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Targeting acidity in diseased tissues: mechanism and applications of the membrane-inserting peptide, pHLIP

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Abstract

pHLIPs are a family of soluble ~36 amino acid peptides, which bind to membrane surfaces. If the environment is acidic, a pHLIP folds and inserts across the membrane to form a stable transmembrane helix, thus preferentially locating itself in acidic tissues. Since tumors and other disease tissues are acidic, pHLIPs' low-pH targeting behavior leads to applications as carriers for diagnostic and surgical imaging agents. The energy of membrane insertion can also be used to promote the insertion of modestly polar, normally cell-impermeable cargos across the cell membrane into the cytosol of targeted cells, leading to applications in tumor-targeted delivery of therapeutic molecules. We review the biochemical and biophysical basis of pHLIPs' unique properties, diagnostic and therapeutic applications, and the principles upon which translational applications are being developed.

Keywords

membrane insertion; tumor targeting; transmembrane; tumor microenvironment

Introduction

pH-Low Insertion Peptide (pHLIP): a soluble peptide with pH-dependent transmembrane activity.

Origins of pHLIPs, from bacteriorhodopsin to tumors

pH-Low Insertion Peptides (pHLIPs) are being developed for applications in several biomedical fields to exploit their specific biodistribution into tumors and other acidic tissue microenvironments, including drug delivery (1–5), *in vivo* imaging (6–17) and diagnostic

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histology (18), as well as in basic research (19–24). pHLIP peptides target acidic tissues such as tumors due to a serendipitous biophysical coincidence. The pH at which pHLIPs are activated to insert into cell membranes approximates the extracellular pH found at cell surfaces in several pathological states. Understanding the unique biophysics of pHLIPs and how the acidic microenvironments of these disease states chemically and physically affect pHLIPs has allowed us to take advantage of these interesting properties towards the development of clinical tools. The story of pHLIPs' origins and the observations that led to their discovery serves as an example of how funding of basic biophysical research can lead to important translational innovations.

In the mid-1990s, research on membrane protein folding had led to the idea that many transmembrane helices ought to be independently stable across bilayers (25). A test of this idea using peptides corresponding to each of the seven transmembrane helices of bacteriorhodopsin identified a single helix (the C-helix) that failed to form a transmembrane helix under typical assay conditions, but could form a helix under acidic condition (26)s. The pH-dependent insertion of the peptide comprising bacteriorhodopsin's C-helix across liposomal membranes was initially used to examine the influence of transmembrane domain sequence on membrane insertion properties, and was followed up by biophysical study of its pH dependent insertion activity (19). Several years after its discovery, the peptide was adapted for the translocation of cell impermeable cargos into cells in culture, including fluorescent small molecules, peptide nucleic acids and the phalloidin toxin, establishing a possible role in the intracellular delivery of therapeutic agents (1). These studies soon led to the prediction that pHLIPs may selectively target acidic tumors *in vivo*, and then to the first demonstration of that activity in tumor-bearing mice (6).

Following these initial studies, pHLIP peptides are now being adapted for numerous clinical applications in cancer biology as well as other acidic pathologies. Their ability to contribute to diagnostic or therapeutic tasks and our ability to adapt them for these functions depends upon our understanding the basic biochemical and biophysical principles that impart the unique traits of pHLIP peptides.

The biochemical basis of pH-dependent transmembrane activity

pHLIPs have the unusual property of being stable as monomers in three different environments: in aqueous solution, across the membrane bilayer and at the interface between them. Partitioning among the three states is a biased equilibrium, with the bias shifted in response to pH. Low pH favors the transmembrane state. The biochemistry of the pH-dependent membrane insertion activity was found to be based on two aspartic acid residues present in the transmembrane domain (Fig. 1), which at physiological pH are negatively charged and therefore pose an energy barrier to their insertion into the hydrophobic core of a membrane bilayer. However at low pH acidic groups become protonated and less polar, making the transmembrane conformation energetically favorable (19).

pHLIPs can be described as exhibiting a three-state activity in biological environments as revealed by fluorescence and circular dichroism measurements *in vitro* (19, 27). pHLIP peptides are soluble in aqueous solutions (State I), and remain monomeric at low micromolar concentrations or exist as soluble multimers at higher micromolar

concentrations (27–30). Due to their hydrophobic transmembrane character, pHLIPs have a high affinity for lipids (31). As a result, pHLIPs tend to reversibly interact with membranes and cell surfaces at neutral and basic pH. In State II, pHLIPs remain predominantly unstructured, bound to the outer leaflet of the membrane. In acidic conditions, where the transmembrane aspartic acids become protonated, pHLIPs rapidly become helically structured and the C-terminus inserts across the membrane as the transient binding interaction of State II transitions to stable insertion as a transmembrane α-helix in State III (Fig. 2).

