalized via clathrin-mediated endocytosis. By comparing side-by-side biopanning experiments and sequencing clones at early rounds of enrichment, peptides specific for raft-mediated and clathrin-mediated endocytosis were identified.

During the panning, it was necessary to perform one round of panning before splitting the output into two groups to achieve consistency in convergent sequences (data not shown). This step is probably necessary due to the large loss of clonal diversity that occurs during the first round of selection.[16] Interestingly both groups converged on a single phage clone HCC15.1. However, both HCC15.1 and H1299.3 had high selectivity values on H1299 cells. This result may be due in part to amplification bias, which is known to occur during phage selections.[16] Additionally, phage amplification is inherently dependent on the ability to isolate viable phage clones able to infect bacteria. It is possible that phage clones localized within particular subcellular compartments are more difficult to identify, as they are degraded or difficult to isolate from the cells. Early sequencing, starting in round 3, appears to partially mitigate this problem by identifying clones enriched in the selection population but which are not the convergent sequence due to amplification bias and other factors that influence clonal selection outside of the desired selection pressure. Interestingly, both isolated clones exhibited high selectivity for H1299 cells without using a negative selection step. Thus, this protocol generates highly selective phage clones for the target cells without the need for an additional negative selection step.

This panning scheme does not allow for determining if a phage can undergo endosomal escape after internalization, and our data do not address the ability of the peptide to be eventually released into the cytoplasm. Endosomal escape is considered an important step in delivery biologically active cargo to cells.[24] This scheme is focused on initial internalization of the phage and selection of the internalization pathway. This may limit the applications of phage/peptide selected using this protocol. However, layering of addition selection criteria may be possible to select for phage that internalize a defined mechanism and escape from endosomes. Additionally, many endosomal escape agents have been reported to improve endosomal release of biologics and can be incorporated into the targeting peptide if necessary.

The data from the panning experiments indicate that HCC15.1 and H1299.3 may use different mechanisms of endocytosis because H1299.3 is present only in the nontreated group, whereas HCC15.1 was present in both the nontreated and chlorpromazine treated groups. Subsequent studies to confirm this observation indicated that HCC15.1 primarily uses a cholesterol dependent mechanism of endocytosis implying a raft-mediated mechanism of endocytosis (Figure 3b). On the other hand H1299.3 is dependent on clathrin for endocytosis (Figure 3). In addition, HCC15.1 accumulates in a perinuclear location[15] whereas H1299.3 accumulation colocalizes with Lamp-1 in the periphery of the cell. Therefore, only H1299.3 is internalized via clathrin-mediated mechanism and subsequently is trafficked into lysosomes.

This study outlines a selection scheme that is fundamentally different than the recent papers published by Pasqualini et al. which also describe a technique to identify peptides which accumulate in organelles.[25,26] Pasqualini et al. selections rely on penetrin to mediate receptor-independent internalization of the phage prior to accumulation in a given organelle. Our technique differs in that internalization is driven by the receptor-mediated internalization process, then the phage accumulates in an organelle via intracellular trafficking pathways. This important difference allows selection of ligands that maintain specificity for cancer cells by preventing nonselective membrane penetration. This difference may enhance the therapeutic window of drug conjugates targeted using peptides selected by the method presented in this study by reducing off-target effects resulting from cytotoxicity of nontargeted, healthy cells.

Both peptides identified from this panning scheme internalize into NSCLC cell lines (Figure 2).[15] Also, both HCC15.1 and H1299.3 demonstrate minimal internalization into a normal bronchial epithelial cells line, HBEC3 implying a potential therapeutic window for both peptides to be used as targeting ligands. However, despite both HCC15.1 and H1299.3 internalizing into a similar panel of NSCLC cell lines, the peptides differentially accumulate into these lines (Figure 2).[15] For example, minimal HCC15.1 accumulation is observed in A549 while these cells internalized the greatest amount of H1299.3. These data imply that these peptides may use different forms of endocytosis, as predicted by the phage panning protocol, and/or the peptides may bind to different receptors to mediate internalization. Currently, the identities of receptors that bind HCC15.1 or H1299.3 are unknown. Further, BLAST searches of H1299.3 did not reveal any strong leads as to the receptor(s) that mediate binding and subsequent internalization into cells. Despite lacking this data, these ligands still exhibit differential trafficking patterns that may be beneficial for designing drug conjugates. For example, HCC15.1 is potentially better suited to delivering sensitive cargo as it appears to avoid organelles known to degrade biologic molecules, whereas H1299.3 is possibly better suited to

delivering toxic chemotherapeutic pro-drugs due to the availability of multiple lysosomal cleavable linkers that will release active drug only intracellularly.

