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Applications of pHLIP Technology for Cancer Imaging and Therapy

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Abstract

Acidity is a biomarker of cancer that is not subject to the blunting clonal selection effects that reduce the efficacy of other biomarker technologies, like antibody targeting. The pH (Low) Insertion Peptides (pHLIPs) provide new opportunities for targeting acidic tissues. Through the physical mechanism of membrane-associated folding, pHLIPs are triggered by the acidic microenvironment to insert and span the membranes of tumor cells. The pHLIP platform can be applied to imaging acidic tissues, delivering cell-permeable and impermeable molecules to the cytoplasm, and promoting the cellular uptake of nanoparticles. Since acidosis is a hallmark of tumor development, progression, and aggressiveness, the pHLIP technology may prove useful in targeting cancer cells and metastases for tumor diagnosis, imaging, and therapy.

Keywords

Acidity; tumor targeting; imaging; drug delivery; nanotechnology

Extracellular Acidity

Acidity is a hallmark and predictor of tumor development and progression

Even in the presence of oxygen, cancer cells use the anaerobic pathway more than normal cells do, a phenomenon known as the Warburg effect [1–3]. Normally, cell metabolism occurs through a low rate of glycolysis followed by oxidation of pyruvate in the mitochondria, whereas anaerobic metabolism is associated with a high rate of glycolysis and

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lactic acid fermentation [4]. Protons are a byproduct of both pathways; however, when cells use glycolysis and lactic acid fermentation at a high rate, excess lactate and protons are produced. In order to maintain homeostasis, a cell must keep its intracellular pH at the physiological level, around pH 7.2–7.4. If the pH inside the cell drops, the cell will pump excess acidity into the extracellular space to maintain physiological pH levels in the cytoplasm. Additionally, cancer cells overexpress surface carbonic anhydrases (CAIX and CAXII), which catalyze the transformation of carbon dioxide and water into carbonic acid (bicarbonate and protons) [5–7]. Thus, excessive amounts of acid and protons accumulate in the extracellular space, especially in poorly perfused tumor regions, reducing the pH of the extracellular space and leading to tumor acidification [8, 9].

Not only is acidity a hallmark of tumor development, but acidity also facilitates tumor growth [10]. The acidification of the healthy tissue surrounding a tumor leads to tissue remodeling that allows for local invasion. Studies in which the pH of the surface of tumors was monitored showed that the regions of the highest **tumor invasion** (see Glossary) corresponded to areas of highest acidity [10]. Therefore, targeting the most acidic regions of tumors is especially beneficial because the most acidic regions are the most aggressive [11].

Bulk extracellular pH versus cancer cell surface pH

Recent studies using a fluorophore with a pH-dependent emission spectrum have shown that the pH in the vicinity of the plasma membrane of cancer cells is about 0.3–0.7 pH units lower than the bulk extracellular pH [12]. In experiments *in vitro*, where the pH of the solution in which the cancer cells are growing (bulk pH) is maintained at pH 7.4, the cell surface pH for highly metastatic cells has been shown to be around pH 6.7. In three-dimensional tumor models, *ex vivo* mouse tumor tissue, and live animals, the cancer cell surface pH has been shown to be as low as pH 6.0. Thus, cancer cells have a “crown of acidity” near their cell surfaces. The pH becomes less acidic with distance from the cell surface and, therefore, the bulk extracellular pH can be relatively high, especially in well-perfused regions. However, the cell surface pH always remains low (i.e., acidic). The bulk extracellular pH correlates with perfusion, while the cell surface pH is expected to be less dependent on tumor tissue perfusion, and to be a predictive marker of tumor development and progression, since more aggressive tumor cells are more acidic.

pHLIP Technology

Peptides of the pHLIP family and their mechanism of action

The pH (Low) Insertion Peptide (**pHLIP**®) was derived from the C-helix of the protein bacteriorhodopsin, and originally was called the BRC peptide [13]. The salient feature of a pHLIP is its ability to sense the pH in the vicinity of the plasma membrane and to spontaneously form a helix and insert across the membrane when the extracellular environment is acidic (Figure 1) [14, 15]. Numerous modifications have been made to the primary sequence of pHLIPs to evaluate and tune the properties of the interaction of the pHLIP with the cell membrane [16]. These modifications include testing a pHLIP consisting entirely of D-amino acids against one containing entirely L-amino acids (no change was observed) [17]; truncating and reversing the wild-type (WT) pHLIP sequence, and in so

doing introducing new pHLIP variants [18, 19]; swapping some or all aspartic acid residues for glutamic acid residues [16, 20, 21], positively-charged lysine residues [18, 22–30], or the protonatable **non-standard amino acids** such as γ -carboxyglutamic acid and α -aminoadipic acid [31]; and the *de novo* design of a pHLIP variant [32].

