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Therapeutic Efficacy of a Family of pHLIP–MMAF Conjugates in Cancer Cells and Mouse Models

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Abstract

The targeting of therapeutics specifically to diseased tissue is crucial for the development of successful cancer treatments. The approach here is based on the pH(low) insertion peptide (pHLIP) for the delivery of a potent mitotic inhibitor monomethyl auristatin F (MMAF). We investigated six pHLIP variants conjugated to MMAF to compare their efficacy *in vitro* against cultured cancer cells. While all pHLIP–MMAF conjugates exhibit potent pH- and concentration-dependent killing, their cytotoxicity profiles are remarkably different. We also show that the lead conjugate exhibits significant therapeutic efficacy in mouse models without overt toxicities. This study confirms pHLIP–monomethyl auristatin conjugates as possible new therapeutic options for cancer treatment and supports their further development.

Graphical Abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.6b00847.

Effects of free MMAF-linker and pHLIP variants on HeLa cell proliferation, chemical stability of pHLIP-(WT)–MMAF, effect of pHLIP–MMAF variants on plasma membrane integrity and lactate dehydrogenase (LDH) release, and the effects of free MMAF-linker on A431 cell proliferation (PDF)

The authors declare no competing financial interest.

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Author Contributions

D.T., K.E.B., and M.K.R. designed the project and wrote the manuscript. D.T. and K.E.B. performed the experiments and analyzed the data. M.K.R. and H.H. performed animal experiments.

Keywords

monomethyl auristatins; peptide drug delivery; targeted drug delivery; tumor acidity; cytotoxicity; in vivo efficacy

INTRODUCTION

Monomethyl auristatins derivatives represent a family of synthetic microtubule depolymerization agents with remarkable toxicity toward cancer cells. However, due to their high potency, they are too toxic for clinical use unless conjugated to a tumor-targeting moiety. Antibody–drug conjugates (ADCs), such as brentuximab vedotin (Adcetris), have harnessed the specificity of monoclonal antibodies to selectively deliver these compounds to tumors in an effort to increase their therapeutic window. However, relying on a single cancer biomarker as the basis for developing a targeted therapy is flawed and destined to result in selection of tumor clones that have lost expression of the target antigen, and eventually promote acquired resistance and disease progression.¹ It is the case for brentuximab vedotin and trastuzumab emtansine (Kadcyla), with which most treated patients develop acquired drug resistance.^{1–6} However, nearly all solid tumors have elevated acidosis (low extracellular pH), which can be exploited for targeted therapy. As acidosis results from alterations in basic cellular metabolism that are inherent to the oncogenic process, it is hypothesized to be less prone to the development.

Here, we use the pH(low) insertion peptide (pHLIP), a peptide that exploits tumor acidity to promote its selective targeting and intracellular delivery of cargo molecules.^{7–14} We recently showed that pHLIP provides localized delivery of monomethyl auristatin E (MMAE) to cancer cells.¹² This previous study shows that pHLIP-mediated translocation of MMAE inhibits the proliferation of cancer cells in a pH-selective and concentration-dependent fashion and that it offers clear advantages over treatment with free MMAE. The same study also shows that the pHLIP–MMAE conjugate effectively targets triple-negative breast tumor xenografts in mice and that pHLIP–MMAE is stable in serum over a relevant time frame for its systemic clearance rate. Altogether, these results indicate that pHLIP–monomethyl auristatin conjugates may provide a targeting mechanism to attenuate systemic toxicity and also support their further development as an efficacious treatment of cancer.

In the present study, with the goal to optimize the selective delivery of monomethyl auristatins to cancer cells and tumors, we evaluate the selective cytotoxicity of a panel of pHLIP variants with different insertion properties in conjugation with monomethyl auristatin F (MMAF), and translate the lead molecule into preclinical testing in subcutaneous xenograft model of cervical cancer.

EXPERIMENTAL SECTION

Materials

N-Hydroxybenzotriazole (HOBt), *o*-benzotriazol-*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 purchased from PCAS BioMatrix Inc. Diisopropylethylamine (DIEA), piperazine, *N,N*-dimethylformamide (DMF), dichloro-methane (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. Pyridyldithiol-activated Py-ds-Prp-monomethylauristatin F (MMAF) was purchased 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. Pierce LDH Cytotoxicity Assay Kit was purchased from Thermo Fisher Scientific Inc. Noncanonical amino acids (Gla and Aad) were purchased from ChemImpex.