Biochemical and Biophysical Characteristics

Sequence determinants of the pK of insertion

pHLIPs' pH-dependent insertion activity is imparted by the presence of the titratable acidic residues interrupting their transmembrane domains. The pH at which 50% of peptides are inserted in State III (pK of insertion, pK_{ins}) for the original bacteriorhodopsin C-helix, referred to as wild-type pHLIP (WT), has been found to be ~6.0 (19). The pK_{ins} values of pHLIPs are notably higher than the pK_A of aspartic acids in solution (~4) (32). These differences are not surprising, since pK_A values are influenced by multiple factors, including the dielectric constant of the medium. Since the dielectric constant near the surface of the membrane is lower than the water surrounding it, interactions with the membrane likely raise the pK_A of pHLIPs (33, 34), as is evidenced by variations to the position of the acidic groups. When the first transmembrane acidic residue, aspartic acid-14, is shifted to position 13, where it is expected to have increased water exposure at State III, the pK_{ins} lowers to 5.5 (30), supporting the hypothesis that the dielectric constant of the medium contributes to raising the pK_A of pHLIPs' acidic side chains. Variations to the WT sequence were investigated in an effort to control pHLIP insertion properties. Replacing either of the transmembrane domain aspartic acids with glutamic acid produced pHLIPs with the higher pK_{ins} = ~6.5 (7, 29), coinciding with the higher pK_A of the glutamic acid side chain, due to the electron donor activity of the extra methylene ($-CH_2-$) group in the side chain in comparison with the aspartic acid side chain (32). Similar modulations to the acidity of these carboxylic acids in the transmembrane domain may be capable of further tuning the pH profile of pHLIP peptide insertion.

It is also interesting to note that the proline at position 20, midway through the transmembrane region, is essential for the pH dependent insertion activity of pHLIP. The proline-20 to glycine variant exhibited α-helical structure in both State II and State III, leading to promiscuous insertion activity at a broad pH range, with a far lower apparent cooperativity of insertion than WT pHLIP (35). Proline, known commonly to destabilize helices due to its conformational constraints, is likely to limit the α-helicity of pHLIP peptides in States I and II, maintaining a high energy barrier to membrane insertion until protonation of the transmembrane acidic residues promotes helix formation during the State II to State III transition.

These three positions, aspartic acids 14 and 25, and proline 20, have been found to be important to pHLIPs' pH-dependent activity, which is further emphasized by the loss of three-state activity when these positions are varied to non-similar residues (30, 36, 37). Loss

of the pHLIP properties includes a loss of solubility in some cases, as well as losses of pH dependent insertion or conformational states.

pHLIPs in perspective with other membrane inserting peptides and proteins

pH-dependent membrane insertion is not a property restricted to pHLIPs. Several examples of proteins with such behavior are found in nature. Examples include the diphtheria toxin (38–40), colicin E1 (41–43), the apoptotic repressor Bcl-xL (44), annexin 12 (45, 46) and viral envelope proteins such as the influenza HA (47, 48). The membrane insertion of each of these proteins is a complex process that in some cases requires active contributions from the cell. For example, the pH-dependent membrane insertion of diphtheria toxin requires first the endocytosis of the protein followed by enzymatic cleavage in the endosome, and finally the processed fragment penetrates the endosomal membrane in response to the acidification of the maturing endosome. Similarly, the influenza hemagglutinin responds to endosomal acidity by a membrane insertion event, but the inserting peptide is sequestered in a larger protein and not in solution prior to the insertion.

By comparison, pHLIP peptides follow a simple path to cross the cell membrane. pHLIP insertion also relies only on physical properties of the membrane and not on active processes. So far as is known, pHLIPs are monomers in each of the three states at low concentration (27), and the equilibration mechanisms do not require other proteins or oligomeric interactions. In many other peptides the insertion events are cooperative and/or require insertion machinery. The lack of any requirement for other proteins is supported by the pure systems used *in vitro*, and *in vivo* by the fact that D-amino acid pHLIP peptides act similarly to L-amino acid peptides, which would not be expected if any specific interactions were involved (6). Because of its relative simplicity, studying the insertion mechanism of pHLIPs may be a useful approach to understanding the underlying principles behind the pH-dependent membrane insertion of natural proteins.

The “cell penetrating peptides” (CPPs), such as the HIV peptide, Tat, the *drosophila* derived penetratin, and the wasp venom peptide, mastoparan, are similar to pHLIPs in that they are short peptides, often isolated from larger proteins, that spontaneously translocate across membrane bilayers and have been used as carriers to deliver cargoes across the membrane barrier (49, 50). Unlike the predominantly acidic pHLIPs, CPPs are rich in the basic residues arginine and lysine, which bear positive charge at physiological pH. Since the outer surface of biological membranes tends to be predominantly negatively charged, CPPs have electrostatic attraction to the membrane surface. While their mechanism of entry remains unclear, there is evidence that CPPs may disrupt the integrity of the bilayer, creating leaks in the membrane (51). By contrast, the insertion of pHLIPs across the membrane produces a known and well-characterized structure, the transmembrane α-helix, which does not produce any leakage across the membrane barrier (52).

Another category of proteins that bear a resemblance to pHLIP are the C-tail-anchored proteins (TA). TA proteins contain a C-terminal transmembrane domain of 25 to 30 residues, which imparts the uncommon ability to post-translationally insert into membranes without the assistance of the translocon (53–55). The membrane insertion occurs at the C-terminal domain, which, similarly to pHLIPs, is not overly hydrophobic, allowing TA

proteins to stably partition into both aqueous and membrane environments. However, this activity is not pH-dependent, and so lacks the essential properties exhibited by pHLIPs.