Variation of the WT pHLIP sequence led to novel pHLIPs, such as Variant 3 (Var3), with significantly improved tumor targeting properties [18, 28, 33–35]. The overall features of the pHLIP peptide sequences are still present in all variants: a middle region interspersed with a combination of hydrophobic residues and residues that are negatively charged at physiological pH but become neutrally charged at low pH, and hydrophilic flanking regions, with the membrane-inserting C-terminus (in most sequences) containing a few additional protonatable residues (Box 1) [9, 36–38]. Var3, in particular, has a truncated membrane-inserting end, which leads to its faster partitioning into the cell membrane to form a transmembrane helix. This variant exhibits the highest difference between the Gibbs free energies of its interaction with the membrane at low and high pHs, which ensures pH-dependent preferential targeting of the cancer cells [18].

Box 1

Peptides of the pHLIP family share the same features in their primary sequences (Figure I) and exhibit the same mechanism of action (Figure 2). These shared characteristics include: i) an N-terminal region (flanking sequence 1) that varies from 3 to 20 residues and consists mainly of polar amino acids that contribute to the overall solubility of the peptide and are used for conjugation with cargo destined for the extracellular space; ii) a middle region (transmembrane sequence) that varies from 15 to 25 residues and consists mainly of hydrophobic residues, but also includes amino acids that are negatively charged at physiological pH but become neutrally charged at low pH due to protonation; and iii) a C-terminal region (flanking sequence 2) that varies from 0 to 10 residues and may contain a few additional protonatable residues, as well as residues for conjugation with cargo that will be delivered across the cellular membrane to the cytoplasm. Single cysteine and/or lysine residues can be used for conjugation with cargo molecules and can be introduced into one or both flanking sequences.

These features are reflected in the sequences of the wild-type (WT) and the Variant 3 (Var3) pHLIPs, seen below, where protonatable residues are shown in bold and the putative transmembrane region is underlined:

WT: AEQNPIY WARYADWLFTTPLLLLDLALLVDAD EGT

Var3: ADDQN PWRAYLDLLFPTDTLLLDLLW

Figure I. The main features of sequences of the pHLIP peptide family

Members of the peptide of pHLIP family follow a similar pattern in terms of peptide sequence: an N-terminal flanking region (yellow) consists mainly of polar residues; a transmembrane region (red) consists mainly of hydrophobic residues, but also includes

protonatable residues which prevent insertion at physiological pH; and a C-terminal flanking region (green), which may not present in all peptides, contains a few additional protonatable residues. Cargo can be conjugated either to the C- or N-terminus via single cysteine or lysine residues.

The mechanism of action of all peptides in the pHLIP family is well understood: membrane-associated folding is triggered by the protonation and increase in hydrophobicity of the peptide when exposed to acidic environments (Figure 2) [15, 19, 22, 39, 40]. At physiological pH, a population of pHLIPs mainly exists as an equilibrium between two states: a solvated state in which the pHLIP adopts a conformation with little secondary structure, and a state in which the pHLIP remains disordered and is adsorbed to the surface of the cell membrane [15]. At low pH, an increased concentration of protons results in the protonation of negatively charged residues at key locations in the peptide. This protonation increases the overall hydrophobicity of the pHLIP, resulting in its folding to form a helix that partitions across the bilayer as a **transmembrane helix**. The inserted peptide leaves its N-terminus in the extracellular space while the C-terminus is translocated across the membrane into the cytoplasm. The lipid composition of the cell membrane can contribute to modulation the binding and insertion into the membrane of a pHLIP [20, 41, 42].