Solid-Phase Peptide Synthesis

Six variants of pHLIP were used in this study, each having a cysteine residue at the C terminus: pHLIP(WT), pHLIP(D25E), pHLIP(P20G), pHLIP(R11Q), pHLIP(R11Q;D14Up), pHLIP-(D14Gla;D25Aad) (Table 1). All six variants were 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 m 250 $\times$ 21.20 mm C8; flow rate 10 mL/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.

Preparation of pHLIP–MMAF Constructs

Each pHLIP peptide was dissolved in DMSO to a concentration of 1 mM, followed by the addition of 1.5 equiv of Py-ds-Prp–MMAF in DMSO (Figure 1C). 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 h. 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, and their identity was confirmed by MALDI-TOF MS: pHLIP(WT)–MMAF: purity > 98%; calculated ($M + H^+$) = 5030; found ($M + H^+$) = 5030. pHLIP(D25E)–MMAF: purity > 98%; calculated ($M + H^+$) = 5045; found ($M + H^+$) = 5053. pHLIP(P20G)–MMAF: purity > 98%; calculated ($M + H^+$) = 4991; calculated ($M + Na^+$) = 5013; found ($M + Na^+$) = 5018. pHLIP(R11Q)–MMAF: purity > 98%; calculated ($M + H^+$) = 5003; found ($M + H^+$) = 5004. pHLIP(R11Q;D14Up)–MMAF: purity > 98%; calculated ($M + H^+$) = 5003; found ($M + H^+$) = 5008. pHLIP(D14Gla;D25Aad)–MMAF: purity > 98%; calculated ($M + H^+$) = 5115; found ($M + H^+$) = 5113. The conjugates were quantified at 280 nm by UV/vis absorbance spectroscopy using the molar extinction coefficient of pHLIP (13,940 $M^{-1} cm^{-1}$) and lyophilized to 10^{-8} mole aliquots.

Preparation of POPC Liposomes

Liposomes were prepared as described previously.¹² Ten milligrams of POPC in chloroform was 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 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 assay, 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).

Tryptophan Fluorescence Measurements

Lyophilized aliquots of pHLIP constructs were resuspended to 10 μ M with 5 mM sodium phosphate, pH 8.0, and incubated for 1 h at room temperature. This stock solution was diluted to a final concentration of 7 μ M and incubated with POPC liposomes at a 1:300 peptide-to-lipid molar ratio for 2 h at room temperature. For pH titration, samples were adjusted to the desired experimental pH values with the addition of small aliquots of a HCl solution to elicit pH-dependent insertion. Samples were allowed to equilibrate at the desired pH for 30 min at room temperature before fluorescence measurements. All measurements were carried out using a Fluorolog FL3-22 fluorescence spectrophotometer (Horiba) with the following settings: excitation wavelength of 295 nm, emission from 310 to 450 nm, slit widths for emission and excitation both set at 5 nm, and excitation and emission polarizers set at 0° and 90°, respectively. The appropriate background spectra were subtracted in all cases. The pH-dependent changes in spectral position of fluorescence maximum ($\lambda_{\max}$) were fitted to the following equation:

$$\lambda_{\max} = \lambda_{\text{pH}4} + (\lambda_{\text{pH}8} - \lambda_{\text{pH}4}) / (1 + 10^{n(\text{pH}50 - \text{pH})})$$

where n is a cooperativity constant, pH_{50} is an apparent constant, and $\lambda_{\text{pH}8}$ and $\lambda_{\text{pH}4}$ are the limiting values of the fluorescence maximum at pH 8 (membrane-bound) and at pH 4 (inserted), respectively.

Cell Culture

Human cervical adenocarcinoma HeLa (ATCC, cat # CCL-2) and human epidermoid A431 (ATCC, cat # CRL-155) 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% CO_2 at 37 °C.

Antiproliferation Assay

HeLa cells were seeded in 96-well plates at a density of 3000 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 s 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 min. 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) and the plate was incubated at 37 °C for 2 h. After treatment, the media was removed, cells were washed once with 100 μ L of complete DMEM, and 100 μ L 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 microcombination pH probe (Microelectrodes, Inc.). Cell viability was determined after 72 h using the colorimetric MTT assay. Briefly, 10 μ L of a 5 mg/mL MTT stock solution was added to the treated cells and incubated for 2 h at 37 °C. The resulting formazan crystals were solubilized in 200 μ L 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. Data were fitted with a sigmoidal dose–response and IC₅₀ values were estimated using Prism 5 for Macintosh (GraphPad, Inc.).

