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Targeting acidic diseased tissue: New technology based on use of the pH (Low) Insertion Peptide (pHLIP)

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Abstract

We discuss a peptide that targets cells in the acidic tissues that result from a range of pathological states, including tumours, and that can also translocate cell-impermeable cargo molecules across cell membranes in a pH-dependent manner. The technology is based on the interactions of a water-soluble membrane peptide, which we call pHLIP (pH (Low) Insertion Peptide), with the lipid bilayers of cell membranes. At the normal pH of healthy tissue it binds to cell surfaces, but at low pH pHLIP inserts as a monomer across the cell membrane to form a stable transmembrane helix. pHLIP holds promise for imaging and drug delivery applications.

INTRODUCTION

It is well established that tissues in pathological states, as in cancer, inflammation, arthritis, stroke, ischemia and others, are associated with an elevated level of extra cellular acidity. In most cases, hypoxia and low blood supply are associated with the pathology, and, as a consequence, the metabolism in cells in diseased tissue becomes partially anaerobic, leading to the production of acid from glycolysis (the Pasteur effect) (1,2). Moreover, malignant cancers have an elevated uptake of glucose even under normal oxygen conditions, known as “aerobic glycolysis” or the Warburg effect (3). For reasons not fully understood, cells exhibiting a Warburg effect continue to catabolize glucose at a high rate, even when mitochondrial oxidative pathways are available (4,5). The consequence of such metabolism in any tissue is the formation of H^+ , which must be removed from the cell to maintain the normal cytoplasmic pH required by many cellular processes having narrow pH optima. pH regulation is carried out by transmembrane proteins that pump protons from the cytoplasm across the plasma membrane to the extra cellular space or the lumen of various organelles (6–8). As a consequence, pH measured in the extra cellular fluid is decreased, for example in tumours compared with normal tissue. Due to the electrostatic potential from the proton gradient across the dielectric membrane, the H^+ ions accumulate near the surfaces of the cells, and the effective pH is even lower in these regions than is measured in the bulk extra cellular fluid. Thus, pH measurements near the surfaces of cells in diseased tissue and targeting of extra cellular acidity could both enhance understanding of the development and progression of diseases and provide an enhanced opportunity to improve the diagnosis and treatment of pathological states if acidic targeting can be made practical. Targeting the general property of tumour acidity may be especially important given the high heterogeneity of the cells in human cancers (9), which makes it problematic to rely on any specific tumour biomarker for targeting, even for one type of cancer. Several nano sized systems have been developed that exhibit pH-sensitive properties. Among them are polymers, dendrimers, micelles, liposomes, and hydro gel nanoparticles (see review by Ganta (10,11–13)). A significant feature of each of these nanocarriers is an ability to release encapsulated therapeutic and/or imaging agents in response to changes in pH. Most

of the pH-sensitive carriers are used for drug release in the acidic environments found in cytoplasmic compartments (endosomes and/or lysosomes) after cellular uptake of the conjugates by endocytosis, rather than for general targeting of acidic tissue.

MOLECULAR MECHANISM OF pHLIP INTERACTION WITH THE LIPID BILAYER OF MEMBRANE

We have discovered a new technology to target extra cellular acidity and translocate cell-impermeable cargo molecules across cell membranes in a pH-dependent manner (14,15). It is based on the action of a membrane peptide that we call pHLIP, for “pH (Low) Insertion Peptide”, which consists of a transmembrane part (the underline shows the helical region in the bacteriorhodopsin helix C structure, the original source of the sequence) and two flanking sequences: AEQNPIYWARYADWLFTTPLLLLDLALLVDADEGT. At neutral and high pHs pHLIP is monomeric, and in equilibrium between unstructured forms in aqueous solution (state I) and bound to the surface of a lipid bilayer (state II) (16). In an acidic environment the equilibrium is shifted toward a monomeric transmembrane helical form (state III), and the process of insertion is accompanied by an energy release of about 1.8–2.0 kcal/mol in addition to the binding energy locating the peptide at the surface (16,17). Therefore, the affinity of the peptide for a lipid bilayer is about 20 times higher at low pH than at higher pHs. In pure lipid vesicles, the pKa of the transition (from state II to III) for variants of pHLIP varies from 6.0 to 7.0 (16, 17, unpublished results). The pathway of pHLIP entry into the membrane and the translocation of molecules into cells is not mediated by endocytosis, by interactions with cell receptors or by formation of pores in the cell membrane (14,15). We have shown that pHLIP insertion is associated with the protonation of two Asp residues in the transmembrane region, which leads to an increase in pHLIP hydrophobicity that immediately (within seconds) triggers folding and insertion of the peptide across a cell membrane (15,16). Conformational states of the peptide in aqueous solution and in membrane at various pHs can be monitored by tryptophan fluorescence and circular dichroism spectroscopy (Figure 1). Biophysical investigation of pHLIP in solution, attached to, and, inserted across, lipid bilayers reveal that the peptide is monomeric in all three states at low concentrations (18). The insertion of pHLIP into a membrane is unidirectional: the C-terminus goes inside a cell or vesicle, and the N-terminus stays outside (14,18). When pHLIP is bound to the surface of a bilayer near neutral pH, membrane integrity is preserved but the elastic properties are changed as reported by an increase of membrane viscosity (19). In the inserted form the peptide does not induce any significant perturbation of the lipids: it does not promote membrane fusion and it does not induce leakage of liposomes, red blood cells, or cancer cells (15,19). Further, pHLIP is not toxic for cancer cells at concentrations up to 16 mM, and does not produce any observed acute toxicity in mice (15).