It is important to distinguish between the pHLIP family, which consists of membrane-inserting peptides, and **cell-penetrating peptides** (CPPs) or **amphipathic pore-forming peptides**. CPPs and pore-forming peptides have entirely different mechanisms of action and different biological performance; they are not part of the pHLIP family.

Targeting extracellular acidity

Targeting cancer cells by exploiting extracellular acidification should be compared with other targeting approaches, which are commonly based on the presence of growth factors or tumor antigens. A problem in targeting these kinds of specific features is that the cancer cell population, both between tumors and within an individual tumor, is extremely heterogeneous and very adaptable [43]. The potential for successfully treating tumors that do not express or exhibit limited expression of the targeted growth factor or antigen is low, and, even in treatment-responsive tumors, minority populations of cancer cells can undergo clonal selection and eventually re-grow into a treatment-resistant phenotype. Subsequent treatments will either have limited efficacy or be entirely ineffective. Additionally, these types of targeting methods have the potential to severely damage healthy tissues where the growth factor or antigen might also be present. Therefore, targeting tumor acidity, a physical characteristic that is not only found across an entire tumor but is found in tumors of all sizes, including metastases, could have advantages over targeting methods based on characteristics that are found only in some tumors or some cancer cell populations [44, 45].

Acidity is produced not only by cancerous tissue, but also by any tissue that is experiencing hypoxia: the lack of oxygen in the tissue triggers cells to switch to a high rate of the anaerobic, glycolytic energy production pathway, consequently resulting in acidification. pHLIPs have been shown to target infections in lungs [46], inflammatory arthritis [22], and ischemic myocardium, a consequence of heart disease, and could be used for diagnosis and

treatment thereof [26]. It is also possible that pHLIPs could be used as targeted treatment in ischemic strokes. In contrast to diseased tissue, healthy tissue is typically not associated with increased acidity except in the gastrointestinal tract and kidney, the pHs of which might be regulated by the implementation of a special diet or supplementary drinks if their acidity proves problematic.

In contrast to other pH-sensitive agents that sense bulk pH, members of the pHLIP family of peptides bind to cell surfaces at all pH values and therefore sense pH at the surfaces of cancer cells, where pH is the lowest, further accentuating the pH sensitivity of the peptides. The pH-dependent behavior and inserted conformation of pHLIPs make them useful for delivering cargo inside cancer cells (by attaching the cargo to the inserting end of the peptides) or for tethering cargo to the cell membrane (by conjugating to the non-inserting end of the peptides) [9]. Additionally, small-angle X-ray scattering experiments have shown that pHLIPs do not form oligomers larger than tetramers, even at very high concentrations [47], a useful property for drugs to be used intravenously, as the local concentration at the injection site is generally much higher than the drug concentration when it reaches the target area, and it is crucial that the drug does not aggregate upon injection. These basic features give rise to a wide variety of possible applications in diagnostic imaging, fluorescence guided surgery, the intracellular delivery of small molecules like toxins and gene regulation agents, the delivery of proteins to the surfaces of cells, and the delivery and uptake enhancement of nanoparticles.

Applications of pHLIP Technology

Diagnostic nuclear imaging

Positron emission tomography (PET) and single-photon emission computed tomography (SPECT) are imaging techniques that rely on the delivery of radioactive imaging agents to targeted tissue. One of the main challenges in PET/SPECT imaging is to obtain images of target tissue that contrast highly with the image background, motivating the use of a targeting agent. When a radionuclide is conjugated to the non-inserting terminus of a pHLIP (Figure 3A), the resulting construct not only targets tumor tissue very well, but also remains securely in the tumor long enough to allow the excess, non-inserted construct to clear from normal tissues, resulting in higher contrast images [17, 23, 25, 29, 33]. Success has been seen using the radioisotopes ^{18}F ($9 \pm 2\% \text{ID/g}$ at 4 h p.i.) and ^{64}Cu ($19 \pm 2\% \text{ID/g}$ at 24 h p.i.) conjugated to Var3 [33]. These constructs are in the process of clinical translation as novel nuclear imaging markers of diseased tissue acidity.