Cell Membrane Integrity Assay

For the trypan blue uptake assay, HeLa cells were seeded in 96-well plates at a density of 3000 cells/well and incubated until confluent (~72 h). For the lactate dehydrogenase (LDH) assay cells were seeded in 24-well plates at a density of 200,000 cells/well and incubated until confluent (~24 h). 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 cells were incubated at 37 °C for 5–10 min. 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 h. After treatment, the media was removed, and cells were detached using trypsin and counted based on trypan blue uptake using an hemacytometer. For the LDH assay, cell media supernatant was collected in a 96-well plate in triplicate after treatment, and LDH reaction mixture was added to each well as recommended by the manufacturer protocol. After a 30 min incubation at room temperature, stop solution was added to each well, and the absorbance at 490 and 680 nm of each well was measured using an Infinite 200 PRO microplate reader (Tecan). Percent LDH release was calculated against control cells treated with media at physiologic pH

Construct Stability in Cell Medium and Mouse Serum

pHLIP(WT)–MMAF was solubilized in an appropriate volume of DMEM without FBS (pH 7.4), so that upon pH adjustment 10 μ M is obtained, or in 25% mouse serum to obtain a 20 μ M solution. Peptide solutions were gently sonicated for 30–60 s using a bath sonicator and incubated at 37 °C and 5% CO₂. One hundred microliters of the reaction mixture was injected and monitored via RP-HPLC at various time points (Agilent Eclipse XDB-C18 5 μ M 9.4 $\times$ 250 mm; for DMEM pH 5.0: flow rate 5 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; for DMEM pH 7.4 and serum: flow rate 3 mL/min, phase A: water, 0.01% TFA; phase B: acetonitrile, 0.01% TFA; gradient 60 min from 95/5 A/B to 0/100 A/B). HPLC peaks were collected and identified via MALDI-TOF MS.

***In Vivo* Efficacy**

HeLa cells were grown as outlined above and harvested by trypsin-treatment, and 5×10^6 viable cells/mouse were injected subcutaneously (s.c.) into the inguinal space of 20, 6–8 week old female NCr nu/nu mice (Taconic Biosciences). Tumor-bearing mice ($n = 17$) were injected intravenously (i.v.) with 200 μ L of either pHLIP–MMAF (20 μ M in PBS + 1% DMSO) or vehicle control. Tumor volume was determined using caliper measurements and the formula volume = length $\times$ width $\times$ (height $\times$ 0.5). A tumor weight of 500 mg was prospectively set as a stopping point for this study. An analogous study was carried out with NCRr nu/nu mice bearing A431 tumors. A431 tumor-bearing mice ($n = 10$ /cohort) were treated intraperitoneally (i.p.) with either pHLIP(WT)–MMAF or vehicle control and evaluated by immunohistochemistry (IHC) using K_i -67 as a biomarker of efficacy. Sections were evaluated, in a blinded manner, for K_i -67 staining by the FCCC animal histopathology core facility, and a Wilcoxon ranked-sum analysis was used to evaluate statistical difference between treatment groups.