DUAL DELIVERY CAPABILITIES OF PHLIP

PHLIP possesses dual delivery capabilities: it can tether cargo molecules to the cell surface and/or it can inject and release cell-impermeable cargo molecules into the cytoplasm (Figure 1). In the first scenario, a cargo molecule is attached to the pHLIP N-terminus via non-cleavable bonds, while in the second it is conjugated to the C-terminus via a bond that is cleaved in the environment of the cytoplasm, such as a disulfide (14). The conjugation of molecules to the N-terminus of pHLIP, which stays outside of the cell, does not affect the process of insertion, so cargo molecules varying in size, polarity and shape can be tethered to the cell surface. Cys or Lys residues can be introduced near the N- or C-termini to facilitate conjugation of cargo molecules to pHLIP.

IMAGING ACIDITY

The pH-selective insertion and folding of pHLIP in membranes has been demonstrated to target acidic tissue *in vivo*, including solid tumours (human and mouse), sites of inflammation and kidneys (14,20,21). Once inserted across cell membranes in acidic tissue, pHLIP remains in this state for several days. We have demonstrated that the N-terminus of pHLIP, conjugated with various fluorescent probes or a ^{64}Cu -DOTA chelate (for PET) imaging, can find and accumulate in tumours of various types established in mice, at various stages of tumour development (14,20,21). The accumulation of pHLIP in tumours directly depends on the extra cellular acidity of the tumour environment, which we have modulated by co-injection of glucose (to reduce pH) and feeding of animals with bicarbonate water (to increase pH) (20 and Yao et al., unpublished data). pHLIP targeting correlates with the aggressiveness of tumours: highly metastatic tumours, which are known to be more acidic (22) than non-metastatic ones are targeted much better by fluorescent pHLIP (Figure 2). Currently, pHLIP conjugated with various radiotracers for PET and SPECT imaging is under development.

NEW PRINCIPLES OF DRUG DESIGN

The design of drugs is often guided by the Lipinski rules of five, which postulate that a successful drug should be hydrophobic and small in order to traverse membranes and reach cytoplasmic targets (partition coefficient Log P from -0.4 to $+5.6$ and MW of 160 to 480 $\text{g}\cdot\text{mol}^{-1}$) (23). We have shown that pHLIP can inject and release large, polar, cell-impermeable cargo molecules attached to its C-terminus via an S-S bond that is cleaved in the cytoplasm (14). Among the successfully injected molecules are organic dyes, phalloidin-rhodamine (a polar, cyclic peptide of about 1300 $\text{g}\cdot\text{mol}^{-1}$), and peptide nucleic acids (PNA, about 2500 $\text{g}\cdot\text{mol}^{-1}$) (14). The energy released in the process of peptide insertion/folding (about 2 kcal/mol) can contribute to the translocation of molecules through the hydrophobic core of the cell membrane lipid bilayer, as can the increase in local concentration from localization at the membrane surface by the stages II and III binding of the peptide. These factors are sufficient to translocate polar, cell-impermeable molecules with Log P ~ -2.5 (24). Our recent data indicate that translocation of polar cargo across the membrane can be enhanced by attachment of hydrophobic molecules to the cargo or to the C-terminus of pHLIP (An et al., unpublished data). pHLIP successfully translocates fluorescently labeled phalloidin across the hydrophobic barrier of a cell membrane, where phalloidin is released by disulfide bond reduction and binds to its cellular target – F-actin (11). Actin filaments stained with fluorescent phalloidin have an unmistakable filamentous pattern, distinct from the appearance of other cellular structures, organelles or membrane staining (Figure 3). The phalloidin translocated and released in the cytoplasm of live cells leads to the inhibition of cell contractility and division. For this reason pHLIP-phalloidin might even be conceptualized as a novel potential antimetastatic drug. Due to the preferential delivery of the toxic effect to the cancer cells, side effects of such a treatment might be significantly reduced and efficacy might be enhanced. This suggestion is offered as a conceptual argument that illustrates the idea of exploring active molecules that have log P's in the -2 to -3 range for targeted delivery.

