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Inhibition of Cancer Cell Proliferation and Breast Tumor Targeting of pHLIP-Monomethyl Auristatin E Conjugates

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Abstract

Localized delivery is vital for the successful development of novel and effective therapeutics for the treatment of cancer. The targeting and delivery described herein is based on the pH(Low) Insertion Peptide (pHLIP), a unique delivery peptide that can selectively target tumors in mice and translocate and release cargo molecules intra-cellularly based solely on the low extracellular pH intrinsic to cancer cells. In this study, we investigate the efficacy of pHLIP to target and deliver the highly potent and clinically validated microtubule inhibitor monomethyl auristatin E (MMAE) to cancer cells and breast tumors. We show that pHLIP-MMAE conjugates induce a potent cytotoxic effect (> 90% inhibition of cell growth) in a concentration- and pH-dependent manner after only 2-hour incubation without any apparent disruption of the plasma membrane. pHLIP-MMAE conjugates exhibit between an 11 and 144-fold higher anti-proliferative effect at low pH than at physiological pH, and a pronounced pH-dependent cytotoxicity as compared to free drug. Furthermore, we demonstrate that a pHLIP-MMAE drug conjugate effectively targets triple negative breast tumor xenografts in mice. These results indicate pHLIP-based auristatin conjugates may have an enhanced therapeutic window as compared to free drug, providing a targeting mechanism to attenuate systemic toxicity.

INTRODUCTION

A myriad of cancer targeting therapies aimed at improving effectiveness and diminishing off-target cytotoxic effects have been developed and hold the promise of being curative options for defined subsets of cancers. One such class of agents is the antibody drug conjugates (ADCs). For example, the anti-CD30-MMAE conjugate brentuximab vedotin and the anti-HER2-mertansine conjugate ado-trastuzumab emtansine are approved for the treatment of Hodgkin's lymphomas and HER2-positive metastatic breast cancer, respectively. However, preclinical and clinical evidence demonstrates that therapy strategies

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ASSOCIATED CONTENT

Supporting Information. Data on inhibition of cell proliferation by MMAE, pHLIP variants, and chemical stability of pHLIP-MMAE conjugates.

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based on the targeting of specific proteins is significantly hampered by tumor heterogeneity, which can promote tumor evolution, and lead to loss of cell surface proteins, and eventually, to therapy resistance and disease progression.¹ Moreover, targeted cancer biomarkers tend to be over-expressed in a tumor-associated, not tumor-specific manner. While over-expression provides a window of selective targeting, targeted uptake into normal tissues is seen,² and has the potential to lead to unacceptable toxicity profiles. Thus, alternatives are desperately needed to circumvent these limitations.

Our approach exploits a fundamental cellular mechanism that is inherent to cancer cells. Indeed, while no specific gene mutation or chromosomal abnormality is common to all cancers, nearly all solid tumors have elevated aerobic glycolysis and acidosis due to various effects (e.g. Warburg effect and poor clearance), regardless of their tissue or cellular origin.^{3,4} Therefore, the extracellular environment of tumors is acidic (pH ~ 6.0–6.9)^{5–8} as compared to normal tissues, but the pH at the surface of cancer cells is likely to be lower.⁹ Importantly, tumors' aggressiveness and metastatic potential are promoted at low extracellular pH,^{10–12} thus targeting tumor acidity is hypothesized to be less prone to the development of acquired resistance than targeting a single protein antigen. For these reasons, acidosis is a hallmark of tumor progression from early to advanced stages and may provide an opportunity for tumor-targeted therapy.¹³

Our delivery strategy herein is based on the pH(Low) Insertion Peptide (pHLIP), a peptide that can selectively target tumors in mice solely based on their acidity rather than on any specific biomarker^{14–16}; in an acidic environment (i.e., pH ~6), pHLIP undergoes a pH-dependent folding that promotes insertion of its C-terminus across the cell membrane to form a transmembrane helix.^{17,18} Notably, the pH at which pHLIP undergoes its insertion corresponds to the extracellular pH of solid tumors. This process has been used for the translocation and release of various payloads, including cell-impermeable model peptides, imaging agents and toxins into cancer cells.^{19–21} Importantly, pHLIP-mediated translocation of cargo molecules across the cell membrane is not mediated either by interactions with cell surface receptors or through formation of pores in cell membranes. Thus, pHLIP releases cargo molecules directly into the cytoplasm without the need to escape endosomes or lysosomes.^{22,23} In animal models, pHLIP can target not only subcutaneous tumor xenografts derived from a variety of tumor types (including breast tumors),^{24,25} but also spontaneous prostate tumors in TRAMP mice and metastatic lesions in lung.¹⁴

