Cy5.5-labeled pH low insertion peptide (pHLIP)

pHLIP-Cy5.5

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Chemical name:	ACEQNPIYWARYADWLFTTPLLLLDLALLVDADEGTG-Cy5.5	
Abbreviated name:	pHLIP-Cy5.5	
Synonym:		
Agent Category:	Peptide	
Target:	Low pH	
Target Category:	Others	
Method of detection:	Near-infrared fluorescence optical imaging	
Source of signal / contrast:	Cy5.5	
Activation:	No	
Studies:	• *In vitro* • Rodents	No structure available.

Background

[PubMed]

The pH low insertion peptide (pHLIP) is a peptide of 37 amino acids that inserts across the cell membrane as an α-helix when the extracellular pH (pH_e) is acidic (1-4). pHLIP labeled with the near-infrared (NIR) fluorescent marker Cy5.5 (pHLIP-Cy5.5) was developed by Andreev et al. for optical mapping of areas (tumor and arthritis) of elevated acidity in the small animals (1).

Tumor microenvironment is characterized by low pH_e (5, 6). Almost all solid tumors have a neutral to alkaline intracellular pH (pH_i), but they develop an acidic pH_e (known as the Warburg effect, Nobel Prize in 1931). The average pH_e could be as low as 6.0 (7-9). A pH

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gradient ($pH_i > pH_e$) exists across the cell membrane in tumors. This gradient is contrary to that found in normal tissues, in which pH_i is lower than pH_e (7.2–7.4) (7-9). Diffusion of the H^+ ions along concentration gradients from tumors into adjacent normal tissues creates a peritumoral acid gradient (10). The mechanisms responsible for the low pH_e include anaerobic glycolysis because of hypoxia, aerobic glycolysis (the Warburg effect), increased metabolic CO_2 production associated with uncontrolled cell growth, and increased activity of ion pumps on the cell membrane (5, 7).

Low pH_e affects many aspects of tumor physiology. It is one of the driving forces in the clonal selection leading to invasive and metastatic diseases (11, 12). Rofstad et al. have shown that lowering culture pH to 6.8 results in a promotion of *in vivo* metastasis of treated human melanoma cells compared with controls (cultured at pH 7.4) after tail vein injection of the cells in mice (13). Exposure of tumor cells to an acidic environment leads to increased expression of various factors that contribute to tumor progression (12). Tumor cells are able to maintain a high proliferation rate in the acidic environment, whereas the peritumoral acid gradient limits immune response to tumor antigens and induces normal cell apoptosis, extracellular matrix degradation, and angiogenesis (7, 11). The passage of noncarrier-mediated weak drugs through the cell membranes is also influenced by the acidic pH_e (14-16). Typically, the drugs in an uncharged state (lipophilic form) pass more efficiently through the cell membranes. This leads to the hypothesis of 'ion-trapping' that weakly basic drugs will concentrate in more acidic compartments (14, 15). The acid pH_e of tumors will therefore hinder weakly basic drugs from reaching their intracellular targets, thereby reducing cytotoxicity (16). Conversely, the acid pH_e of tumors will improve uptake of weak acids into the relatively neutral intracellular space (17). The currently used chemotherapeutic drugs such as mitoxantrone, doxorubicin, daunorubicin, anthracyclines, anthraquinones and vinca alkaloids are all weak bases (pKa 5.5–6.8), while cyclophosphamide, 5-fluorouracil and chlorambucil are weak acids (pKa 7.8–8.8) (15). Both *in vitro* and *in vivo* studies have shown that the activities of those weak bases are inhibited by the low pH_e (14-16). On the contrary, the actions of the weak acids are enhanced by the low pH_e. The pH gradient in tumors exerts a protective effect upon the cells from weak-base drugs as well as acts to potentiate the action of weak acid drugs (17). Studies have consistently shown that selective tumor alkalinization *in vivo* is likely to result in an enhancement in the anti-tumor activity of weakly basic chemotherapeutic drugs (18, 19). Low pH_e has also been shown to impair the effectiveness of some drugs such as paclitaxel in that their chemical structures do not predict pH-dependent ionization (7). In addition, radiation therapies are known to be significantly less effective at the hypoxic and acidic regions of tumor (20).

An understanding of the mechanisms involved in tumor-specific low pH_e leads to the development of targeted therapeutic approaches (6, 7). Low pH_e is also considered a promising marker for tumor targeting detection (4, 8). The pHLIP interacts with the surface of membranes as an unstructured peptide at neutral pH_e, but at acidic pH_e (<7.0) it inserts across the membrane and forms a stable transmembrane α-helix (1, 2, 21, 22). The pHLIP affinity for membranes at low pH (5.0) is 20 times higher than that at high pH (8.0). Studies by Zoonens et al. showed that the pHLIP could translocate cell-

impermeable cargo molecules across a cell membrane and release them in the cytoplasm (23). The process is mediated by the formation of a transmembrane α-helix because of increased peptide hydrophobicity associated with the protonation of Asp residues at low pH (1, 22). Replacement of the two key Asp residues located in the transmembrane part of pHLIP with Lys or Asn leads to the loss of pH-sensitive membrane insertion (3). Andreev et al. labeled the pHLIP with Cy5.5 and tested its feasibility for optical mapping of tumor and arthritis which were characterized by elevated acidity (1).