In conclusion, we developed a phage display biopanning scheme to identify peptides that both accumulate specifically in cancer cells and internalize via a defined mechanism of endocytosis. We demonstrate proof of principle for this approach by identifying two peptides that were both specific for cancer cells but internalized via different mechanisms of endocytosis. In the future we plan to expand the phage display biopanning methodology to additional cell lines as well as endocytic inhibitors in order to identify additional targeting ligands and expand the utility of the method. Clearly, other specific inhibitors, such as nystatin, filipin, or siRNA toward endocytic machinery, for example, could be readily applied into the panning scheme potentially resulting in the ability to tune this method to select peptides specific for any type of endocytosis.[14,27] Future studies using H1299.3 as a targeting ligand in direct drug conjugate constructs will determine the efficacy of H1299.3 as a targeting ligand. Finally, further characterizing the details of H1299.3 subcellular trafficking and identify the receptor H1299.3 uses for internalization may enhance the utility of H1299.3 as a targeting ligand.

METHODS

Cell Culture. Nonsmall cell lung cancer (NSCLC) cell lines were graciously provided by the Hamon Center for Therapeutic Research (UT Southwestern) and cultured according to previously reported conditions.[15] All cell lines were DNA fingerprinted and routinely tested for *Mycoplasma* contamination.

Phage Display Biopanning. Phage display was performed with slight modification from established protocols.[4,15,28] The library for this study consisted of an M13 phage displaying a random 20-mer peptide fused to the pIII protein.[28,29] Phage display was performed using established protocols with the exception of adding a 15 min preincubation period with 100 μM chloroquine prior to addition of library.[28] Chloroquine is used to neutralize lysosomal pH with the assumption that neutralizing the low pH environment of the lysosome will enhance recovery of intact phage which traffic to the lysosome. As outlined in Figure 1, the phage output from round 1 was split and side-by-side biopanning was performed in the presence of 125 μM chlorpromazine or without inhibitor. In round 1, 1×10^{10} phage articles were added to H1299 cells. The library has an approximate diversity of 1×10^{8} unique phage clones; thus, this represents 100 library equivalents, i.e., 100 copies of every unique phage present in the library. For rounds 2−5 chlorpromazine was preincubated with H1299 cells for 15 min prior to addition of phage. The cells from each group were then incubated with phage for 1 h, then washed 2× with PBS^{+}+0.1%BSA (10 min total), washed 3× with acid wash pH = 2.2 (10 min total) to remove extracellular bound phage, and finally washed 2× with PBS^{+}+0.1%BSA (2 min total). Cells from each group were lysed using 30 mM Tris +0.05% Triton-X-100, pH = 8, according to standard protocol.[28] Lysates were mixed with K91 *E. coli* to amplify internalized phage after each round. A total of 6−12 phage clones were sequenced from the output of each group in rounds 3−5 using established colony PCR-based protocol.[28] Phage selectivity was measured by calculating the fold increase in internalization of the selected phage compared to a control phage in H1299 cells using previously established methods.[28]