The ability of pHLIP to effectively function as an imaging agent supports its development as a drug delivery vehicle. However, while pHLIP has been used to inhibit cancer cell proliferation by the delivery of mushroom phallotoxins,^{20,21} to our knowledge, it has not been tested to target and deliver a clinically relevant therapeutic agent with proven efficacy *in vitro* and *in vivo*. In the present study, we investigate the efficacy of pHLIP to deliver the highly potent and clinically validated microtubule inhibitor monomethyl auristatin E (MMAE) to cancer cells *in vitro* in a pH dependent manner. We show that pHLIP and one of its variants chosen to optimize targeting, can induce a potent cytotoxic effect in cancer cells, including triple negative breast cancer cells. Gene expression profiling divides breast cancer into at least five different subtypes²⁶ that are broadly classified into three different treatment groups: Hormone responsive²⁷, HER2/ERBB2 amplified²⁸ and triple negative breast

cancer²⁹. Triple negative breast cancer (TNBC), so named for its lack of estrogen receptor, progesterone receptor and HER2 expression, is over-represented in younger and African-American patient populations.³⁰ TNBC has a high rate of early recurrence in response to chemotherapy treatment and in contrast to the other treatment groups there are no effective targeted therapies.^{31,32} Furthermore, we demonstrate that the pHLIP-MMAE drug conjugates effectively targets human TNBC xenografts in mice. This study represents the first demonstrated example of pHLIP-dependent delivery of a clinically relevant cytotoxic to tumor cells both *in vitro* and *in vivo*.

EXPERIMENTAL SECTION

Materials

N-Hydroxybenzotriazole (HOBt), *o*-benzotriazol-*N*, *N*, *N*, *N'*, *N'*-tetramethyluronium hexafluorophosphate (HBTU), and all *N*-fluorenyl-9-methoxycarbonyl (Fmoc) protected L-amino acids were purchased from GL Biochem Ltd. H-Rink Amide-ChemMatrix solid support resin was from PCAS BioMatrix Inc. Diisopropylethylamine (DIEA), piperazine, *N*, *N*-dimethylformamide (DMF), dichloromethane (DCM), trifluoroacetic acid (TFA), methanol, acetonitrile, dimethyl sulfoxide (DMSO) and Dulbecco's modified Eagle's medium (DMEM) were all from Thermo Fisher Scientific Inc. Fetal Bovine Serum (FBS) was purchased from Atlanta Biologicals Inc. Penicillin-Streptomycin was purchased from Sigma-Aldrich. monomethylauristatin E (MMAE), and pyridyldithiol-activated MMAE (Py-ds-Prp-MMAE) were from Concortis. 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was from Avanti Polar Lipids Inc. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from EMD Millipore. AlexaFluor 750 NHS ester was purchased from Life Technologies.

Solid-Phase Peptide Synthesis

Two variants of pHLIP were used in this study: pHLIP-Cys (pHLIP(WT)) with a cysteine residue at its C terminus and pHLIP-D25E-Cys (pHLIP(D25E)) where the transmembrane Asp25 residue is replaced with Glu. pHLIP-Cys was either purchased from GL Biochem Ltd (with a free C-terminal carboxylic acid) or prepared in our laboratory by solid-phase synthesis. pHLIP(D25E) was exclusively prepared in our laboratory. Briefly, peptides were prepared by Fmoc solid-phase synthesis, using H-Rink Amide resin affording an amidated C-terminus and purified via reverse-phase high performance liquid chromatography (RP-HPLC) (Phenomenex Luna prep 10 μ 250 $\times$ 21.20 mm C8; flow rate 10mL/min; phase A: water 0.1% TFA; phase B: acetonitrile 0.1% TFA; gradient 60 min from 95/5 A/B to 0/100 A/B). The purity of the peptides was determined by RP-HPLC (Agilent Zorbax Eclipse 5 μ m 4.6 $\times$ 50 mm XDB-C8; flow rate 1 mL/min; phase A: water 0.01% TFA; phase B: acetonitrile 0.01% TFA; gradient 45 min from 95/5 A/B to 0/100 A/B) and their identity was confirmed via matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry. It is important to note that no significant difference in behavior (e.g., secondary structure, pH-dependent properties) were observed between C-term amidated and free carboxylated peptides (data not shown).