RESULTS AND DISCUSSION

Since the pHLIP–monomethyl auristatin conjugate (pHLIP–MMA) is a two-component system (i.e., targeting/delivery peptide and drug cargo), we investigate herein the effect of varying both pHLIP and the monomethyl auristatin moieties to enhance the potency toward cancer cells and tumors when compared to our original pHLIP(WT)–MMAE conjugate.¹² Advantageously when compared to other type of targeting and delivering methods, the properties of small peptides can be adjusted relatively easily by modifying their amino acid sequence. The pHLIP peptides shown in Table 1 have been chosen to cover a wide range of properties with the objective to tune/improve selectivity and translocation in cultured cells and tumor localization in vivo. One criterion for selecting pHLIP variants is their apparent pH_{50} of insertion in lipid membranes (i.e., the pH at which 50% of pHLIP are in the α -helical inserted state). Indeed, it is hypothesized that an increased pH_{50} might be beneficial to pHLIP-based drug delivery, as most solid tumors exhibit an average extracellular pH 0.5–0.8 units lower than in healthy tissues.^{15–18} For example, the apparent pH_{50} of insertion across 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) membrane for pHLIP(WT) is about 6.1 (Table 1).^{11,19–21} Of the six pHLIP variants considered here, three have a larger pH_{50} than pHLIP(WT): (1) pHLIP(D25E), where Asp25 is substituted by the more hydrophobic glutamic acid,²⁰ (2) pHLIP(P20G), where the Pro to Gly substitution increases pH_{50} by enhancing the propensity of the peptide to form an α -helix,²² and (3) pHLIP(D14Gla;D25Aad), where Asp14 is substituted by an γ -carboxyglutamic acid (Gla), which has an additional side chain carboxylic acid, and where Asp25 is substituted for the more hydrophobic α -aminoadipic acid (Aad). This variant not only shows an increase in pH_{50} (6.79) but also a slight increase in insertion cooperativity (i.e., sharper pH-response).¹¹ While the variants with increased pH_{50} of insertion might match better the tumor microenvironment pH, one cannot exclude that a decreased pH_{50} of insertion may provide better selectivity in vivo by increasing pHLIP's sensitivity to acidic environments, thereby decreasing off-targeted delivery. It is therefore not clear, at this very early stage of the development of pHLIP as a therapeutic, whether a high or low pH_{50} is preferable for effective targeting and delivery in vivo. Moreover, the pH is lowest at the surface of cells

compared with the bulk extracellular pH (~6.8) and increases with distance from cellular membrane.²³ In addition, variants with reduced aggregation tendency may result in a larger pool of conjugate available for targeting.²¹ For these reasons two other variants were investigated as well: pHLIP(R11Q) and pHLIP(R11Q;D14Up), where substitutions not only lower p*H*₅₀ (5.8 and 5.6, respectively) but also improve response to high ionic strength and reduce aggregation tendency.²¹ From the panel of pHLIP variants studied here, only pHLIP(WT) and pHLIP(D14Gla;D25Aad) have been previously reported to translocate molecules in cultured cancer cells. Apart from our own work with pHLIP(WT)–MMAE, pHLIP(D14Gla;D25Aad) has been shown in conjugation with taxol against A549 cultured cells but with marginal effect, and pHLIP(WT) in conjugation with a peptide nucleic acid showed promising therapeutic efficacy in mice.^{10–12}

The chemico-physical properties of MMAs can also be exploited to enhance pHLIP–MMA conjugate potency. For instance, MMAF (Figure 1A,B) has a higher aqueous solubility and lower lipid permeability than MMAE due to the charged C-terminal phenylalanine group (Log *P*_{o/w} = 0.7 vs 2.2, Figure 1B), making it of great interest for targeted delivery. Indeed, MMAF would have a lesser tendency to diffuse out of the cells after delivery and thus be more effective than MMAE. This property could also limit side effects associated with possible degradation of conjugate in serum with concomitant release of free drug; release of free drug is thought to contribute to toxicities associated with ADCs.²⁵ In addition, it has been shown that MMAF (unlike MMAE) can sustain significant modification to the N-terminal position (i.e., position of conjugation to pHLIP) and still retain its potency.^{1,26} For these reasons, we conjugated MMAF (modified with a pyridyldithiol-based linker) to the C-terminus of pHLIP variants via a cleavable disulfide bond (Figure 1C) to allow for the release of MMAF into the cytoplasm of cancer cells upon translocation and reduction of the disulfide bond. It is important to note that conjugation of MMAF does not appear to affect significantly the p*H*₅₀ of insertion of pHLIP, as exemplified in Figure S1.

Inhibition of Cell Proliferation

The ability of pHLIP–MMAF conjugates to inhibit cancer cell proliferation was evaluated in human cervical cancer HeLa cells. Similarly to our previous study,^{2–6,12} cells were treated with pHLIP conjugates for only 2 h to prevent cell death that may result from prolonged exposure to low pH and to possibly better mimic the transient exposure of tumor cells to pHLIP–MMAF anticipated to occur *in vivo* (i.e., pHLIP clears with approximately a 6 h half-life).¹² Furthermore, low pH treatments were performed at pH 5.0 to keep the treatment pH lower than the variants' p*H*₅₀ of insertion (Table 1) and push the equilibrium toward insertion. While tumor pH is likely not as acidic as pH 5.0, such treatment allows for the direct comparison of pHLIP variants with different p*H*₅₀ of insertion. Indeed, if p*H*₅₀ was the only factor involved in pHLIP-mediated targeting and delivery, pHLIP variants with the highest p*H*₅₀ should always exhibit the largest effect as more peptide would be inserted in the membrane (and thus more MMAF translocated). Importantly, it should be the case at any pH. This choice of treatment pH is further discussed below in the context of our results. Noteworthy, this low pH treatment did not have any significant harmful effect on cell proliferation in itself, as shown by treatment without conjugates (Figure 2; 0 μ M Control).