Sequence determinants of insertion kinetics

pHLIP is one of very few examples where the energetics of protein folding in a membrane has been measured and one of even fewer where kinetic analysis has been possible (56–63). The topology of pHLIP insertion recapitulates the native orientation of pHLIPs' biological source, the bacteriorhodopsin C-helix (64). During the State II to State III transition, the C-terminus inserts across the membrane (1, 26). Thus the composition of the C-terminus has a large influence on the kinetics of pHLIP insertion. Variations in its size, charge, and polarity have been investigated in an attempt to tune the insertion properties for specific applications. In the WT pHLIP sequence the C-terminus is long enough to insert completely across the membrane, into the lumen of a vesicle or the cytosol of a cell. The inserting end of the WT sequence contains multiple acidic groups (-DADEGT-C_{term}). WT pHLIP therefore bears a significant obstacle to insertion, as the large, charged C-terminus must either be pulled through while charged or, much more likely, be protonated before insertion. Once inside the neutrally charged cytosol of a cell, the C-terminus is likely to deprotonate and again become charged, thus the C-terminus may have an additional effect after insertion, potentially acting as a charged anchor to resist reversal to State II. By truncating the inserting C-terminus of pHLIPs to reduce or eliminate its charge potential, the kinetics of the State II to State III transition increase dramatically, but with the corollary effect of losing retention time in State III in acidic environments and faster signal clearance due to the loss of the charged intracellular anchor (Table 1). In concert with modifications to the transmembrane sequence, such truncated pHLIP variants have been optimized for specific clinical applications (15, 65, 66).

Kinetic studies of pHLIP insertion using fluorescence and circular dichroism indicate that multiple intermediate states exist in the folding of pHLIPs into the membrane to become a transmembrane helix, but that they vary with sequence features in the flanking regions (37, 67, 68). These measurements also indicate that exit from the membrane occurs by a different path than insertion. The leading hypothesis for what governs the rate of the intermediate steps in pHLIP insertion is the sequential protonation of acidic groups (68). While the exit of pHLIPs from the membrane has been shown to occur more rapidly and with fewer steps, experimental approaches for measuring exit are flawed in that the inserted C-terminus is exposed to the same conditions as the N-terminus, which does not accurately represent conditions when pHLIPs are inserted into cell membranes. These experimental data are therefore useful for understanding pHLIPs as a model for protein folding in membranes but partly fail to give insight into biological applications of pHLIPs. Experiments using low to intermediate pH transitions, where the inserted C-termini are expected to partially deprotonate and the liposomal pH equilibrates, has allowed some investigation into the exit of pHLIPs in physiological conditions. These observations indicate that deprotonated acidic groups in the C-terminus of pHLIP are likely to drastically slow the exit of inserted pHLIPs from biological membranes (65).

The transmembrane sequence is of obvious significance to pHLIPs' insertion activity. The protonation of the acidic groups driving pHLIP insertion is a cooperative process, as is the coil to helix transition. As a consequence, the transition from State II to State III occurs over a pH range of ~1.5 to 2 units (69, 70). Interestingly, the slope of the insertion curve varies linearly with the number of acidic groups that insert in the membrane. Since the curve slope is related to the cooperativity of the insertion/folding process, this indicates that the different acidic groups are protonated in a concerted fashion (70).

The WT transmembrane sequence also contains a long region rich in hydrophobic residues from leucine-21 through valine-30 that is divided by aspartic acid-25, which is largely responsible for the pH-dependence of the transmembrane state. In acidic conditions, when the aspartic acids are protonated, this region has the propensity to form α-helical secondary structure. While it is interrupted by the charged state of aspartic acid in neutral or basic conditions, this hydrophobic stretch is thought to be responsible for reversible aggregation and precipitation of pHLIPs at high micromolar concentrations in acidic conditions where the aspartic acid is uncharged (30). In State II WT pHLIP remains predominantly unstructured at physiological pH, but forms α-helical structure rapidly on the membrane surface when the pH is acidic, as a step in the insertion process. Thus, unlike other membrane-active proteins, the stabilization of secondary structure in pHLIPs is a pH-dependent process, rather than a result of membrane binding. This hydrophobic region, existing within a molecule that can exist in both aqueous and membrane environments, contributes to pHLIPs' affinity for the membrane surface in State II. Membrane binding in State II has significant ramifications in physiological environments, as discussed below, and so pHLIP variants containing more polar transmembrane regions have been developed which maintain pH-dependent membrane insertion to State III, while only transiently interacting with the membrane surface in State II (15).

Influence of lipid composition on pHLIP insertion

The membrane insertion of pHLIP is also influenced by the physical properties of the bilayer. While the pHLIP variant experiments established the importance of peptide sequence features in membrane insertion activity, the lipidic environment was held constant. POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) is often used to prepare liposomes, and was chosen for pHLIP-insertion experiments as this lipid has net neutral surface charge, contains unsaturated acyl chains and readily form bilayers of similar thickness to biological membranes. In order to gain an understanding of pHLIP insertion activities, this neutral background was initially used; however, the influence of lipid headgroup composition on the insertion events may also be significant.

Liposomes can be prepared with varied compositions in order to impart different physical characteristics, including size, chemical diversity, surface charge and bilayer fluidity. pHLIP binding and insertion have been tested in multiple liposome backgrounds to investigate the influence of lipids on their insertion activity. In State II, pHLIPs produce increased α-helical structure when interacting with liposomes composed of lipids with long acyl-chains, whereas no change is observed in helicity on liposomes with varied cholesterol concentration. A possible explanation is that these two formulations differ in their

membrane's elastic bending modulus. While the longer acyl-chain liposomes exhibit an increased stiffness, cholesterol concentration has no effect on the bending modulus of monounsaturated membranes (71, 72). A likely conclusion, therefore, is that the elastic properties of the bilayer influence helix formation on the membrane surface (35). Cell experiments suggest that membrane fluidity is more determinant that membrane thickness in controlling pHLIP's pK_{ins}. These insights reveal that membrane composition can significantly affect pHLIP insertion activity, so it is important that biologically relevant target membrane compositions be investigated to rationally tune pHLIP insertion properties for target membrane interaction. For example, it has been found that solid tumors frequently possess increased fluidity in their membranes (73–78). Increased membrane fluidity likely contributes to tumor invasiveness and might also contribute to pHLIPs' propensity for tumor accumulation *in vivo* (15). This suggests that the influence of membrane fluidity on the stability of membrane proteins may play a role in tumor pathology, and warrants further investigation.