Preparation of pHLIP-MMAE Constructs

Each pHLIP peptide was dissolved in DMSO to a concentration of 1 mM, followed by the addition of 1.5 eq. of Py-ds-Prp-MMAE in DMSO. To facilitate disulfide exchange, 100 μ L of 1 M Tris buffer, pH 8.0 was added to the solution and allowed to mix at room temperature for 2–3 hours. The N-terminally modified AlexaFluor 750-pHLIP(WT)-MMAE conjugate was obtained by dissolving 1 mg of the NHS activated AlexaFluor 750 in 200 μ L of DMF, followed by the addition of 1 eq. of pHLIP(WT)-MMAE in DMF, in presence of DIEA affording amide bond conjugation to the N-terminal amine of pHLIP. The desired pHLIP conjugates were isolated using the same techniques described for the pHLIP peptides. The purity of the peptide-drug conjugates was determined by RP-HPLC as listed, and their identity was confirmed by MALDI-TOF MS: pHLIP(WT)-MMAE: purity >98%; calculated ($M+H^+$) = 5046; found ($M+H^+$) = 5047. pHLIP(D25E)-MMAE: purity >85%; calculated ($M+H^+$) 5031; found ($M+H^+$) = 5032. Alexa750-pHLIP(WT)-MMAE: purity >95%; calculated ($M+H^+$) ~5915; found ($M+H^+$) = 5912. The conjugates were quantified at 280 nm by UV/Vis absorbance spectroscopy using the molar extinction coefficient of pHLIP ($13,940 \text{ M}^{-1} \text{ cm}^{-1}$) and lyophilized to 10^{-8} mole aliquots.

Preparation of POPC Liposomes

Ten milligrams of POPC in chloroform were dried and then held under vacuum overnight. The dried lipid film was rehydrated with 1 ml of 5 mM sodium phosphate, pH 8.0, and mixed by vigorous vortex. The resulting multilamellar vesicle solution was freeze-thawed in liquid nitrogen for seven cycles and were extruded through a polycarbonate membrane with 100 nm diameter pores using a mini extruder (Avanti Polar Lipids) to produce large unilamellar vesicles. Liposomes were used immediately following their preparation. Lipid concentration was checked using the Marshall's assay³³ and the size distribution was verified by dynamic light scattering analysis performed at a scattering angle of 90° using an ALV/GSC-3 goniometer system equipped with ALV/ALV-7004 correlator (ALV-GmbH, Langen, Germany).

Sample preparation for Circular Dichroism and Tryptophan Fluorescence Measurements

Prior to CD or fluorescence measurements, lyophilized aliquots of pHLIP constructs were resuspended to 20 μ M with 5 mM sodium phosphate, pH 8.0, and incubated for 1 hour at room temperature. This stock solution was diluted to a final concentration of 7 μ M and, when appropriate, incubated with POPC liposomes at a 1:300 peptide-to-lipid molar ratio for 30 minutes at room temperature. Subsequently, samples were adjusted to the desired experimental pH values with the addition of aliquots of a HCl solution to elicit pH-dependent insertion. Samples were allowed to equilibrate at the desired pH for 30 minutes at room temperature before spectroscopic measurements.

Tryptophan Fluorescence Spectroscopy

All measurements were carried out using a Cary Eclipse Fluorescence Spectrophotometer (Varian), and performed at 25 $^\circ\text{C}$ with pHLIP construct concentrations equal to 7 μ M. Samples were excited at 295 nm and the emission spectra were taken from 300 to 500 nm, with the slit widths for emission and excitation both set 5 nm.

Circular Dichroism Spectroscopy

Far-UV CD spectra of pHLIP constructs were recorded on Jasco J-815 CD spectrometer equipped with a Peltier thermal-controlled cuvette holder (Jasco, Inc.). All measurements were performed in 0.1 mm quartz cuvette at 25 °C with pHLIP constructs concentrations equal to 7 µM. CD intensities are expressed in mean residue molar ellipticity $[\theta]$ calculated from the following equation:

$$[\theta] = \frac{\theta_{\text{obs}}}{10 \times l \cdot c \cdot n} (\text{in degrees cm}^2 \cdot \text{dmol}^{-1})$$

where, θ_{obs} is the observed ellipticity in millidegrees, l is the optical path length in centimeters, c is the final molar concentration of the peptides, and n is the number of amino acid residues. Samples were measured in a 0.1 cm path length quartz cuvette and raw data were acquired from 260 nm to 190 nm at 1 nm intervals with a 100 nm/min scan rate, and at least five scans were averaged for each sample. The spectrum of POPC liposomes was subtracted out from all construct samples.

Cell Culture

Human cervical adenocarcinoma HeLa cells and human breast adenocarcinoma MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C.