Cytotoxicity assays with HeLa cells show that, while all six pHLIP–MMAF conjugates display clear pH-selective and concentration-dependent killing, their respective cytotoxicity profiles at both low and physiological pHs are remarkably different (Figure 2). The IC₅₀ values at pH 5.0 and 7.4 for each conjugate are reported in Table 2. Remarkably, the pHLIP-(WT)–MMAF conjugate shows the highest and most selective killing with IC₅₀ values of 29 and 633 nM at pH 5.0 and 7.4, respectively (Figure 2A). It represents a 22-fold higher antiproliferative effect at low pH than at physiological pH. Although not a direct comparison, it also represents a significant improvement over our original pHLIP(WT)–MMAE conjugate (~100-fold more potent).¹² For example, treating at 40 nM results in 100% survival at pH 7.4 but leads to ~80% cell killing at low pH. It also displays the sharpest transition at pH 5.0 between low and high cytotoxicity. It is also a clear improvement over free MMAF-linker, which exhibits a rather modest antiproliferative effect, with an IC₅₀ value of ca. 5 μ M (Figure S2A; 2 h treatment), likely because of its impaired intracellular access. However, in an attempt to compare the activity of pHLIP–MMAF conjugates to that of free MMAF-linker fairly, Figure S2A also shows 72 h treatments with MMAF. Indeed, our treatment protocol, where pHLIP-constructs are removed after a 2 h treatment, is more stringent than the several days of exposure typically used with free drugs, from which IC₅₀ values are typically derived (including the ones presented in Figure 1B).

As expected, MMAF shows a higher antiproliferative effect than with 2 h treatments with an IC₅₀ of 834 $\pm$ 40 nM (Figure S2; 72 h treatment). Interestingly, the maximum killing observed with MMAF-linker is only about 50% for the 2 h treatment at 10 μ M. However, it does not mean that HeLa cells are resistant but rather that not enough MMAF, because of its low cell permeability, has time to cross the cell membrane before being washed out. Indeed, this low permeability effect is alleviated when HeLa cells are treated for 72 h in the presence of MMAF (Figure S2A). pHLIP therefore appears to improve MMAF potency at lower pH by facilitating its translocation across the cell membrane in a mechanism more favorable than passive diffusion. Similar to our previous study with pHLIP–MMAE conjugates,^{12,15–18} pHLIP(D25E)–MMAF shows less potency than the pHLIP(WT) conjugate with an IC₅₀ at pH 5.0 of 368 nM. It also displays the smallest pH selectivity of all conjugates, with less than 3-fold improvement at low pH over physiological pH.

Even though the other four conjugates are less potent than pHLIP(WT)–MMAF, with IC₅₀ values at pH 5.0 ranging from 83 to 471 nM, they exhibit some very interesting features. For instance, they all are less cytotoxic at physiological pH than the pHLIP(WT) and pHLIP(D25E) conjugates, as seen by their respective IC₅₀ and their lower cytotoxicity at high concentration (Table 2 and Figure 2).

These four pHLIP variants may possibly interact less with the plasma membrane than pHLIP(WT) and pHLIP(D25E), making them less prone to cell uptake. Indeed, while pHLIP-mediated translocation of cargo molecules is not believed to be endocytosis-dependent, the observed cytotoxicity at physiological pH might be due to partial endocytic uptake by the cells induced by the interaction of pHLIP with the plasma membrane at normal pH.^{11,19–21,27,28} Weaker lipid interaction may also result in more conjugate being washed out after the 2 h treatment, explaining the differences in toxicity observed at high concentrations (Figure 2). Moreover, Weerakkody and coworkers showed that tuning of the