Fluorescence-guided surgery and ex vivo imaging

Fluorescence-guided surgery utilizing pHLIP technology could ease the challenges of tumor resection, such as visualization of all cancerous lesions, including flat lesions, ultimately reducing the number of surgeries that result in **positive margins**. These features could improve surgery outcomes and reduce tumor recurrences. Excellent targeting and labeling by different fluorescent pHLIPs of primary tumors and sub-millimeter-sized metastatic lesions has been demonstrated on various tumor models, including transgenic breast, prostate, melanoma and pancreatic mice models [11, 24, 28, 34, 35, 48].

Excellent staining and visualization of urothelial tumors has been observed in excised human bladders using an **ICG** pHLIP imaging agent [48]. This imaging agent marked high-grade urothelial carcinomas, both muscle-invasive and non-muscle-invasive lesions, and, most importantly, flat lesions, which are the most difficult to identify and commonly necessitate further surgery. Carcinoma *in situ* was accurately diagnosed in eleven cases, whereas only four cases were identified using white light. The sensitivity and specificity of targeting by the ICG pHLIP imaging agent were found to be 97% and 100%, respectively, excluding staining of necrotic and previously treated tissue.

Another application of fluorophore-labeled pHLIPs is the *ex vivo* staining and imaging of biopsy samples or tissue after surgical resections. Fluorophore-conjugated pHLIPs have shown utility in accurately identifying head and neck cancer, proving their potential to be useful tools for the early identification and evaluation of dysplasia and neoplasia [49, 50]. In these studies, both the normal and suspect biopsy samples were stained *ex vivo* with Alexa Fluor647 pHLIP. Imaging was performed before and after staining, and the ratio of intensity increase of suspect tissue to normal tissue was calculated; a two- to six-fold increase in fluorescence intensity ratio was observed in cancerous tissue compared to normal tissue (with no ratio increase for the inflamed tissue).

The conjugation of the pH-sensing fluorescent dye **SNARF** to a pHLIP results in a compound that can measure pH at the surface of individual cells, which differs greatly from the more easily and commonly measured bulk pH [12]. Currently, approaches for cell surface pH mapping using SNARF-pHLIP in liquid and solid biopsy samples are being developed, which might result in an opportunity to get information about the metabolic status of tumors, ultimately aiding in the prediction of tumor aggressiveness and the tailoring of therapy. SNARF-pHLIP might also be used in longitudinal studies as a very useful tool in the investigation of the development and progression of diseases.

Intracellular delivery of drug-like and polar therapeutic cargo molecules

pHLIP translocate their C-termini into the cytoplasm of tumor cells, allowing the intracellular delivery of therapeutic cargo molecules to treat primary tumor tissue as well as metastases. The ability of pHLIPs to target metastases is especially important: in cancer cases, the primary tumor is generally not the reason for poor prognoses; more often, it is tumor invasion, unseen by traditional diagnostic procedures, that proves fatal.

Extensive biophysical investigations, including kinetics and thermodynamics studies, have been conducted to determine the activation energy required for the membrane insertion/folding (as well as unfolding/exit) of pHLIPs [19–21, 39, 51]. These studies set the foundation for the development of novel approaches in drug delivery that are based on employing the energy of membrane-associated folding and the kinetically controlled pH-triggered translocation of cargo molecules across the lipid bilayer of the cell membrane (Figure 3B). Cargoes can be linked to the C-terminus, for example, by including a cysteine in the pHLIP sequence and attaching the cargo via a disulfide that is cleaved in the cytoplasm, releasing the cargo. Following fundamental studies, numerous *in vitro* and *in vivo* investigations have demonstrated that pHLIPs can deliver several different payloads, such as fluorescent dyes, toxins, drugs, peptides, and **peptide nucleic acids (PNAs)** [14, 52–

61]. The many molecules delivered into cells by pHLIPs can be conceptualized in two categories: **drug-like**, cell-permeable molecules; and polar, cell-impermeable cargoes. In the first case, pHLIPs might improve the pharmacokinetics and favorably alter the biodistribution of drugs, resulting in enhanced tumor accumulation. In the second case, pHLIPs, in addition to tumor targeting, can deliver polar molecules that cannot cross the plasma membrane via **passive diffusion**.