CONCLUSIONS

pHLIP provides an opportunity for the detection of extra cellular acidity by binding, and the possibility of local measurement using pH sensitive probe molecules positioned by pHLIP at the cell surface. Thus, acidity might be measured at cell surfaces in contrast to the current methods that measure the average pH in the extra cellular space (including blood vessels, where the pH is normal). Acidity measurements might be used to follow pathological progression, including in cancers, to monitor therapeutic outcomes, and possibly to predict the course of pathology (for example, tumour growth). Since fluorescently-labelled pHLIP marks tumour

borders with high accuracy, it might be used for fluorescence-guided surgery. Also, pHLIP conjugated with radiopharmaceuticals might be considered for use in a general scan to reveal acidic regions that could be further investigated as potential pathologies.

We believe that the pHLIP delivery platform could preferentially target and deliver significantly polar molecules to the cytoplasm of cancer cells, which would open a desirable area for therapeutic design. Such molecules would not cross the membranes of cells unless the delivery system was active, significantly reducing side effects, and would be preferentially targeted to acidic tissue. They would be less likely to be substrates for efflux pumps, and would be trapped in a cell once released from pHLIP. And, the chemical space for molecular design would be greatly expanded.

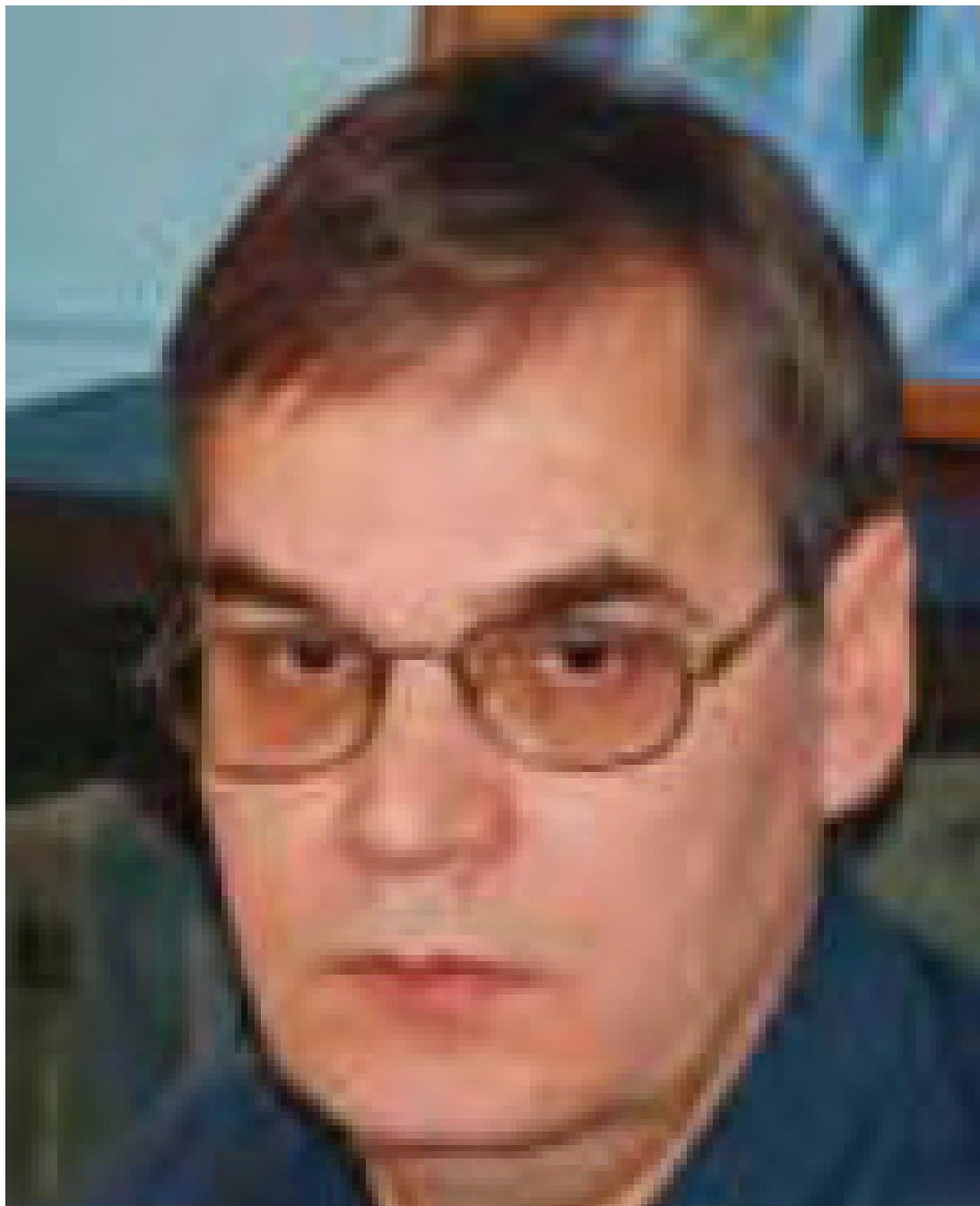
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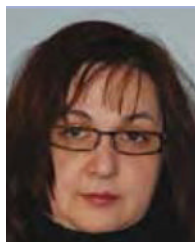
Biographies