Biophysical studies of pHLIP insertion into membranes have predominantly used liposomes as an experimental membrane system. In these studies virtually all the lipids used to form liposomes have been neutral in charge. While this eliminates variables in the experimental system it also fails to accurately represent significant physical traits present in biological membranes. Lipid headgroup charge and lateral pressure profile could modulate the both States II and III of pHLIP.

It has been hypothesized that in State II interactions, pHLIP peptides binding to the membrane surface disrupt the ordered packing of lipids by displacing lipid headgroups in order to bury their hydrophobic side chains in the hydrophobic core of the membrane. The reordering of these lipids in State III may account in part for the energetic favorability of State III following the protonation of the transmembrane acidic groups (1, 31, 52, 65) (Fig. 2). Given the potential influence of charge and fluidity on these factors, they are the subject of ongoing investigations.

Summary of the effects of pHLIP sequence variation on targeting properties

- *Truncated C-terminus* – Accelerates the kinetics of the State II to State III transition, but have the potential to decrease State III stability
- *Polar transmembrane variations* - Bias State II vs. State I equilibrium towards State I, but have the potential to decrease dose efficiency
- *D to E variations in the transmembrane domain* - Increase the pK_{ins}, but have the potential to decrease specificity, *in vivo*

Biological Targeting Activity

Biological systems must maintain strictly controlled chemical environments for the essential reactions of life. The human body maintains a narrow range of both intracellular and extracellular pH's for this reason, with the predominant extracellular environment at around pH 7.4. However, several pathological states are known to significantly disrupt this pH balance, such as cancer, ischemia and inflammation.

Tumors exhibit a set of metabolic and physical traits that produce an acidic extracellular environment. Since pHLIPs insert at low pH, it was hypothesized that pHLIPs would accumulate in tumors *in vivo*. The pH range at which pHLIPs anchor into cells and thus potentially accumulate in tissues is known to be around pH 6.0 to 6.5, depending on the pHLIP's sequence. Tumors become hypoxic as they outgrow their blood supply. Further, as they avidly consume glucose, tumors exhibit the Warburg effect, overwhelming their mitochondrial capacity and shifting towards glycolytic metabolism even when oxygen is present. These metabolic shifts result in heightened production of anaerobic metabolites, such as lactic acid, which accumulate in the surrounding tissue as a result of insufficient blood clearance from the outgrown blood supply. As the tumor cells engage in rapid metabolism, they produce elevated levels of CO_2, and express carbonic anhydrases at their surfaces to clear the waste, still further acidifying the extracellular space. Because of the transmembrane potential, protons accumulate near the cell surface, and the local pH at cell surfaces is even lower than in the bulk. These conditions combine to produce a bulk extracellular pH in tumors of around 6.5 (9, 79, 80), and a lower pH at the cell surfaces, where pHLIPs bind. Given the close correspondence of the pH of the tumor microenvironment to pHLIPs' pK_{ins}, pHLIPs preferentially accumulate in tumors relative to healthy tissues, which generally exhibit local environments of pH 7.4. Fluorescently labeled pHLIPs have so far been shown in eleven published studies to accumulate in tumors with a high degree of contrast to healthy tissues (6–12, 15–18).

pHLIPs have also been found to target several other acidic conditions *in vivo*. For example, ischemic tissues produce acidic extracellular environments due to hypoxia and the buildup of metabolic byproducts. While the pH produced in ischemic tissues is not as low as for tumors, it has been shown to be sufficiently acidic to cause the accumulation of pHLIPs in ischemic heart muscle (14). Also, certain inflammatory responses have been shown to produce an abnormally acidic extracellular environment. In a rat model of rheumatoid arthritis localized to the knee, acidification due to inflammatory immune cell infiltration was shown to accumulate pHLIPs specifically around the knee joint after intraperitoneal injection of fluorescent pHLIPs (6). Similarly, at sites of influenza virus infection in mouse lung pHLIPs accumulated in sites with increased levels of immune cell infiltration (13). These studies indicate that immune inflammation also produces sufficiently acidic environments for pHLIP targeting *in vivo*.

Certain healthy physiological processes also produce sufficiently low pH to trigger pHLIP accumulation. For example, the keratinized skin produces an acidic surface mantle of below pH 6 as part of its role as a microbial and environmental barrier (81). It is hypothesized that this effect contributes to an occasionally elevated background signal observed in healthy skin during imaging experiments *in vivo* (8). However, the primary acidic physiological process targeted by pHLIP occurs in the kidney, where blood pH balance is maintained by the transport, exchange and excretion of protons and bicarbonate ions in the urine.

In many experiments pHLIP appears to accumulate in the kidneys with comparable intensity to the targeted tumors. The factors influencing this biodistribution are likely two-fold. pHLIP peptides are eliminated from the body through renal filtration and excretion in the urine. During experiments involving the injection of fluorescently labeled pHLIPs *in vivo*,

collected urine has been observed to produce a high degree of pHLIP-specific fluorescence. It is therefore unsurprising that pHLIPs' biodistribution is high in the kidneys, as it has been found that many diagnostic and therapeutic agents biodistribute most strongly to their organ of primary clearance (82). Also, the activity of the kidney in blood pH-balance acidifies the luminal environment when excess acidity in the blood must be eliminated *via* proton excretion in urine. The acidification of the urine may account for accumulation of pHLIPs in the kidney following filtration. Fortuitously, WT pHLIP accumulation in both physiological targets and diseased tissues has been found to be completely non-toxic and non-immunogenic (1) making pHLIPs an excellent candidate for use as a diagnostic or therapeutic delivery platform.