biophysical properties of pHLIP–lipid bilayer interactions alters tumor targeting, distribution in organs, and blood clearance.^{20,29} Remarkably, pHLIP(P20G)–MMAF does not show any significant cytotoxicity at physiological pH and thus represents the most pH-selective conjugate of all constructs used in this study. While being 16-fold less potent than pHLIP(WT)–MMAF (IC₅₀, 435 nM vs 29 nM), this unique feature may be an advantage in *in vivo* settings, where it may prevent off-target cytotoxicity. This drastic difference in cytotoxicity at physiological pH may be due to the difference in how pHLIP(P20G) interacts with lipid membranes when compared to pHLIP-(WT).²² However, the exact reasons for this behavior are unknown and are the subjects of current investigations in our group. Additionally, pHLIP(D14Gla;D25Aad)–MMAF, while being 3-fold less potent than pHLIP(WT)–MMAF at low pH, shows a remarkable 67-fold improvement at low pH over physiological pH (Table 1). However, treating at 40 nM results also in 100% survival at pH 7.4 but, unlike pHLIP(WT)–MMAF, leads to only ~27% cell killing at low pH (vs 80% for pHLIP(WT)–MMAF). Noteworthy, these results show that pH₅₀ of insertion does not necessarily correlate with cytotoxicity. While pHLIP(WT)–MMAF has an IC₅₀ of 28 nM, pHLIP(P20G) and pHLIP(D14Gla/D25Aad) conjugates, which have the highest pH₅₀, show IC₅₀ of 435 and 81 nM, respectively. Moreover, pHLIP(P20G) and pHLIP(R11Q) have similar IC₅₀ despite having very different pH₅₀. It suggests that other factors (e.g., membrane binding, higher tendency for aggregation) are in play. Therefore, even though it may not ideally mimic the tumor pH, the low pH treatment used in this study provides valuable information on the behavior of pHLIP–MMAF conjugates and their potential as a selective therapeutic. It is also important to note that the cytotoxicity observed when cells are treated at higher concentrations and at pH 7.4 is not due to any intrinsic peptide cytotoxicity or to pHLIP insertion itself, as none of the pHLIP variant peptides have any detrimental effects on the cell viability at 10 μ M (Figure S2B). This is consistent with previous observations that pHLIP is minimally toxic to HeLa cells.^{11–13} In addition, cell killing does not appear to be due to chemical instability of the linker in cell media either, as no degradation products are observed after normal and low pH treatments with pHLIP-(WT)–MMAF (Figures S3 and S4). It is consistent with our previous observation that the disulfide bond between pHLIP and MMAE is stable in cell media over the time frame of the cell treatment.^{12,21}

Even though previous studies show that pHLIP–MMAE conjugates, pHLIP alone, nor low pH treatments disrupt the plasma membrane, including that of MDA-MB-231 cells.^{12,24,30} We also evaluated here whether the pHLIP–MMAF conjugates may cause cytotoxicity through disruption of the plasma membrane. We quantified the uptake of Trypan blue, which can be taken up by cells only if their plasma membrane is disrupted, and the release of the intracellular enzyme lactate dehydrogenase (LDH) from potentially damaged cells.^{21,31} Figure S5 shows that neither the conjugates nor low pH treatment causes significant uptake of Trypan blue or release of LDH, confirming that the conjugates do not cause any dramatic disruption of the cell membrane. Nevertheless, our results show that all six pHLIP–MMAF conjugates can inhibit cancer cell proliferation in a pH-selective and concentration-dependent manner and offer clear advantages over treatment with free MMAF-linker, albeit with different efficacy. However, pHLIP(WT)–MMAF displays the highest efficacy at low

pH and one of the largest pH-selectivity of all the conjugates. We therefore selected it as the lead agent for further efficacy evaluation in xenograft-based animal studies.