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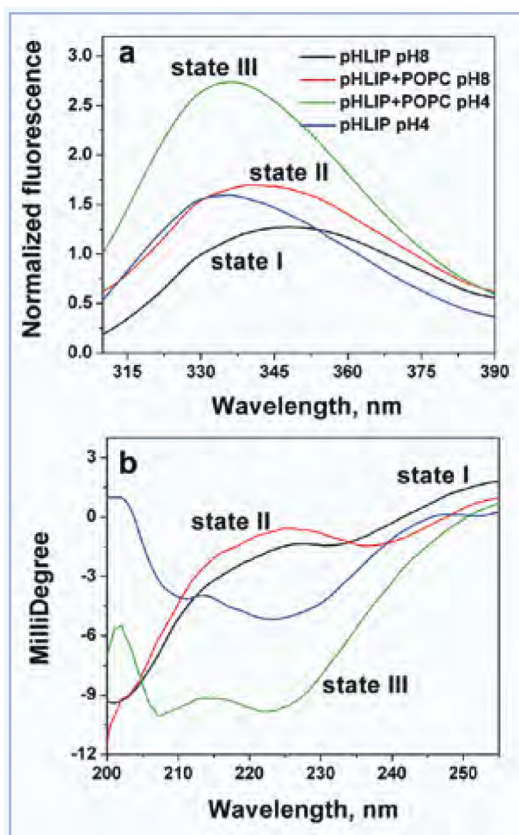


Figure 1.

Tryptophan fluorescence (a) and circular dichroism (CD) (b) spectra of $7\mu\text{M}$ of pHLIP in 10 mM phosphate buffer are shown in the absence and presence of POPC liposomes at various pHs. The fluorescence and CD spectra of pHLIP at pH8 (black line) indicate an unstructured configuration with tryptophan residues fully exposed to solvent. However at low pH (pH4) (blue line) the peptide tends to aggregate, leading to a shift of position of the fluorescence maximum and the appearance of some elements of secondary structure. Incubation of pHLIP with POPC liposomes at pH8 (red line) induces the burial of tryptophan residues inside the lipid bilayer without helix formation. Decreasing the pH to 4.0 by the addition of HCl (green line) induces the insertion of pHLIP and the formation of helical conformation. The transmembrane orientation of helix was confirmed by FTIR spectroscopy (16).