Imaging Applications

The first demonstrations of tumor targeting *in vivo* were performed using pHLIPs modified at their N-termini with near-infrared fluorescent cyanine and Alexa Fluor dyes. The ability of near-infrared light to penetrate tissues allowed these pHLIP-conjugated dyes to be used to produce images of subcutaneous tumors in mice. These probes have been used extensively over the last decade to characterize the targeting activity of pHLIPs in several pathological model systems (Table 2). These experiments have revealed several features relevant to the use of pHLIPs as a diagnostic imaging platform.

Clinical imaging requires several basic conditions. First, effective imaging requires the development of sufficient contrast between targeted and untargeted tissues to reliably differentiate the signal from the background. Second, the applied imaging modality must be capable of penetrating tissues at a depth equal to the depth of the targeted pathology. In the human body, this may require penetrating tens of centimeters of tissue. For this reason radiotracers and magnetic resonance imaging modalities are frequently used in clinical imaging, as the body's thickness does not significantly alter their transmission. Third, the timeframe during which imaging will occur must coincide both with the clinically available time window and with the useful activity of the radiotracer or contrast agent applied. Given the short half-lives (minutes to hours) of the isotopes used in PET and SPECT, imaging must be within minutes to a few hours of administration. Finally, imaging probes must be biocompatible and minimally toxic in order to be safely used in the body.

Imaging contrast

In experiments imaging tumors in mice, basic fluorescent probes have produced a typical fluorescence contrast ratio between tumors and background muscle tissues of around 5 to 1. New tools are being developed that lower the non-inserted background, such as pHLIP-FIRE, which unquenches its fluorescent label only after insertion by the reduction of a disulfide bond to release a quenching agent. Using this approach *in vivo*, the background signal normally associated with the slow to clear State II pHLIPs can be greatly reduced, giving significant improvements in overall signal contrast (83). This contrast is coupled with high resolution, as metastatic lesions of smaller than 1 mm in diameter have been identified in mouse studies (10). In tumor imaging, the resolution and contrast ratios observed should be sufficient for pHLIP to serve as an effective diagnostic tool.

Imaging modalities

pHLIPs' initial testing with fluorescent agents established a technique that could be used in small animal models to visualize acidic tissues *in vivo*. However, visible and near-infrared light is unable to penetrate thick tissues. The use of fluorescent probes in the clinic must therefore be relegated to superficial imaging tasks, such as in the skin, where pHLIP-targeted Alexa Fluors have been used to effectively label melanomas *in vivo* (unpublished data), or as a guide during surgeries to aid in the identification of tumor borders (8). In order to use pHLIP-targeted imaging in larger bodies and for deeper targets, PET and SPECT tracers have been adapted for pHLIP delivery (9, 11, 84). Experiments using chelated radionuclides covalently bound to the N-termini of pHLIPs have shown delivery of these clinically useful cargoes to tumors (9, 11, 12, 85); however, maximal contrast was not achieved, due to the short half-lives of the isotopes and the slow clearance of pHLIPs *in vivo*. Improved imaging was enabled by using pHLIP sequences that clear more rapidly, and these are under study (see below).

Impact of lipid interactions on clearance

The affinity of pHLIPs for membrane surfaces in State II produces a long retention time of pHLIPs and pHLIP-conjugated cargos *in vivo*. The primary route of pHLIP clearance *in vivo* is by renal filtration. As a result, pHLIPs clearance is likely to be primarily *via* pHLIPs in solution in State I. Given the high affinity of pHLIPs for membranes and given the ubiquitous presence of membrane surfaces *in vivo*, the clearance process necessarily occurs slowly. This feature of pHLIPs is beneficial for certain applications, such as therapeutics delivery, but it is a problem in short-lived imaging modalities. C-terminal truncated variant pHLIPs with polar variations to the transmembrane domain show significantly more rapid clearance and have better compatibility with PET and SPECT imaging: however, this comes at the cost of stability in State III, which also clears more rapidly, lowering the net target signal (14, 15).

During fluorescence imaging experiments it was determined that, while peak tumor signal occurs at around 4 to 5 hours after administration, optimum tumor-targeted contrast is achieved when using pHLIPs with acidic C-termini, such as WT pHLIP, at around 24 to 48 hours after injection. To accommodate this timeframe, radionuclides with longer half-lives, such as ^{111}In ($t_{1/2}$ = 2.8 days), have been used (Jason Lewis, unpublished correspondence). In order to accommodate the use of more clinically suitable probes, a method was developed involving the pre-targeting of pHLIPs to tumors *in vivo* bearing on their N-termini the reactive moiety, trans-cyclooctene. After injection, time was allowed for background clearance followed by the injection of tetrazine-decorated liposomes loaded with the PET tracer ^{18}F, which were designed to covalently anchor to pHLIP-decorated tumors by the Diels-Alder cycloaddition of tetrazine to trans-cyclooctene (12).

While these approaches have opened the door to the potential application of pHLIP-targeted PET or SPECT imaging as a diagnostic tool, additional improvements must be made before this technology is able to reach the clinic. Current work is aimed at rapid clearance of imaging agents in States I and II. For these applications a variant of pHLIP with a truncated C-terminus as well as an altered and foreshortened transmembrane sequence has been

developed. Referred to as Variant 7, this pHLIP maintains excellent tumor targeting properties, but has much faster blood clearance. This allows optimal contrast to be achieved far earlier after administration, which is convenient for imaging applications, however also leads to correspondingly rapid loss of signal from the tumor. Variant 7 is now being investigated for possible applications in diagnostic imaging (5, 15, 85, 86).