Assessing the Therapeutic Efficacy of pHLIP(WT)–MMAF *in Vivo*

To evaluate the therapeutic efficacy of pHLIP(WT)–MMAF *in vivo*, female NCr nu/nu mice bearing HeLa xenografts were randomized into cohorts with statistically similar tumor sizes. Agents (pHLIP(WT)–MMAF or vehicle control) were administered intravenously (i.v.) on days 1, 3, 5, and 8 postrandomization. Consistent with *in vitro* data demonstrating that HeLa cells are sensitive to pHLIP(WT)–MMAF (Figure 2), administration of the pHLIP-based conjugate slowed tumor growth compared to vehicle control when measured both as a fraction of initial tumor size (Figure 3A) and as an increase in absolute tumor weight (data not shown). By day eight of the study, tumors in pHLIP(WT)–MMAF treated animals had grown to sizes that were $168.1 \pm 17.5\%$ of initial volumes. This was statistically smaller ($P = 0.04$) than tumors in vehicle-treated animals, which expanded to $247.4 \pm 29.9\%$ of their initial volumes. The statistically significant difference was maintained through day 10 ($P = 0.01$). Although loss of statistical significance ($P = 0.24$) was observed after day 13 (5 days after treatment was stopped), the trend in tumor growth inhibition was retained. We hypothesize that the first three injections postrandomization (days 1–5), coupled with the agents rapid pharmacokinetics,¹² only allow for therapeutic concentrations to be present in tumor for short period of time after injection. Four injections of pHLIP(WT)–MMAF were unable to fully control the tumor xenografts, and therefore, tumors were able to rebound after stoppage of treatment. The impact of dose level, schedule, and route of injection on overall efficacy is the subject of ongoing investigations. Nevertheless, this trend in tumor inhibition was also reflected in the overall survival of animals treated with pHLIP(WT)–MMAF. A tumor weight of 500 mg was prospectively set as a stopping point for this study. As depicted in Figure 3B treatment of mice with pHLIP(WT)–MMAF increased rates of overall survival as compared to the vehicle-treated cohort. At the end of the three-week study, four animals in the control cohort had been removed from study due to exceeding the 500 mg tumor weight cutoff. This is in contrast to pHLIP(WT)–MMAF where only a single animal had a tumor size that required its removal from study. Noteworthy, the efficacy of pHLIP(WT)–MMAF does not appear to be due to chemical instability of the conjugate, as no degradation products are observed by HPLC or mass spectroscopy after incubation in mouse serum (Figure S6), which is consistent with what we observed previously with pHLIP(WT)–MMAF.¹²

The ability of pHLIP(WT)–MMAF to elicit antitumor activity *in vivo* was also observed when NCr nu/nu mice bearing epidermoid carcinoma A431 tumors were treated using an intraperitoneal (i.p.) route of delivery. K_i -67 immunohisto-chemical staining was used as a marker of tumor cell proliferation. A range of 2016–3662 (median = 2513) cells was counted in each of four fields for three tumors from each cohort. Although able to slow cell proliferation in this model, the effect was modest and unable to slow overall tumor growth rates (data not shown). This could be due to inherent resistance of A431 cells to auristatin-based cell killing (Figure S7), inefficient dosing, route of administration, or a combination. While pHLIP(WT)–MMAF was dosed at same level through both the i.v. and i.p. routes, it is possible that i.p. injection limited the bolus effect typically observed with i.v. injection,

preventing sufficient uptake of pHLIP(WT)–MMAF in those A431 tumors and limiting its effect as measured by tumor growth volume change. However, consistent with MMAF mechanism of action and with the i.p. injection having some therapeutic efficacy, tumors from pHLIP(WT)–MMAF treated animals displayed a statistically significant ($P < 0.05$) decrease in K_i -67 positive cells as compared to those from vehicle-treated animals ($72.7 \pm 1.2\%$ vs $79.7 \pm 1.3\%$ K_i -67 positive). Finally, critical to the hypothesis that pHLIP selectively targets tumors, animals receiving either i.p. or i.v. administration of pHLIP(WT)–MMAF exhibited no overt signs of toxicities, consistent with the conjugate stability in serum over a relevant time frame for its systemic clearance rate.¹² Nevertheless, full toxicity analysis is a necessary component of any preclinical program designed to progress pHLIP–MMAF toward clinical development and is the focus of ongoing studies.

CONCLUSION

We have shown that all pHLIP–MMAF conjugates tested here display clear pH-selective and concentration-dependent toxicity against cancer cells, albeit with very different efficacy. Our results highlight that, despite their hypothesized advantages, the pHLIP variants considered so far are not as efficient as pHLIP(WT) in these particular experimental settings. However, it is possible that their respective efficacy may differ when tested against different cancer cell lines, at different pH, or in combination with other therapeutics. Possible differences in pharmacokinetics and biodistribution may also arise in animal settings. These are the focus of current investigations in our laboratory. Importantly, while the therapeutic efficacy of pHLIP(WT)–MMAF in animal setting was modest, it was nevertheless significant. In addition, no overt toxicities were observed, implying that the dosing could be increased and could potentially lead to improved efficacy over that seen here. This study therefore presents new therapeutic opportunities and supports the further development of pHLIP–MMA conjugates for cancer treatment.