Additionally, it is possible to tune the intracellular delivery of cargo by altering the C-terminal sequence, by choices of the link connecting cargo to a pHLIP, and/or by attaching modulator molecules [52–54]. Changing the efficiency of cargo translocation leads to a change in biological response: systematic studies in which polar toxins are conjugated to pHLIPs via cleavable linkers of different hydrophobicity show subtle changes in the insertion properties of the constructs and, therefore, in the resulting cytotoxicity. Thus, depending on cargo polarity, the translocation of cargo across cellular membranes could be optimized by the proper selection of pHLIPs, linkers and modulator molecules.

At low pH (pH <6.5), pHLIPs promote delivery of cell-permeable therapeutic cargoes such as the **tubulin**-binding toxin monomethyl auristatin E (MMAE) and the DNA intercalator doxorubicin, and reduces the cellular entry of these cargoes into healthy tissue at normal physiological pH [57, 58]. pHLIP MMAE conjugates have cytotoxic effects at low pH, inhibiting cell growth by more than 90%, and targeted **triple-negative breast cancer** tumors in mice [58].

One of the most attractive therapeutic applications of pHLIPs is their ability to deliver polar molecules. Studies have been conducted using polar cyclic toxins from the death cap mushroom (*Amanita phalloides*): phalloidin, phalloidin and amanitin. Phalloidin and phalloidin bind to **F-actin**, while amanitin is an inhibitor of **RNA polymerase II**. All toxins, when conjugated to pHLIPs, have shown a pH-dependent ability to induce cell death [52, 53]. α -amanitin is the most potent cargo studied so far, and, when conjugated to a pHLIP and exposed to cancer cells for less than two hours, it displayed a four- to five-fold higher antiproliferative effect at low pH (pH 6.5) compared with physiological pH [54].

Also, pHLIPs have been proven to deliver peptides to interfere with the biological action of protein receptors or to disrupt mitochondrial cellular membranes [59, 60]. **PAR1** is a protein receptor that is overexpressed in many cancer cells. A PAR1 protein fragment was shown to be translocated across the cell membrane by a pHLIP, where it interacted with the intracellular domain of the PAR1 receptor, rendering the protein receptor inactive and inducing pH-dependent cytotoxicity [59].

Intracellular delivery of gene-regulation agents

There is an expectation that future therapy (including cancer therapy) will be based on drugs that induce genetic interference in cancer cells. Efficiently modulating gene expression inside cancer cells and either reprogramming them to be normal cells or inducing apoptosis will be a significant breakthrough. Oncogenic **microRNAs** (oncomiRs) are miRNAs whose overexpression is associated with cancer development; thus, it is desirable to inhibit an

oncomiR, which can be achieved using complementary synthetic RNA strands, such as PNAs [56, 62, 63].

The main obstacle to the successful use of PNA antimiRs is that they do not cross cell membranes. pHLIPs, however, have been shown to deliver PNAs to cancer cells by virtue of their inherent targeting capability and folding energy. pHLIP-mediated intracellular delivery of PNAs targeting the miR-155 oncomiR was shown in a mouse model of large B-cell lymphoma [56]. Treatment of lymphadenopathic miR-155 mice with pHLIP anti-oncomiR PNA leads to tumor burden reduction and prevention of lymphocyte metastases.

Another approach to gene therapy involves the intracellular delivery of plasmid DNAs (pDNAs). In one study, a pDNA expressing growth factor-interfering RNA was attached to a dendrimer via electrostatic interaction, and the dendrimer-pDNA complex was conjugated to a pHLIP. The study showed higher cellular uptake at low pH compared to normal pH and resulted in an 86% inhibition of the targeted growth factor [64].

Targeting of therapeutic agents to cell surfaces

Another interesting use for pHLIPs is the delivery of therapeutic agents to the extracellular surfaces of cells, which might selectively activate various signaling pathways. An example of this principle is shown in the use of **tissue factor (TF)**, a protein that anchors to the plasma membrane and induces blood coagulation. However, targeting is crucial for the successful use of tissue factor to accomplish thrombotic occlusion; otherwise, harmful side effects would be common. The tissue factor protein naturally has a transmembrane domain that anchors it to cell surfaces. A study in which the transmembrane domain of the natural tissue factor was truncated and replaced with a pHLIP (resulting in tTF-pHLIP) showed targeting of the acidic tumor vasculature, and the tTF-pHLIP induced local blood coagulation. The administration of the tTF-pHLIP selectively induced thrombotic occlusion in tumor-bearing mice, impairing tumor growth without any obvious side effects [65].