Therapeutic Applications

pHLIPs are capable of translocating polar, normally membrane-impermeable molecular cargoes across the cell membrane by virtue of the energetic favorability of pHLIPs' formation of a transmembrane α-helix in acidic extracellular environments. pHLIPs' unique membrane insertion properties set this family of peptides apart from their most functionally similar analogues, the cell-penetrating peptides. While many cell-associated targeting approaches rely on endocytic uptake to enter cells, these peptides can additionally cross cell membranes without active transport. Traditional cell-penetrating peptides, such as Tat and Penetratin are thought to deliver cargoes that would normally be repelled by the cell membrane by disrupting the membrane itself (88). This sort of disruption may cause destructive effects in cells, such as membrane depolarization, ion gradient disruption, and can cause cell death (50). Importantly, delivery *via* cell-penetrating peptides occurs indiscriminately, with little to no targeting bias. The pHLIPs improve upon these properties significantly by nondestructively inserting into cell membranes and by accumulating selectively in acidic tissues, allowing a degree of target specificity.

As carriers for the delivery of therapeutic agents to tumors, pHLIP-conjugates have significant benefits over the recently booming field of antibody-drug conjugates. Antibody-drug conjugates rely on cell-surface markers specific to a subcategory of tumors in order to localize their drug cargo to the targeted tumor. The delivery of the cargo into the cell is then often dependent upon engulfment of the antibody and its associated receptor into an endosome, where the covalent linkage between antibody and drug can be cleaved by the highly acidic and catabolic conditions of the maturing endosome or its eventual target, the lysosome. The drug must then survive this environment to penetrate the endosomal membrane and finally enter the cytosolic compartment of the targeted cell. pHLIP improves upon this approach first by targeting tumors *via* a physical characteristic, extracellular acidity, rather than a target with heterogeneous expression, such as a cell-surface marker. While therapies targeted to cell-surface markers have made improvements over the systemic delivery of free chemotherapeutic agents, such targeting may give rise to resistant tumors by selection from cells in heterogeneous tumors. Also, pHLIPs' delivers cargos directly into the cytosolic compartment of the cell, avoiding exposure to destructive lysosomal conditions.

In order to insert into tumor cells and release cargoes into the cytosolic compartment, therapeutic cargoes must be covalently conjugated to pHLIP using a reversible linker activated by conditions unique to the cytosol. Disulfide bonds between cysteine residues in the C-terminus of pHLIPs and thiols in the therapeutic cargoes have been used successfully for this purpose. Disulfide bonds provide covalent linkages that are stable in the blood, but respond to the strongly reducing environment inside the cell by cleaving and reproducing their parent thiol groups. This approach has been applied to the intracellular delivery of

large, normally cell impermeable cargoes, such as peptide nucleic acids, fluorescent dyes and the toxins phalloidin and α-amanitin (Table 3) (1–3, 89). In these studies, pHLIP-targeted delivery of phalloidin and α-amanitin caused cell death in cultured cancer cells in a pH-dependent manner, paving the way for future *in vivo* studies using pHLIP-delivered agents as tumor-targeted chemotherapies.

Certain therapeutic targets in cancer have been underutilized due to the difficulty of delivering effective agents into cancer cells. For example, oncogenic microRNAs (oncomiRs) are known to contribute to a range of cancers when overexpressed due to the ability of microRNAs to influence the expression of a wide range of genes, however effective antagonism of oncomiRs requires the delivery of antisense nucleic acids into tumor cells. Nucleic acids are large, polar polymers not suitable for drug-like administration. pHLIPs have recently been used to deliver nucleic acids with the less polar peptide backbone, or Peptide Nucleic Acids (PNAs) as antisense therapies to oncogenic microRNAs *in vivo*. This approach was used to treat a mouse model of miR-155 addicted large B-cell lymphoma, using an anti-miR-155 PNA delivered as a disulfide cargo on pHLIP. The pHLIP delivery of this large (~8000 MW) and polar cargo achieved sufficient efficiency to both reverse established tumors and inhibit the development of metastases, as well as preventing accumulation of PNAs in the liver, where free PNAs are commonly sequestered. This success is the first example of pHLIP-delivered therapy *in vivo*, and serves to demonstrate the advantages pHLIPs have in anticancer drug delivery (Cheng, et al. 2014. Nature. Accepted for publication).

Alternatively, pHLIP has been applied as a tool for anchoring therapeutic cargoes to the outside of cells, such as nanoparticles and liposomes. pHLIPs have been successfully used to anchor relatively large cargoes to the cell surface, such as nanogold particles (16, 90) and proteins. Cell-surface decoration of tumor cells with pHLIP anchored antigens has been used to recruit an acquired immune response in several model systems (Unpublished data) and several approaches are being evaluated for the use of pHLIPs to deliver antigens to tumors *in vivo*. Similarly, pHLIP-decorated liposomes have been used to deliver membrane incorporated therapeutic cargoes such as the cytotoxic signaling lipid ceramide and the pore-forming protein Gramicidin A, to cancer cells by anchoring liposomes to acidic tumor cells. In this approach, anchoring is followed by pHLIP-insertion promoted fusion of the toxin-loaded liposomal membrane with the cell membrane, incorporating the toxin into the tumor cell membrane or disgorging any liposomal contents into the cytosol. In the examples tested, both ceramide and Gramicidin A, delivered in pHLIP-targeted liposomes, produced pH-dependent apoptosis in cultured cancer cells (Table 3) (4, 5).