Peptide Synthesis. All peptides were synthesized using standard FMOC solid phase synthesis techniques and purified to >95% purity using reverse phase HPLC as previously reported.[4,18,28] Peptides were synthesized on NovaPeg Rink amide resin (NovaBiochem) and had a polyethylene glycol (PEG_{10}) spacer (Polypure) between the c-terminus of the peptide identified from the phage and the cysteine used to multimerize the peptides (HCC15.1 − ATEPRKQYATPRV-FWTDAPG-(PEG_{10})-C-NH_2, H1299.3 − LQWRRDDN-VHNFGVWARYRL-(PEG_{10})-C-NH_2). The expected/observed mass for HCC15.1 and H1299.3 is 2991.52/2991.32 and 3305.06/3305.07, respectively. The peptides were multimerized using tri and monolysine cores functionalized with maleimide, as previously described, to create tetrameric and dimeric presented peptides, respectively.[17,30] Biotinylated-glutamate or cysteine (NovaBioChem) was synthesized into the core in order to conjugate dyes and cargos to the peptides using streptavidin or cysteine-maleimide chemistry, respectively as previously reported.[15,17] H1299.3 dimeric peptide was conjugated to C5-maleimide AF546 for imaging studies. H1299.3 and C5-maleimide AF546 were dissolved in phosphate buffer (13 mM KH_2PO_4, 54 mM $NaHPO_4$, pH 7.4. 9), then shaken for 1 h at RT. The precipitate was washed 3 times in phosphate buffer then dried in a desiccator O.N. The purity of the dimeric dye labeled peptide was >95% as measured by rpHPLC with the expected/observed mass found to be 8265.68/8265.12. The peptide was suspended in DMSO then culture media for imaging studies. H1299.3 peptide solutions were made fresh from lyophilized, stock stored at −20 °C, for each assay due to poor stability in neutral pH solutions.

Flow Cytometry Assays. Established flow cytometry protocols were used to determine the specificity of endocytic pathway inhibition, demonstrate peptide internalization, and determine primary mechanism of endocytosis.[15] To determine specificity of endocytosis, human Transferrin-AF488 (Life Technologies) was used to measure clathrin-mediated endocytosis according to manufacturer's protocol and LacCer-BODIPY (Life Technologies) to monitor raft-mediated endocytosis as previously reported.[31,32] Nysatin (10−50 nM) and Chlorpromazine (50−150 μM) was titrated on H1299 cells and internalization of fluorescent markers of endocytosis were used to determine selective inhibition of endocytosis. Inhibitors were preincubated with cells for 15 min at 37 °C, 5% CO_2, then fluorescent markers were added in the presence of inhibitor and incubated for 30 min at 37 °C, 5% CO_2. Cells were washed 2× in PBS^{+}+0.1% BSA then fluorescent markers were removed from the cell surface using acid wash (3× 10 min, pH = 2.2) or washing with defatted BSA (Sigma) as previously described.[15,31] Internalization was detected using flow cytometry to measure the level of florescence in a single cell. Treatment with 125 μM chlorpromazine resulted in 63% reduction in clathrin specific internalization with minimal cytotoxicity (Supporting Information Figure S1, data not shown).

For peptide internalization assays, peptides were conjugated to streptavidin-phycoerythrin (SA-PE) via the multimerization core using previously established protocols.[15] For assays using HCC15.1, 10 nM of tetrameric peptide was incubated with 1:1 molar ratio SA-PE for 30 min in PBS with gentle agitation. Excess streptavidin binding sites were quenched by the addition of RPMI 1640 which contains 0.2 mg/L D-biotin. For assay using H1299.3, 10−50 nM of dimeric or tetrameric peptide was incubated at 1:1 molar ratio of SA-PE for 30 min in phosphate buffer (13 mM KH_2PO_4, 54 mM $NaHPO_4$, pH 7.4) with gentle

agitation and then quenched with RPMI 1640. Peptide SA-PE conjugates were incubated with cells for 60 min at 37 °C, 5% CO_2 to allow for peptide internalization. Surface bound extracellular peptide was removed using acid wash (pH = 2.2), then cell fluorescence was analyzed via flow cytometry. As determined by titration of peptide, 50 nM of H1299.3 fluorescence conjugate resulted in sufficient signal-to-noise ratio for detecting internalization by the flow cytometry assay and thus this concentration is used for subsequent internalization assays.

For inhibitor studies cells were preincubated with 125 μM chlorpromazine or 30 nM nystatin for 15 or 60 min, respectively, as previously described,[14] then treated with either 10 nM of HCC15.1 or 50 nM of H1299.3 peptide SA-PE conjugate in the presence of inhibitor for 30 min at 37 °C, 5% CO_2. Surface bound extracellular peptide was removed using either acid wash (pH = 2.2) or trypsinization (0.25% Trypsin with 2.21 mM EDTA, Corning), then cells were analyzed for internal fluorescence using flow cytometry.