Anti-proliferation Assay

HeLa and MDA-MB-231 cells were seeded in 96-well plates at a density of 3,000 cells/well and incubated overnight. Before treatment, construct aliquots were solubilized in an appropriate volume of DMEM without FBS (pH 7.4) - so that upon pH adjustment the desired treatment concentration is obtained - and gently sonicated for 30–60 seconds using a bath sonicator (Branson Ultrasonics). After removal of cell media, this treatment solution was added to each well and incubated at 37 °C for 5–10 minutes. The pH was then adjusted to the desired value using a pre-established volume of DMEM, pH 2.0 buffered with citric acid (final volume = 50 µL) and the plate was incubated at 37 °C for 2 hours. After treatment, the media was removed, cells were washed once with 100 µL of complete DMEM, and 100 µL of complete medium was added to each well before returning the plate to the incubator. Treatment solutions were collected and their pH values measured using a micro-combination pH probe (Microelectrodes, Inc.). For physiologic pH treatments a small down-drift (~0.2 pH unit) was usually observed, whereas an up-drift was observed for low pH treatments (e.g., pH 7.4 → pH 7.2, and pH 5.0 → pH 5.2). Cell viability was determined after 72 hours using the colorimetric MTT assay. Briefly, 10 µL of a 5 mg/mL MTT stock solution was added to the treated cells and incubated for 2 hours at 37 °C. The resulting formazan crystals were solubilized in 200 µL DMSO and the absorbance measured at 580 nm using an Infinite 200 PRO microplate reader (Tecan). Cell viability was calculated against control cells treated with media at physiologic pH.

Cell Membrane Integrity Assay

HeLa and MDA-MB-231 cells were seeded in 96-well plates at a density of 3,000 cells/well and incubated until confluent (~ 72 hours). Before treatment, construct aliquots were solubilized in an appropriate volume of DMEM without FBS (pH 7.4) so that upon pH adjustment 10 μ M is obtained. Cell media was removed, the treatment solution was added to each well and cell were incubated at 37 °C for 5–10 minutes. The pH was then adjusted to the desired value using a pre-established volume of DMEM, pH 2.0 buffered with citric acid (final volume = 50 μ L). The plate was incubated at 37 °C for 2 hours. After treatment, the media was removed, cells were detached using trypsin and counted based on trypan blue uptake using an hemacytometer.

Construct Stability HPLC in Cell Media and Mouse Serum

pHLIP(WT)-MMAE and pHLIP(D25E)-MMAE were solubilized in DMEM without FBS (pH 7.4) to obtain a 10 μ M solution, and gently sonicated for 30–60 seconds using a bath sonicator. Peptide solutions were incubated at either pH 7.4 or pH 5.0 (37 °C and 5% CO₂) and 100 μ L aliquots were monitored via RP-HPLC at various time points (Agilent Eclipse XDB-C18 5 μ M 9.4 $\times$ 250 mm; flow rate 2 mL/min; phase A: water 0.01% TFA; phase B: acetonitrile 0.01% TFA; gradient 45 min from 95/5 A/B to 0/100 A/B). HPLC peaks were collected and identified via MALDI-TOF MS. The stability of Alexa750-pHLIP(WT)-MMAE was determined in mouse serum by solubilizing the construct to 10 μ M, incubating it at 37 °C and monitoring via RP-HPLC at specific time points.

Tumor Targeting *In Vivo*

Tumor targeting of Alexa750-pHLIP(WT)-MMAE was evaluated in NCr nu/nu mice harboring MDA-MB-231 tumor xenografts (n = 3). Tumor cells were implanted subcutaneously (3×10^6 / animal) and allowed to grow until tumors reached volumes of approximately 100 mm³. Alexa750-pHLIP(WT)-MMAE was then administered intravenously (100 μ L, 5 μ M in PBS pH 7.2) and its uptake in tumor and normal tissues was monitored over time by optical imaging on an IVIS Spectrum (Perkin Elmer). Average Radiant Efficiency (ARE, expressed as [p/s/cm²/sr] / [μ W/cm²]) was calculated for regions of interest (ROIs) drawn around regions encompassing tumor, kidney, and background blood pool.