pHLIPs' ability to insert normally cell-impermeable cargoes across membranes has greatly expanded the range of size and polarity for agents that might be used in cancer therapy. Additionally, pHLIPs' pH-dependent control over membrane insertion could also be used to regulate the delivery of existing, cell-permeable therapeutics, such as traditional chemotherapeutic agents. For this application pHLIP has several beneficial properties. First and most importantly, the primary obstacle to effective chemotherapeutic treatment of cancers is the toxicity of the administered agent(s) to healthy tissues in the patient's body. These side effects are frequent, often severe, and in many cases can themselves be life

threatening. By imparting the low-pH dependent insertion activity of pHLIPs on these agents, most off-target delivery events should be attenuated, reducing toxicity to healthy cells while maintaining cancer toxicity, and significantly improving the therapeutic indices of the agents. Second, the cancers for which a given chemotherapeutic agent is approved are defined in large part due to the native biodistribution of the agent, limiting each agent to a small number of anatomical targets within which tumors will be exposed to a high enough fraction of the administered dose to achieve a therapeutic effect. By imparting pHLIPs' biodistribution on these agents it should be possible to expand their applications to a greater range of tumors to which the agent alone would not natively biodistribute. pHLIP-conjugation should also impart the activity of targeting metastatic legions, which pHLIPs have been shown to target *in vivo* (10). Lastly, while the slow clearance of pHLIPs in State II is a confounding factor for imaging applications, it is complementary to drug-delivery in that it enhances dose efficiency. Traditional drug administration relies on a very low percentage of the total administered agent being absorbed by the tumor in the short time window available before the vast majority of the drug is cleared from the body. Several techniques, such as encapsulation and PEGylation, have been applied to therapies in order to enhance blood retention and improve the pharmacokinetics and uptake of the drug (91, 92). pHLIPs improve this step by providing a significantly longer retention time during which the pHLIP-drug conjugate may have many more passes at delivery to the tumor, improving dose efficiency and potentially improving overall tumor accumulation.

While obstacles exist in coupling traditional chemotherapeutic agents to pHLIPs, this application opens the door to the co-development of new drug chemistries which can both take advantage of pHLIPs' ability to insert larger and more polar cargos as well as basing new agents on thiol-bearing scaffolds which will allow for simple, cytosolically reducible, reversible conjugation to pHLIPs.

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Abbreviations

CD	circular dichroism
CPP	cell-penetrating peptides
LUV	large unilamellar vesicles
pHLIP	pH-Low Insertion Peptide
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
TM	transmembrane helix
TA	C-tail-anchored proteins

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Figure 1.

WT pHLIP is a 36 amino acid peptide. The putative transmembrane domain is shown in red, and the N-and C-terminal flanking regions are shown in blue. For convenience, all references to the sequence are numbered with respect to this peptide.

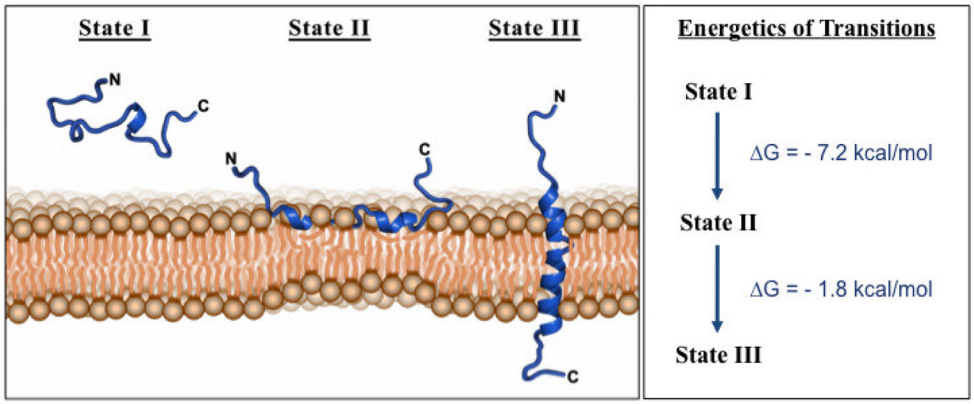

Figure 2.

pHLIP peptides exhibit three distinct states. In State I, pHLIPs are soluble and unstructured in aqueous solution. In State II, pHLIPs bind reversibly to the outer leaflet of membrane bilayers, remaining largely unstructured at physiological pH. In acidic extracellular environments a pHLIP inserts its C-terminus across the membrane to form State III, a stable transmembrane α-helix. The transition from State I to State II in the presence of lipids proceeds spontaneously with a negative Gibbs free-energy change and State II greatly predominates at equilibrium. The transition from State II to State III in acidic environments also proceeds spontaneously with a negative Gibbs free-energy change (31).

Table 1
pHLIP insertion and exit kinetics in lipid vesicles

Order of magnitude approximations are given for the characteristic times, τ (s), of the rate limiting steps in each transition. These data were collected under stopped-flow assay conditions by fluorescence and circular dichroism measurements of pHLIP interactions with liposomal membranes during transitions between high and low pH. Intermediate pH transitions reveal that both insertion and exit kinetics are dependent upon the number of protonatable groups in the inserting end of pHLIP. Inside the liposomal membrane the pH equilibrates rapidly with the pH of the outside solution, so both the inserted C-terminus and non-inserted N-terminus are exposed to the same initial conditions. It is important to note for State III to State II transitions that in physiological conditions the two termini would be exposed to different pH environments in State III. Therefore the effects of the charged C-terminal anchor in the WT peptide may have a significant impact on retention in cells that would not be observed in this experimental system. Data are adapted from (65).