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
MMAF	monomethyl auristatin F

MS	mass spectroscopy
pHLIP	pH (low) insertion peptide
POPC	1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-phosphocholine
ROI	regions of interest
RP-HPLC	reverse-phase high-performance chromatography
Trp	tryptophan

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Figure 1.

(A,B) Structure of Dolastatin 10 and some of its synthetic monomethyl auristatin derivatives: MMAE, MMAF, and MMAF-OMe. ^(a)LogP_{o/w} represents the partition coefficient between octanol/water. More hydrophobic the compound (more cell permeable), larger the LogP_{o/w}. Values were calculated using QikProp 3.0 from the structures of each compound. ^(b)Data taken from refs 25 and 32. ^(c)Data taken from ref 26. (C) Structure of MMAF modified with a pyridyldithiol-based linker and conjugation to pHLIP via disulfide exchange as well as the structure of MMAF as released intracellularly after pHLIP insertion and reduction of the disulfide bond.

Figure 2.

Inhibition of cell growth. (A–F) HeLa cells treated with pHLIP–MMAF conjugates. Blue lines represent cells treated at pH 7.4, and cells treated at low pH are shown in red. For each condition, 3000 cells/well (96-well plate) were seeded, allowed to adhere overnight, treated for 2 h, washed once with media, and incubated for 72 h in complete medium at physiologic pH. Cell viability was assessed with the MTT assay. All measurements were normalized to the media control (0 μ M, pH 7.4), as 100% cell viability. Results are shown as mean $\pm$ SEM ($n = 9$ –12).

Figure 3.

(A,B) Inhibition of tumor growth in vivo. HeLa cells were injected subcutaneously into the inguinal space of female NCr nu/nu mice. Tumors were allowed to develop until they were approximately 75 mm³ in size. On day 0, animals were randomized into cohorts with statistically similar tumor sizes; pHLIP(WT)– MMAF (76.8 ± 19.3 mm³; *n* = 8) and vehicle control (73 ± 15.6 mm³; *n* = 9) cohorts. On days 1, 3, 5, and 8 (arrows), animals were injected i.v. with 200 μL of either pHLIP(WT)– MAF (20 μM in PBS + 1% DMSO) or vehicle control (PBS + 1% DMSO). Tumors were measured with calipers routinely for approximately 3 weeks.

Table 1

Properties of pHLIP Peptides Used in This Study

pHLIP variants	sequence	pH ₅₀ ^a
WT	AAEQNPYIYARYA <u>D</u> WLFTTPLLLL <u>D</u> LALLVDADEGTCG	6.1 ^b
D25E	AAEQNPYIYARYADWLFTTPLLLL <u>E</u> LALLVDADEGTCG	6.5 ^b
P20G	AAEQNPYIYARYADWLFTT <u>G</u> LLLLDLALLVDADEGTCG	6.7 ^c
R11Q	AAEQNPYIWA <u>Q</u> YADWLFTTPLLLLDLALLVDADEGTCG	5.8 ^d
R11Q/D14Up	AAEQNPYIWA <u>QYDA</u> WLFTTPLLLLDLALLVDADEGTCG	5.6 ^d
D14Gla/D25Aad	AAEQNPYIYARYA <u>Gla</u> WLFTTPLLLL <u>Aad</u> LALLVDADEGTCG	6.8 ^b

^aMeasured in POPC unless otherwise noted.^bData taken from ref 16.^cDetermined in this article (see Supporting Information).^dData taken from ref 15.

Table 2*In Vitro* Cytotoxicity of pHLIP–MMAF Conjugates

conjugate	IC ₅₀ at pH 5.0 (nM) ^a	IC ₅₀ at pH 7.4 (nM) ^a	selectivity ^b
pHLIP(WT)–MMAF	29 ± 2.7	633 ± 84	22
pHLIP(D25E)–MMAF	368 ± 54	935 ± 14	2.5
pHLIP(P20G)–MMAF	435 ± 59	n.a.	n.a.
pHLIP(R11Q)–MMAF	471 ± 74	2487 ± 230	5.3
pHLIP(R11Q/D14Up)–MMAF	155 ± 11	2582 ± 80	16.4
pHLIP(D14Gla/D25Aad)–MMAF	83 ± 9.9	5585 ± 313	67

^aErrors are shown as standard errors.^bSelectivity represents the ratio of the IC₅₀ at pH 7.4 over the IC₅₀ at pH 5.0.