RESULTS AND DISCUSSION

Synthesis of pHLIP-drug Conjugates

Auristatins are synthetic analogs of the natural peptide dolastatin 10, a highly potent microtubule depolymerization agent originally isolated from the sea organism *Dolabella auricularia*.³⁴ A number of synthetic derivatives have been produced, such as monomethyl auristatin E (MMAE), monomethyl auristatin F (MMAF), and monomethyl auristatin F-OMe (MMAF-OMe), with the goal to optimize properties such as solubility, potency, pharmacokinetics, and chemical linkers for delivery vehicles.^{35,36} Auristatins have an IC₅₀ that is 52- and 197-fold more toxic than the clinically relevant chemotherapies vinblastine and doxorubicin, respectively.³⁵ However, a targeting mechanism is necessary to deliver

these potent cytotoxic agents to reduce their off-target effects and to increase their concentrations into tumors.³⁷ For instance, ADCs have exploited the targeting specificity of monoclonal antibodies to selectively deliver MMAE to tumors in an effort to increase the therapeutic window of these highly toxic agents.³⁸ Toward this end, we conjugated MMAE, modified with a succinimide nitrophenyl-based linker, to the C-terminus of pHLIP and pHLIP(D25E) (Figure 1A) via a cleavable disulfide bond and purified the conjugates by HPLC and determined via MALDI-TOF MS (see Experimental Section). This linking strategy allows for the release of MMAE into the cytoplasm of cancer cells after translocation and reduction of the disulfide bond (Figure 1B).^{19,25} Cleavage of the disulfide bond results in release of an N-terminally modified form of MMAE (Figure 1B). The resulting MMAE derivative and the parental MMAE exhibit IC₅₀ ~ 125 nM and 4 nM against HeLa cells, respectively, when incubated with the cells for 72 hours (Figure S1).

Biophysical Characterization of the Interaction of pHLIP-MMAE Conjugates with Lipid Bilayers

Prior to evaluating the pHLIP-mediated translocation of MMAE into cancer cells, the interaction of the pHLIP-MMAE conjugates with lipid bilayers was studied (Figure 2). Far-UV circular dichroism spectroscopy (CD) was used to determine the secondary structure of pHLIP-MMAE conjugates in the presence of POPC liposomes at both normal and low pH. Figure 2A shows the typical pH-dependent transition of pHLIP from an unstructured configuration at pH 7.4 (blue line) to an α -helical structure when the pH is lowered to 5.0 (red line). Tryptophan fluorescence was employed to determine the insertion of pHLIP into the vesicle bilayers based on the sensitivity of the fluorescence emission of two tryptophan residues present in the sequence of pHLIP to the polarity of the environment. At pH 7.4, the Trp fluorescence emission maxima of pHLIP is centered at 348 nm (Figure 2b, blue line), reflecting the exposure of Trp residues to polar, aqueous environments. Lowering the pH to 5.0 results in a 14 nm λ_{max} blue shift (from 348 nm to 334 nm; red line), which is characteristic of Trp residues buried in hydrophobic environments, suggesting pHLIP's insertion into the membrane. Taken together, these results indicate that the presence of MMAE at the C-term of pHLIP does not significantly affect the pH-mediated insertion of pHLIP.

With the goal of optimizing the targeting and delivery of MMAE by pHLIP, we also considered the variant pHLIP(D25E) (Figure 1A), which has a higher pK_a of insertion than native pHLIP(WT) (6.49 vs. 6.0).³⁹ The higher pK_a of insertion may match better the extracellular pH of tumors (i.e., pH 6.5 to 6.9) and favor pHLIP insertion and MMAE translocation into cells. Similarly to pHLIP(WT)-MMAE, CD and tryptophan fluorescence measurements indicate that conjugating MMAE to pHLIP(D25E) does not interfere with pHLIP's characteristic shape-shifting behavior. Indeed, a transition from an unstructured conformation to an α -helix, and a clear blue shift (from 347 nm to 329 nm) are observed by CD (Figure 2C) and Trp fluorescence (Figure 2D), respectively. Thus, both pHLIP(WT)-MMAE and pHLIP(D25E)-MMAE conjugates can insert in lipid bilayers under acidic conditions to form stable transmembrane α -helices in a manner similar to that of pHLIP alone. The pK_a of insertion of the pHLIP-MMAE conjugates is not expected to differ significantly from pHLIP alone (pK_a~6) because 1) MMAE has no ionizable group that

could influence the pKa of insertion, and 2) conjugation of phalloidin and amanitin toxin derivatives to pHLIP was shown to not change the pK of insertion (6.14 and 5.9, respectively).^{20,40}

Inhibition of Cell Proliferation

The ability of pHLIP to move MMAE across the cell membrane and to inhibit cancer cell proliferation in concentration and pH-dependent manners was evaluated. Cell treatments for both pHLIP(WT)-MMAE and pHLIP(D25E)-MMAE constructs were carried out with conjugate concentrations ranging from 1.25 to 10 μ M at either pH 7.4 or 5.0. After a 2-hour incubation at 37 °C, the treatment media was replaced by fresh media, and cells were grown for an additional 72 hours at normal pH before assessing cell viability using the MTT assay. This treatment protocol was chosen to prevent cell death that may result from prolonged exposure to low pH. It may also better mimic the transient exposure of tumor cells to pHLIP-MMAE anticipated to occur *in vivo*. It is worth noting that this treatment protocol is quite stringent when compared to the typical multi-days exposure treatments used with free drugs (Figure S1).