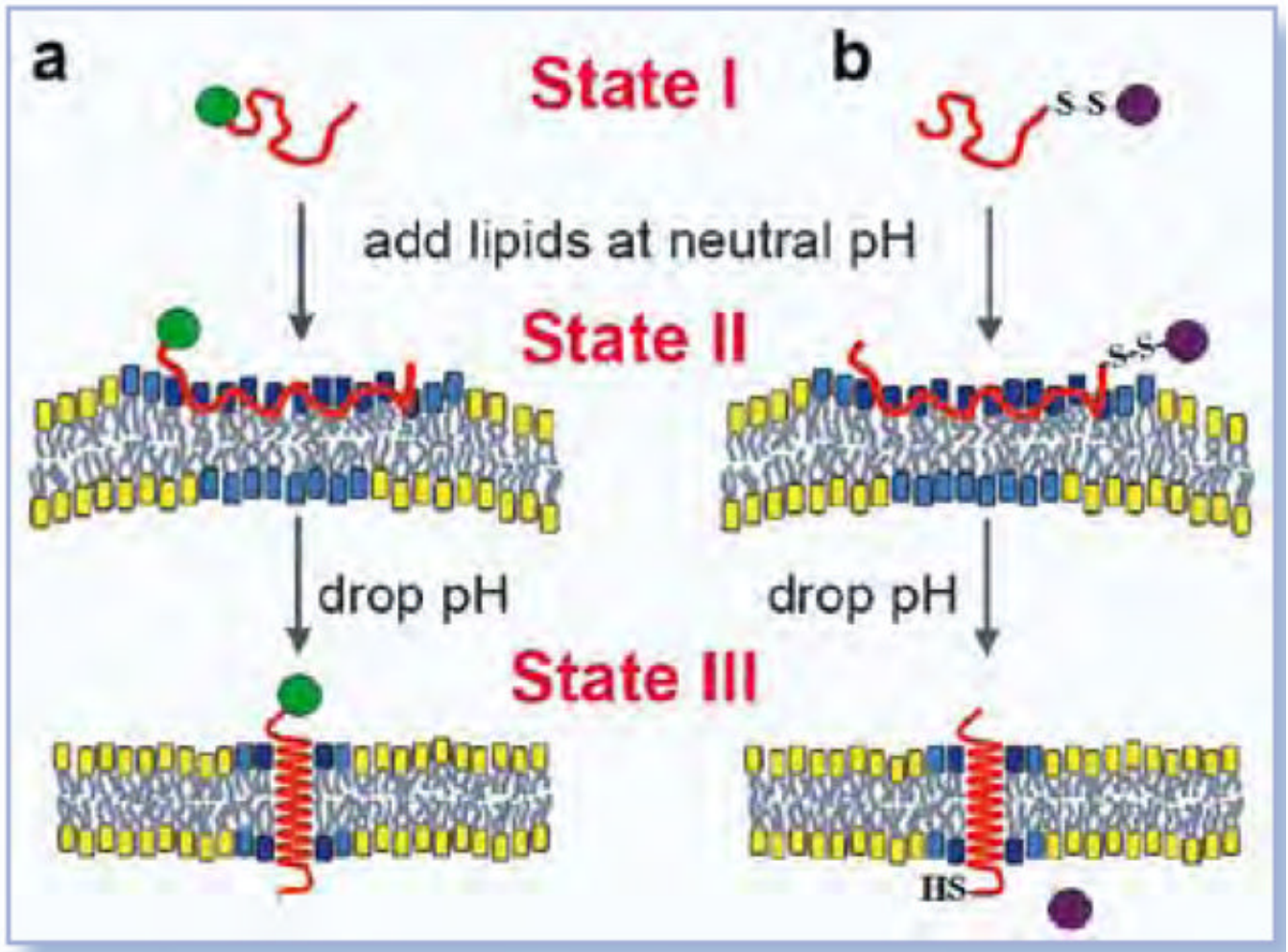


Figure 2.

A schematic representation of pHLIP dual delivery capabilities is shown: a) tethering of cargo molecules to the surface of cells with low extra cellular pH and b) translocation of cell-impermeable polar cargo molecules across the membrane lipid bilayer. State I corresponds to the peptide in solution at normal and basic pHs. By addition of vesicles, the unstructured peptide is adsorbed on the membrane surface, raising the local concentration (State II). A drop of pH leads to the protonation of Asp residues, increasing peptide hydrophobicity, and resulting in the insertion and formation of a transmembrane alpha-helix (State III). Lipids interacting with the peptide directly are marked with blue head groups, lipids influenced by the interaction but not interacting with the peptide directly have cyan head groups, and lipids that are not involved in the interaction with pHLIP have yellow head groups.