Nanotechnology

In the realm of nanotechnology, pHLIPs might find a variety of uses [66]. In nanotechnology applications, multiple pHLIPs decorate a single nanoparticle, which can range in size from a few nanometers to hundreds of nanometers, in contrast to the single peptide-cargo conjugates described above (Box 2). Nanocarriers decorated with pHLIPs are biocompatible, can target tumors, and demonstrate enhanced cellular uptake by cancer cells. Some pHLIP-coated nanoparticles that have been investigated are lipid, polymer, and metal-based nanomaterials [27, 67, 68].

Box 2

Not only can pHLIP conjugates be constructed in a one-to-one ratio between pHLIP (blue) and cargo (red), but the pHLIP can also be used to coat molecules in order to increase biocompatibility, targeting, and to induce cellular uptake (Figure 1). Nanoparticles, for instance, have a wide variety of uses in medical applications, including as drug-laden nanocarriers, as particles that enhance another form of therapy, and as therapeutic agents themselves. Decorating nanoparticles with multiple pHLIPs not only

results in targeting specificity and greater uptake by cells in acidic, diseased environments, but can also bestow biocompatibility on what might be an otherwise non-biocompatible nanoparticle. pHLIPs could be used as decorations on a wide variety of nanoparticles, and have already been studied in lipid, polymer, and metal-based nanomaterials.

Figure 1. pHLIP delivery capability

pHLIPs are used to increase the tumor targeting and delivery of various types of cargoes, such as imaging agents and therapeutics, by conjugating cargo to pHLIP in a one-to-one ratio (A). pHLIPs can also be used to decorate nanoparticles (B), to increase the biocompatibility, tumor targeting and promote cellular uptake of nanoparticles by cancer cells.

In pHLIP-coated liposomes, pHLIPs promote lipid exchange and fusion between the lipid bilayer of the liposome and the cellular membrane [67]. Fusion with cellular or endosomal membranes allows the direct release of polar payloads into the cytoplasm, or the transfer of hydrophobic payloads into the membrane lipid bilayer (Figure 4) [67]. The principle of pHLIP-mediated liposomal fusion was used to deliver the hydrophobic peptide gramicidin A, a monovalent ion channel. Introducing pores into the targeted cell membrane disrupts ion balance, resulting in apoptosis [67]. This case demonstrates that pHLIPs might be used not only to improve liposome targeting over **passive targeting methods**, but also to enhance payload delivery into cells by promoting the fusion of the liposome with the cell membrane.

Gold nanoparticles have found various applications in medicine, including imaging, radiation therapy enhancement, and hyperthermia localization. Gold nanoparticles have been shown to enhance cancer cell uptake by over 600% at low pH compared with cells treated with gold nanoparticles alone [27], and to promote the internalization of gold nanoparticles in human platelets [69]. Tumor targeting specificity was observed in studies of pHLIP-coated gold nanoclusters, ultimately resulting in the enhancement of radiation effects and increased cytotoxicity [70]. Additionally, pHLIP-coated hollow gold nanospheres (HAuNS) containing chlorin e6 (Ce6), a chemical used to amplify the response of tumor cells to photodynamic therapy, were developed. The HAU NS-pHLIP-Ce6 particles, when irradiated with near-infrared light, treated tumors more effectively than hollow gold nanospheres alone because of the photosensitizer-enhanced hyperthermia due to Ce6 [71, 72].

Concluding Remarks

pHLIPs have been shown to target cancer tumor acidity. Targeting that exploits acidity, a physical characteristic ubiquitous to tumors, may have advantages when compared to other current approaches. Passive targeting, which relies on the abnormal vasculature present in large tumors, and active targeting, which relies on specific traits of cancer cells, have disadvantages due to the inconsistent presentation of passively-targeted traits, the heterogeneity of the cancer cell population, and the presentation of targeted traits in healthy cells. These problems can result in failure to target some cancer cells, the selection of resistant tumor cells, and, ultimately, ineffective diagnosis, surgery, and therapy due to relapse, the development of treatment-resistant cancer, and significant damage to healthy tissue.