	α-helix formation	State II to III (insertion)	State III to II (exit)
WT pHLIP	0.1 sec	1 – 10 sec	0.1 sec
Truncated C-term pHLIP	0.1 sec	0.1 sec	0.1 sec

Table 2

Published reports of imaging agents delivered by pHLIPs to acidic disease sites *in vivo* or *ex vivo*.

Year	Modality	Agent(s)	Model(s)	Reference
2007	Fluorescence	Cyanine 5.5 and Alexa Fluor 750	JC mouse mammary adenocarcinoma, established by *s.c.* injection in C3D2F1 mouse, and rheumatoid arthritis established by Freund's adjuvant injection in rat knee	Andreev, et al. (6)
2009	Fluorescence	Alexa Fluor 750	GFP-expressing M4A4 human melanoma, established by *s.c.* injection in athymic mouse	Andreev, et al. (7)
2009	Fluorescence	Cyanine 5.5	GFP-expressing HeLa tumor, established by *s.c.* injection in athymic mouse	Segala, et al. (8)
2009	PET	^{64}Cu-DOTA	PC-3 and LNCaP human prostate carcinomas, established by *s.c.* injection in athymic mouse	Vavere, et al. (9)
2011	Fluorescence	Cyanine 5.5 and Alexa Fluor 750	GFP-expressing HeLa tumors, M4A4 human melanomas and NM2C5 human melanomas, established by *s.c.* injection in athymic mouse. Metastatic M4A4 legions established by *i.v.* injection. TRAMP mouse prostate tumor, allowed to develop spontaneously.	Reshetnyak, et al. (10)
2012	PET	^{18}F-*o*-pyridine	PC-3 and LNCaP human prostate carcinomas, established by *s.c.* injection in athymic mouse	Daumer, et al. (84)
2012	SPECT	^{99m}Tc-AH114567	Lewis lung carcinoma, established by *s.c.* injection in C57Bl/6 mouse	Macholl, et al. (11)
2013	PET	^{18}F-liposomes	SKOV3 human ovarian adenocarcinoma, established by *s.c.* injection in athymic mouse	Emmetiere, et al. (12)
2013	Fluorescence	Alexa Fluor 647	Influenza virus infected mouse lung	Li, et al. (13)
2013	Fluorescence	Alexa Fluor 647	Human tumor biopsies	Loja, et al. (18)
2013	Fluorescence	Alexa Fluor 488 and Liposomal Rhodamine	*In situ* infarct and *ex vivo* global low-flow myocardial ischemia in mouse	Sosunov, et al. (14)
2013	Fluorescence	Alexa Fluor 750	GFP-expressing HeLa tumor, established by s.c. injection in athymic mouse	Weerakkody, et al. (15)
2013	Optical	Nanogold	GFP-expressing HeLa tumor, established by s.c. injection in athymic mouse	Yao, et al. (16)
2014	Fluorescence	Alexa Fluor 488 Alexa Fluor 546 Alexa Fluor 647	4T1 mouse mammary tumors, established by *s.c.* injection in BALB/c mouse and transgenic mouse mammary tumors	Adochite, et al. (86)
2014	Fluorescence	Alexa Fluor 546 and Alexa Fluor 647	Luciferase-labeled Capan-2 human pancreatic ductal adenocarcinoma, established by injection into mouse pancreas	Cruz-Monserratte, et al. (17)
2014	PET	^{68}Ga-DOTA and ^{64}Cu-NOTA	PC-3 and LNCaP human prostate carcinomas, established by *s.c.* injection in athymic mouse	Viola-Villegas, et al. (85)
2014	Fluorescence	Alexa Fluor 647	Human tumor biopsies	Luo, et al. (87)
2014	Fluorescence	TAMRA	4T1 mouse mammary tumors, established by *s.c.* injection in BALB/c mouse	Karabadzhak, et al. (83)

Table 3

Intracellular delivery of normally cell impermeable cargos by pHLIPs.

Year	Agent(s)	Model(s)	Reference
2006	12-mer Peptide Nucleic Acid, Dansyl, and phalloidin-TRITC	*In vitro* cultures of HeLa, TRAMP mouse prostate adenocarcinoma, and JC mouse mammary adenocarcinoma.	Reshetnyak, et al. (1)
2010	Rhodamine and aminophalloidin	*In vitro* cultures of HeLa, JC mouse mammary adenocarcinoma, and M4A4 human melanoma cells	An, et al. (2)
2011	Phallacidin	*In vitro* cultures of HeLa cells.	Wihesinghe, et al. (89)
2013	α-amanitin	*In vitro* cultures of HeLa, M4A4 human melanoma, U2OS human osteosarcoma, and MDA-MB-231 human breast adenocarcinoma cells	Moshnikova, et al. (3)
2013	Liposomal ceramide and liposomal propidium iodide	*In vitro* cultures of HeLa, and A549 human lung carcinoma cells	Yao, et al. (4)
2013	Liposomal Gramicidin A *	*In vitro* cultures of HeLa, M4A4 human melanoma, and A549 human lung carcinoma cells	Wijesinghe, et al. (5)
2014	Anti-miR-155 PNA	Mouse model of miR-155 addicted large B-cell lymphoma	Cheng, et al. (Nature, accepted)

* Gramicidin A delivery by transfer into cell membrane, not intracellular.