When HeLa cells were treated with pHLIP(WT)-MMAE at low pH, cell proliferation was severely disrupted. Up to 93% growth inhibition is observed (Figure 3A). The anti-proliferative effect is concentration-dependent with cell viability ranging from 80% to 7%, with increasing treatment concentration ranging from 1.25 to 10 μ M, respectively (Figure 3A, grey bars). As anticipated, inhibition of cell growth is also pH-selective: Treatment with pHLIP(WT)-MMAE at pH 7.4 under the same conditions had no or a moderate effect on cell proliferation (Figure 3A; black bars). This lack of anti-proliferative effect at normal pH is consistent with the notion that delivery of MMAE is mediated by the pH-dependent insertion of pHLIP across the plasma membrane. It is important to note that the low pH treatment in itself did not have any detrimental effect on the proliferation of HeLa cells, as shown by treatment without pHLIP(WT)-MMAE (0 μ M treatment). We also tested pHLIP(WT)-MMAE with human MDA-MB-231 breast cancer cells (Figure 3B). A similar concentration and pH-dependent anti-proliferative effect is observed: Up to 88% cell growth inhibition is observed with low pH treatment, while no significant inhibition of proliferation is observed for cells treated at normal pH. However, MDA-MB-231 seem less sensitive to pHLIP(WT)-MMAE treatment, as shown by a higher cell viability with the 2.5 μ M treatment. This effect may be due to the apparent higher resistance of MDA-MB-231 cells to MMAE when compared to HeLa cells (Figure S1A and S1B).

When HeLa and MDA-MB-231 cells were treated with pHLIP(D25E)-MMAE, cell proliferation was also disrupted in a concentration- and pH-dependent fashion (Figure 3C and 3D). pHLIP(D25E)-MMAE appears to be more effective at inhibiting cell growth than pHLIP(WT)-MMAE. It is especially evident when comparing treatments at 1.25 μ M (Figures 3C and 3D), with which greater cell killing is attained with pHLIP(D25E)-MMAE than with pHLIP(WT)-MMAE (MDA-MB-231 cells: 49% versus 100%, respectively). However, while 5 and 10 μ M treatment exhibit nearly 100% growth inhibition at low pH (Figure 3C and 3D; grey bars), non-negligible cytotoxicity is observed at pH 7.4, with about 75% and 40% cell viability, respectively (Figures 3C and 3D; black bars). This relatively

high cytotoxicity at higher concentrations is not due to any intrinsic peptide cytotoxicity or to pHLIP insertion itself, as neither pHLIP(WT) or pHLIP(D25E) peptides have any harmful effects on the cell viability of either cell line at 10 μ M (Figure S2). It is consistent with previous observation that pHLIP is minimally toxic.^{20,22} Cell killing does not seem to be due to instability of the linker (or any other chemical instability) in cell media either, as no degradation products are observed by HPLC or mass spectroscopy after low pH treatment with pHLIP(WT)-MMAE or pHLIP(D25E)-MMAE (Figure S3). We also assessed whether either pHLIP-drug constructs may cause cell death through disruption of the plasma membrane: Cells were counted after a 2-hour treatment based on the uptake of trypan blue, which can only be taken up by cells if their plasma membrane is disrupted. Figure 4 shows that neither conjugates, nor low pH treatment, caused disruption of the membranes of either cell line. While pHLIP-mediated translocation of cargo molecules is thought to not be mediated by endocytosis, we cannot exclude the possibility that the observed cytotoxicity of pHLIP(WT)-MMAE and pHLIP(D25E)-MMAE at higher concentrations might be associated with partial endocytotic uptake by the cells promoted by the interaction of pHLIP with the plasma membrane at low pH.⁴⁰

Nevertheless, our results indicate that pHLIP-mediated translocation of MMAE can inhibit the proliferation of cancer cells in a pH-selective fashion, and offers clear advantages over treatment with free MMAE: (1) pHLIP prevents the high toxicity of MMAE at physiological pH. For instance, in Figure 3A, 5 μ M of pHLIP(WT)-MMAE shows only marginal cytotoxicity against HeLa whereas MMAE alone exhibits about 70% cytotoxicity (Figure 3E). Similar results are observed with pHLIP(D25E)-MMAE and MDA-MB-231 cells. (2) Conjugation to pHLIP enhances MMAE anti-proliferative effects at low concentrations when treated at low pH. It is especially evident when comparing cell viability at 2.5 μ M: While free MMAE has a marginal cytotoxicity effect at these concentrations (20–30%; Figures 3E and 3F), pHLIP-MMAE conjugates are systematically more toxic at low pH (up to 87.5% cytotoxicity; Figures 3A–D).