Not only do pHLIPs target both primary tumor tissue and metastases well, but also the characteristics of the insertion of pHLIPs into cancer cells may make them a useful tool for many applications (see Outstanding Questions). pHLIPs have been used to tether imaging agents to cell surfaces for diagnostic imaging and fluorescence guided surgery. Additionally, the energy of insertion of pHLIPs has been used to deliver many different types of cargoes into the cytoplasm: small molecules such as toxins, chemotherapeutic agents, and protein fragments; PNA and pDNA for gene therapy; and nanomaterials such as liposomes, gold nanoparticles for therapy and imaging, and drug-laden porous nanoparticles. The pHLIP family of peptides presents novel opportunities in cancer diagnosis and targeted therapy due to the customizable nature of the peptides for uses in a wide variety of applications.

Outstanding Questions

- **How can pHLIP® be more effectively used in therapeutic applications?** One of the greatest strengths of pHLIP® is its ability to deliver polar and moderately hydrophobic cargo molecules across the cellular membrane directly into the cytoplasm, avoiding the endocytotic pathway and reducing drug resistance. Optimized pHLIP® drug conjugates could be extremely useful in therapeutic applications, especially for the treatment of metastatic cancers and cancers which are difficult to treat, such as triple-negative breast cancers, pancreatic cancer, infiltrating and diffusive glioblastomas, and potentially many others.
- **How can pHLIP® and/or the link between peptide and cargo be optimized to exclusively target cancer cells in order to achieve intracellular delivery of highly toxic cargoes?** Off-target delivery of highly toxic chemotherapeutic agents (e.g., amanitin) has serious consequences and should be reduced even if the number of molecules delivered to the target tissue is consequently reduced. Typically, the delivery of just a few very potent drug molecules is enough to induce a cytotoxic effect.
- **How can pHLIP® and/or the link between peptide and cargo be optimized for the efficient intracellular delivery of large quantities of less toxic cargoes?** For some cargoes, like those that alter gene expression (e.g.,

PNA), the cargo itself is very specific to its target within cancer cells, and off-target delivery does not pose significant problems. At the same time, these types of cargoes need to be delivered in larger quantities to induce a noticeable biological effect.

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Glossary

Amphipathic pore-forming peptides

Peptides possessing both hydrophobic and hydrophilic regions that insert and assemble to form pores in the cellular membrane

Cell-penetrating peptide (CPP)

A peptide, typically with a strong positive charge, that binds to the phospholipids of the cell membrane and is taken up by the cell

Drug-like molecule

A molecule that exhibits properties similar to those of established drugs; for example, per Lipinski's Rule of Five: no more than five hydrogen bond donors, no more than ten hydrogen bond acceptors, molecular weight below 500 Da, and an octanol-water partition coefficient $\log P \leq 5$

F-actin

Filaments that are part of the cell cytoskeleton and contribute to structural stability and cell movement

ICG

Indocyanine green, an FDA-approved fluorescent dye used to mark blood vessels in angiography and perfusion diagnostics, but does not target the tumor itself

MicroRNA

Small molecules of RNA that play a functional role in gene expression by blocking messenger RNA translation

Non-standard amino acids

Amino acids that are not encoded in the human genome

PAR1

Proteinase-activated receptor 1, a member of the large family of G protein-coupled receptors that plays a functional role in the blood coagulation process

Passive diffusion

The ability of molecules to cross cell membranes on their own

Passive targeting methods

Targeting methods that rely on naturally occurring biological characteristics, such as non-intact tumor vasculature, to induce the localization of cargo within a tumor

Peptide nucleic acid (PNA)

Artificial DNA- or RNA-like molecule with a peptide backbone that forms a sequence-specific base-paired complex with DNA or RNA

pHLIP®

The pH (low) insertion peptide; pHLIP is a registered trademark

Positive margin

The condition that exists after surgical resection when the surgery was not successful in removing all cancerous tissue

RNA polymerase II

A key enzyme that catalyzes transcription

SNARF

seminaphtharhodafleur, a fluorescent dye with two pH-dependent emission peaks, making possible the use of SNARF to measure pH through spectrography or ratiometric imaging

Tissue factor (TF)

Protein that is exposed to the blood upon tissue damage, triggering blood clot formation

Tubulin

A family of proteins that polymerize into microtubules, which are part of the cytoskeleton and contribute to structural stability, cytoplasmic transport, cell movement, and cell division