Optical fluorescence imaging is increasingly being used to monitor biological functions of specific targets in small animals (24-26). However, the intrinsic fluorescence of biomolecules poses a problem when fluorophores that absorb visible light are used. Near-infrared (NIR) fluorescence detection avoids the natural background fluorescence interference of biomolecules, providing a high contrast between target and background tissues in small animals. NIR fluorophores have a wider dynamic range and minimal background fluorescence as a result of reduced scattering compared with visible fluorescence detection. NIR fluorophores also have high sensitivity, attributable to low background fluorescence, and high extinction coefficients, which provide high quantum yields. The NIR region is also compatible with solid-state optical components, such as diode lasers and silicon detectors. NIR fluorescence imaging is a non-invasive alternative to radionuclide imaging in small animals (27, 28).

Synthesis

[PubMed]

Four peptides (L-pHLIP, D-pHLIP, N-pHLIP, and K-pHLIP) were designed by Andreev et al. (1). L-pHLIP and D-pHLIP were synthesized with the same sequence but from L- and D-amino acids, respectively. The pHLIP peptide sequence was ACEQNPIYWARYADWLFTTPLLLLDLALLVDADEGTG. N-pHLIP and K-pHLIP were used as controls. In N-pHLIP and K-pHLIP, Asn and Lys replaced Asp residues, which are protonated at low pH and drives pHLIP insertion into membrane. All peptides were prepared with solid-phase peptide synthesis using standard 9-fluorenylmethyloxycarbonyl chemistry and purified with reverse-phase chromatography. NIR fluorescent dye Cy5.5 was conjugated with Cys or Lys residues placed on the pHLIP N terminus, which remains outside the cell when the peptide inserts across the lipid bilayer. The detailed purity and degree of labeling of the pHLIP-Cy5.5 conjugate were not reported.

In Vitro Studies: Testing in Cells and Tissues

[PubMed]

Andreev et al. analyzed the molecular mechanism of pHLIP insertion *in vitro* using liposomes and human red blood cells (RBCs) (1). In the presence of liposomes, pHLIP inserted into the lipid bilayer with perpendicular orientation and formed transmembrane α-helix at low pH (4.5) (1, 29). The control K-pHLIP did not show secondary structure changes and remained outside the membrane at pH 4.5 and pH 8.0. N-pHLIP formed an

α-helical structure and was found primarily in the membrane over a wide pH range (4.0–8.0). With concentrations up to 10 μM, pHLIP did not cause lysis of the human RBCs at pH 6.0 or pH 7.4 after incubation with RBCs for 1 h at room temperature. Morphologically, incubation of the RBCs (1% suspension) with pHLIP (5 μM) for 15 min resulted in the appearance of spicules on the surface of 67% of the cells at normal pH (7.4), but only on 13% of the cells at low pH (6.0). K-pHLIP (5 μM) induced formation of echinocytes at both pH levels (57% of cells at pH 7.4 and 55% of cells at pH 6.0), and N-pHLIP (5 μM) did not induce shape changes in RBCs (74% and 83% of the cells were discocytes at the respective pH levels). The results indicate that pHLIP inserts into the membrane at low pH (6.0), whereas K-pHLIP weakly binds to the membrane surface and N-pHLIP interacts with lipid vesicles and forms α-helical structure at both pH levels.

Animal Studies

Rodents

[PubMed]

Andreev et al. tested the ability of pHLIP-Cy5.5 to accumulate in tumors with low pH_e and inflammatory arthritis as another model of low pH (1). Mouse tumors were established with subcutaneous injection of murine breast adenocarcinoma cells (CRL-2116) in the right flank of female C3D2F1 mice (number of animals not reported). pHLIP-Cy5.5 (500 μg/kg) was given as a single intraperitoneal injection into the left side of the mice. Optical imaging showed that pHLIP-Cy5.5 was redistributed and concentrated in tumors within 20 h after injection. pHLIP-Cy5.5 was able to mark tumors of various sizes with high accuracy. The fluorescent signal in tumors was stable for 4 days and was ~5 times higher than in the healthy counterpart tissue ($n > 20$ animals). No specific accumulations were observed in tumors for Cy5.5 dye alone, K-pHLIP-Cy5.5, and N-pHLIP-Cy5.5. Arthritis was induced in the right knee joints with the use of methylated bovine serum albumin and Freund's complete adjuvant. After intraperitoneal injection of pHLIP-Cy5.5 (30 μg/kg), pHLIP-Cy5.5 targeted the inflammatory site in the right leg with no accumulation in the left (control) leg. The fluorescent signal was 4–5 times higher in the right than in the left knee joints.

No significant difference was observed between the accumulation of L-pHLIP-Cy5.5 and D-pHLIP-Cy5.5 in tumor, inflammatory foci, and kidney, indicating that the peptide insertion process does not depend on amino acid chirality. Buffering the feed water to pH 8.2 resulted in ~50% reduction of the fluorescent signal in the kidneys of mice (number of animals not reported) There was no significant accumulation in the liver or other organs. A preliminary toxicity study showed no physiological or behavioral changes within 2 months after intravenous injection of L-pHLIP and D-pHLIP (4 mg/kg) in 4-week-old female and male mice (number of animals not reported).

Andreev et al. concluded that pHLIP nanotechnology offers an approach for mapping areas of elevated acidity in the body, possibly enabling the study of pathological processes,

diagnosis of diseases, treatment by delivery of molecules to affected areas, and monitoring of therapeutic outcomes. The authors have reported similar results by labeling pHLIP with NIR dye Alexa750 (detailed data not shown) (1). Vāvere et al. labeled the same peptide with ^{64}Cu (^{64}Cu-DOTA-pHLIP) and used it for positron emission tomography imaging of low pH_e in prostate tumor xenografts (4).

Other Non-Primate Mammals

[PubMed]

No references are currently available.

Non-Human Primates

[PubMed]

No references are currently available.

Human Studies

[PubMed]

No references are currently available.

NIH Support

P20RR016457, GM070895, GM073857

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