Our results suggest that, on one hand, MMAE is effectively sequestered outside the cells by pHLIP at normal pH, preventing its passive diffusion into cells and its cytotoxicity. On the other hand, pHLIP improves MMAE potency by actively translocating it into the cytoplasm at lower pH in a mechanism more favorable (and/or faster) than passive diffusion, likely resulting in a higher effective MMAE concentration inside cells. All together, these effects participate in reducing the effective dose of MMAE necessary to observe cell death, which may alleviate off-target effect.

***In vivo* targeting**

The pH-dependent anti-proliferative selectivity observed with pHLIP-MMAE and its low cytotoxicity at higher concentration in comparison with pHLIP(D25E)-MMAE prompted us to test the hypothesis that pHLIP can selectively deliver MMAE to tumors. As seen in Figure 5, intravenously administered Alexa750-pHLIP(WT)-MMAE is selectively retained in MDA-MB-231 breast cancer xenograft tumors over a 48 hour imaging time course. As tested, tumors were clearly visualized by 4 – 6 hours post-injection with maximum tumor: background ratios of 2.3 being observed at the 28 hour post-injection time point (Figure 5C).

Consistent with a renal clearance mechanism, Alexa750-pHLIP(WT)-MMAE rapidly accumulates in kidney with concomitant clearance from the periphery. Background levels dropped 4.5-fold by 28 hours and 6-fold by 48 hours (Figure 5C). The conjugation of MMAE to pHLIP did not discernibly alter the tumor targeting properties of pHLIP as compared to that seen with other Alexa-pHLIP constructs.²⁴ The current imaging studies were carried out with sub-therapeutic doses of Alexa750-pHLIP(WT)-MMAE to assess its tumor targeting capability and set the stage for in-depth studies aimed at determining the therapeutic efficacy and toxicity profiles associated with this novel drug conjugate. The clinical efficacy of antibody-drug conjugates (ADCs) comprising intact IgG molecules linked to potent cytotoxics, such as auristatins and maytansine, has resulted in significant interest in delineating the pharmacologic parameters underlying efficacious ADCs and using that information to guide development of next generation targeting vehicles.⁴¹ A comprehensive analysis of tumor and normal tissue exposures to auristatin upon SGN-75 (anti-CD70::MMAE) treatment supports selective tumor targeting and retention, as well as serum stability, as being critical characteristics.⁴² Advances in antibody engineering techniques have led to development of antibody-based fragments that exhibit rapid tumor targeting coupled with rapid systemic clearance. Those antibody-based molecules have proven to be effective delivery vehicles for other therapeutic agents.^{43–45} The rapid and selective uptake of Alexa750-pHLIP(WT)-MMAE into tumors is analogous to that observed with rapidly cleared antibody-based agents. This data, coupled with our demonstration that pHLIP-MMAE is stable in serum over a relevant time frame for its systemic clearance rate makes us hypothesize that appropriate dosing of pHLIP(WT)-MMAE will promote tumor control and limit the toxicities associated with free MMAE. As detailed above, unlike ADCs, the mechanism of action that drives tumor targeting of pHLIP-based constructs is independent of target antigen expression. We hypothesize that this makes pHLIP(WT)-MMAE particularly relevant to diseases such as TNBC, for which efficacious ADC target antigens have not yet been established.