Transmembrane helix

The formation by a polypeptide of a helix that spans the lipid bilayer of a cellular membrane

Triple-negative breast cancer

Any breast cancer that does not express the estrogen receptor, progesterone receptor, and epidermal growth factor receptor 2 (HER2/neu). Triple-negative breast cancer is difficult to treat due to the reliance of typical chemotherapies on the expression of one or more of these receptors

Tumor invasion

The spread of cancer cells (metastasis) from the primary tumor site to adjacent tissue or blood

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Trends Box

- The pH (Low) Insertion Peptides (pHLIPs[®]) target cancer cells in primary tumors as well as metastases based on the acidic environment that is universal to tumor tissue.
- Acidity-based targeting is not diminished by the phenomena that blunt the efficacy of other biomarker-based targeting methods.
- pHLIPs act as monomers and remain anchored across the membrane, leaving it intact, which distinguishes them from pore-forming and cell-penetrating peptides.
- A growing family of pHLIP variants gives choices for the delivery of various cargoes, including imaging agents for nuclear diagnostic imaging and fluorescence guided surgery, cell-permeable and impermeable therapeutic agents for intracellular delivery, and different types of nanoparticles.
- pHLIPs show promise for many medical applications, and the clinical translation of several pHLIP conjugates is currently underway.

Figure 1. pHLIP membrane interaction and insertion

The peptides of the pHLIP family (blue) exist in equilibrium between solvated and membrane-adsorbed conformation at the normal extracellular pH found in healthy tissue, whereas peptides insert across cellular membrane at the low extracellular pH found in acidic, diseased tissue.

Figure 2. Mechanism of pHLIP insertion into the cellular membrane

When the pHLIP (blue) encounters healthy tissue where the extracellular pH is around pH 7.4, the protonatable residues of the pHLIP (red circles) remain deprotonated and negatively charged, and the peptide resides at or near the hydrophilic surface of the cellular membrane. Weakly bound to the membrane, the pHLIP is washed from the membrane via normal perfusion and continues to circulate through the body. Cancer cells, however, produce excess acidity as a consequence of their malfunctioning metabolisms and overexpression of certain surface proteins, and pump these acidic byproducts out of the cell interior in order to maintain comfortable conditions inside the cell, resulting in the acidification of tumor tissue. When the pHLIP encounters tumor tissue, it senses the low extracellular pH at the cancer cell surface (i.e., the concentration of protons (cyan circles) at the surface of the cellular membrane is high), and the protonatable residues and negatively charged C-terminal carboxyl group of the pHLIP become neutrally charged (green circles). The protonation leads to an increase in the overall hydrophobicity of the pHLIP, increasing the affinity of the peptide to the hydrophobic core of the cellular membrane and triggering the pHLIP to spontaneously fold into a helix and insert across the membrane, resulting in the formation of a transmembrane helix. When the C-terminal protonatable residue and carboxyl group are then exposed to the normal intracellular pH of the cell, they are deprotonated, again becoming negatively charged, and anchor the pHLIP in the membrane.

Figure 3. Two uses for pHLIPs: tethering cargo to the cell surface or translocating cargo across the membrane into the cytoplasm

A pHLIP (blue) can be used to target and tether cargo molecules to the surfaces of cells in low pH environments (A). The cargo could be an optical marker, a PET or SPECT imaging agent, or an antigen or protein delivered to induce certain cellular processes. A pHLIP can also be used for the intracellular delivery of payloads, translocating cargo (green) across the membranes of cells with low extracellular pH, such as those cells found in acidic, diseased tissue (B). These payloads are conjugated to the membrane-inserting end of the pHLIP, typically via a cleavable link (magenta), and could include toxins, chemotherapeutic agents, or agents to alter gene expression.

Figure 4. A postulated pathway for fusion and cellular uptake of pHLIP-coated liposomes pHLIP-coated liposomes interact with the membrane and promote either direct fusion with the lipid bilayer of the membrane (**A**), or uptake via the endocytotic pathway (**B**), followed by fusion with the endosomal membrane. Both uptake mechanisms result in the cytoplasmic delivery of encapsulated polar cargo (green), or delivery of lipid membrane-incorporated hydrophobic payloads to the membrane (red). Schematic is not to scale.