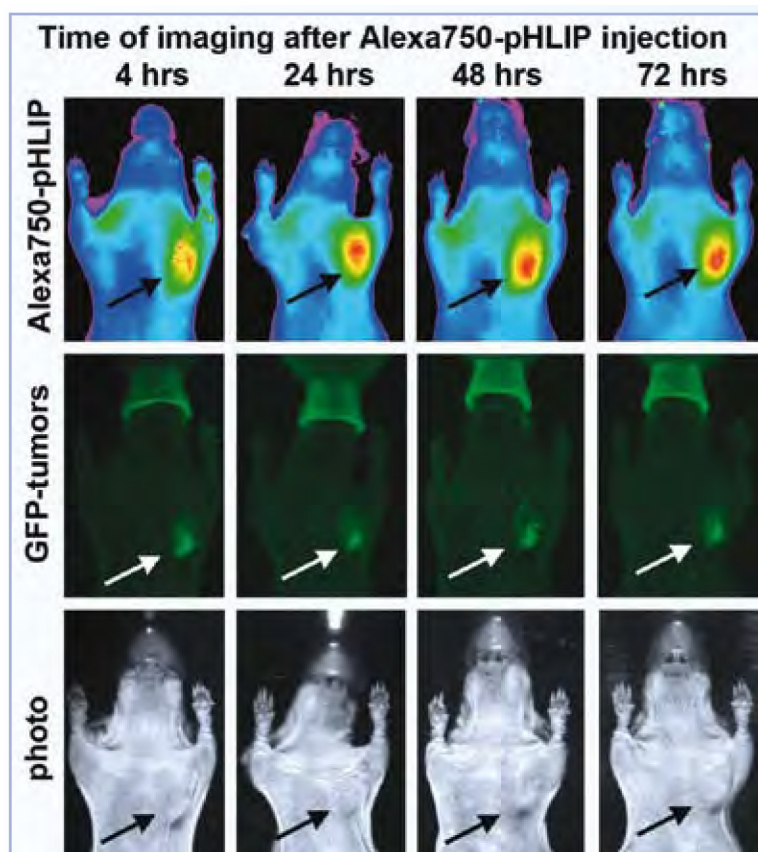


Figure 3. Whole-body NIR (Alexa750-pHLIP), GFP fluorescence and light images of mouse bearing tumour are shown. Highly metastatic M4A4 cells derived from the human melanoma cancer cell line, MDA-MB-435, were used to establish tumour. Fluorescently labelled pHLIP (Alexa750 was covalently attached to the Cys residue at the N-terminus of peptide) was given as a single iv injection (0.8 mg/kg). The images were obtained on the FX Kodak in-vivo image station combined with a gas anaesthesia system at 4, 24, 48 and 72 hours post-injection. Tumour indicated by arrows. Tumour targeting was observed already after 4 hours after injection of fluorescent pHLIP, and peptide stays in tumor during several days. Images obtained at various time points presented with maximum contrast between tumor and other tissue.

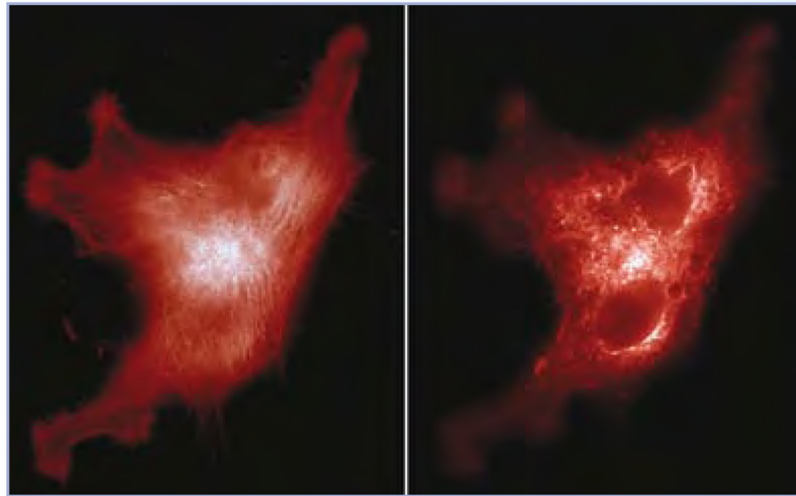


Figure 4.

The delivery of phalloidin-rhodamine into cells by pHLIP: fluorescence images obtained at different focal planes of HeLa cells incubated with a pHLIP-S-S-phalloidin-rhodamine cleavable construct at pH 6.5 are shown. Cells were incubated for 1 hour with 2 μ M of construct at pH6.5 followed by washing with PBS at pH7.4. Pictures are obtained on an Olympus IX71 epifluorescence inverted microscope.