CONCLUSION

The development of tumor-targeting vehicles capable to selectively deliver cytotoxic agents hold the promise of being curative cancer therapies. Here, we have demonstrated that pHLIP provides localized delivery of MMAE, a clinically relevant cytotoxic agent. In combination, pHLIP-MMAE drug conjugates synergistically provide greater selectivity and inhibit cancer cell proliferation in a concentration- and pH-dependent manner *in vitro*. Moreover, the tumor homing abilities of pHLIP-MMAE conjugates were presented in MDA-MB-231 triple negative breast cancer xenograft tumors *in vivo*. Together this data supports the further development of pHLIP-MMAE as an efficacious treatment of cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ADC	Antibody drug conjugate
CD	Circular dichroism
MALDI-TOF	Matrix-assisted laser desorption/ionization Time of Flight
MMAE	Monomethyl Auristatin E
MS	Mass spectroscopy
pHLIP	pH (Low) Insertion Peptide
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
ROI	Regions of interest
RP-HPLC	Reverse-phase high performance chromatography
Trp	Tryptophan

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(A) **pHLIP (WT)** : AAEQNPIYWARYADWLFTTPLL~~LL~~DLALLVDADEGTCG
pHLIP (D25E) : AAEQNPIYWARYADWLFTTPLL~~LL~~**EL**ALLVDADEGTCG

Figure 1.

(A) Sequences of the pHLIP peptides used in this study. The amino acid substitution for the variant pHLIP(D25E) is shown in bold, and the cysteine available for disulfide drug conjugation is shown underlined. (B) Structure of MMAE with a succinimide nitrophenyl-based linker, conjugation to pHLIP via disulfide exchange and the structure of MMAE as released intracellularly after pHLIP insertion and reduction of the disulfide bond.

Figure 2. Interactions of pHLIP-MMAE Constructs with Lipid Bilayers
 (A–B) pHLIP(WT)-MMAE CD spectroscopy and Trp Fluorescence, respectively. (C–D) pHLIP(WT)-MMAE CD spectroscopy and Trp Fluorescence respectively. State I corresponds to the peptides in an aqueous environment is shown in black, state II: peptides in the presence of lipid membranes at pH 7.4, shown in blue and state III: peptides in the presence of lipids at pH 5.0 is shown in red. [peptide] = 7 μ M and peptide-lipid ratio = 1:300 with POPC liposomes.

Figure 3. Inhibition of cell growth

(A), (C) and (E): HeLa cells treated with pHLIP(WT)-MMAE, pHLIP(D25E)-MMAE, and MMAE(linker), respectively. (B), (D) and (F): MDA-MB-231 cells treated with pHLIP(WT)-MMAE, pHLIP(D25E)-MMAE, and MMAE(linker), respectively. Black columns represent cells treated at pH 7.4 and cells treated at low pH are shown in grey. For each condition, 3,000 cells/well (96-well plate) were seeded, allowed to adhere overnight, treated for 2 hours, washed once with media and grown for 72 hour in complete medium at physiologic pH. Cell viability was assessed with the MTT assay. All measurements were normalized to the media control (0 μM, pH 7.4), as 100% cell viability. Results are shown as

mean $\pm$ SD (n=6–9). To assess statistical significance two-tailed Student's t test analyses at 95% confidence level were performed for the comparison of pH 7.4 and pH 5.0 treatments (**** p< 0.0001; *** p=0.0004; ** p=0.0009 and * p=0.011). For Figures (E) and (F), none of the differences between treatment at pH 7.4 and pH 5.0 were statistically different (p > 0.01).

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Figure 4. pHLIP-MMAE conjugates do not disrupt the cell membrane

The integrity of the plasma membrane is assessed by the uptake of trypan blue dye. 3,000 cells/well of HeLa (A) and MDA-MB-231 (B) cells were seeded, allowed to adhere overnight and treated with 10 μ M conjugates or media alone (no peptide) for 2 hours at pH 7.4 (black bars) or pH 5.0 (grey bars). Cells were detached and counted based on trypan blue uptake with an hemacytometer: % of intact cells corresponds to the number of cells not showing any dye uptake over the total number of cells. Results are shown as mean $\pm$ SD (n=4).

Figure 5. Optical imaging of Alexa705-pHLIP(WT)-MMAE in vivo targeting
 Clearance and tumor targeting of Alexa750-pHLIP(WT)-MMAE in NCr nu/nu mice harboring MDA-MB-231 tumor xenografts visualized over a 48 hour time course post-injection. (A) Imaging was performed with the minimum and maximum average radiant efficiency set to 1.1×10^7 and 1.4×10^8 [p/s/cm²/sr] / [μ W/cm²], respectively for all time points. Color intensity reflects absolute levels of probe. Arrow and chevron noted on 28 hr time point in (A) denote position of tumor and kidney, respectively. The third region displaying relatively high fluorescence corresponds to the epi-fluorescence originating from the region corresponding to the knee joint. (B) Contrast set in automatic mode to optimize visualization of tumor targeting at each time point. (C) Quantitation of average radiant efficiency [p/s/cm²/sr] / [μ W/cm²] for ROIs drawn around tumor, kidney, and background (blood pool). Results are shown as mean $\pm$ SD.