Time Course Study. To measure internalization of H1299.3 over time, the H1299.3 dimer was conjugated to IRDye800CW (Li-Cor) using cysteine−maleimide chemistry as outlined above. The purity of the dimeric dye labeled peptide was >95% as measured by rpHPLC with the expected/observed mass found to be 8422.31/8421.12. H1299-dye conjugate (50 nM) was incubated with cells in black-walled, clear bottom 96 well plate for the indicated time; surface bound conjugate was removed using an acid wash. Fluorescence was read using an Odyssey CLx imager (Li-Cor).

Clathrin siRNA Assays. H1299 cells were treated with siRNA oligos against clathrin heavy chain (CHC) or control siRNA oligo using previously established protocols.[33−35] Briefly, H1299 cells were seeded in 12 well plate at a density that allowed >72 h of growth before confluence. Cells were treated with 5.5 μL of 20 μM oligo solution and 6.5 μL of RNAiMax (Invitrogen) in total of 200 μL of RPMI 1640. Cells were washed with RPMI 1640 then treated with 100 μL of siRNA oligo solution for 4 h at 37 °C, 5% CO_2. Cells were washed once, then placed back in complete media. This procedure was repeated again the following day. At 72 h post initial treatment with siRNA oligos, cells were treated with peptide SA-PE conjugates, described above, to measure peptide internalization or utilized for Western blot assays to measure relative protein levels. CHC knock down was confirmed by Western blot using monoclonal antibody against CHC (TD.1, Santa Cruz-12734) as primary and goat anti-mouse-HRP secondary antibody using established protocols.[36]

Fluorescent Confocal Microscopy. Laser scanning confocal microscopy was used to monitor live cell accumulation of peptide as well as identify subcellular localization of peptide accumulation using established protocols.[30] A549 cells were transfected with Golgi, ER, or Lysotracker GFP fusion constructs using Bacman 2.0 (Life Technologies) system, according to manufacturer's protocol. Briefly 200 μL of trypsinized and washed A549 were incubated with 1:10 dilution of baculovirus encoding for the fusion protein at 37 °C 5% CO_2 for 10 min with gentle mixing. The cell/virus mixture was plated in polylysine coated glass bottom dishes containing 1 mL of complete media. A549 cells were incubated overnight at 37 °C, 5% CO_2 to allow for cells to adhere to the dish. The next day cells were washed and treated with 100 nM of H1299.3 dimeric peptide, directly conjugated to Alexa Fluor 546 (Life Technologies) via cysteine-maleimide chemistry according to manufacturer's protocol, for 1 h at 37 °C, 5% CO_2. Cells were washed 2× with complete media, then stained for 10 min with Hoechst 33342 (Molecular Probes) according to manufacturer's protocol. Live cells were imaged using a Nikon TE200-E laser scanning confocal microscope, through a 30 μm pinhole visualizing the blue, green, and red channels using 408, 488, and 543 nm lasers for excitation, respectively.

Statistics. Statistical analysis were performed using GraphPad Prism software or Microsoft Excel. Flow cytometry values are presented as mean with standard deviation or as percentage of mean with standard deviation. In vitro experiments were performed a minimum of 3 independent times using multiple replicates per experiment. Reported p-values are paired or unpaired, two tailed t tests (where appropriate). A p-value ≤ 0.05 was considered statistically significant. Nonsignificant results are indicated throughout the text as N.S. Colocalization studies were analyzed for Mander's coefficient using ImageJ software with the JaCoP plugin[37]

■ ASSOCIATED CONTENT

Supporting Information

Figures quantifying specificity of clathrin inhibition in H1299 cells using pharmacological inhibitors, and a complete list of phage clones sequenced in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

NSCLC, non small cell lung cancer; SA-PE, streptavidin-phycoerythrin; PEG, polyethylene glycol; PBS, phosphate buffered saline; CHC, clathrin heavy chain; GFP, green fluorescent protein; AFU, arbitrary fluorescence unit

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