Novel Generation of pHLIP Peptides for Targeted Delivery of Imaging and Therapeutic Payloads to Tumors

Craig Klumpp[1], Marissa Iraca[1], John Belanger[1], Anna Moshnikova[1], Oleg A. Andreev[1], Yana K. Reshetnyak[1]

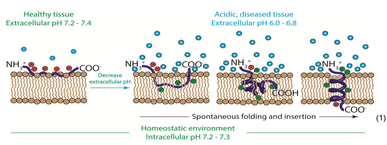
[1]Physics Department, University of Rhode Island, Kingston, RI, United States

Abstract

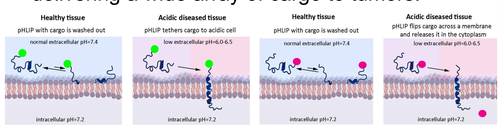
- A drug's ability to target cancer cells allows for a higher therapeutic index resulting in a more effective treatment of disease with lower risk for the patient. One such method of targeting aims at the lower pH region of the tumor microenvironment. pHLIP (pH (low) Insertion Peptide) is a novel way of targeting tumors based on this biomarker. Now a second generation of pHLIP peptides are being developed to further increase tumor targeting efficiency for future clinical use.

Introduction

- The metabolism of the cells within the TME results in an excess expression of H^+ and a bulk tumor pH of 6.5 - 6.9 with surface pHs ranging from 6.0 – 6.5.
- pHLIP peptides are constructed to fold into a transmembrane alpha helix when negatively charged side chains get protonated.



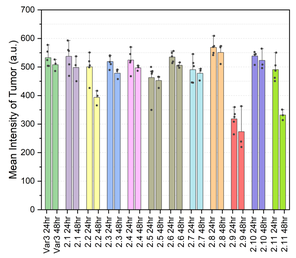
- Cargo can be conjugated to pHLIP's inserting end via a disulfide bond for intracellular delivery or attached to pHLIP's non-inserting end for cellular surface expression. The current generation Var3 peptide has shown success in delivering a wide array of cargo to tumors.



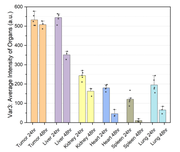
- When developing the next generation of pHLIP peptides, biophysical studies were performed on liposomes to confirm the new peptides expressed the correct properties for tumor targeting.
- Fluorescence spectroscopy shows insertion into a membrane. Circular Dichroism (CD) shows that the folded structure of pHLIP is an α-helix. Fluorescence kinetics shows the insertion rate of the peptide into the lipid membrane. Binding to human serum albumin (HSA) allows us to study pharmacokinetics. *In vivo* work allowed us to determine which peptide had the best tumor targeting capability and lead us to identify the frontrunners of the generation.

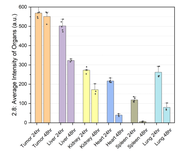
Methods & Results

Biodistribution

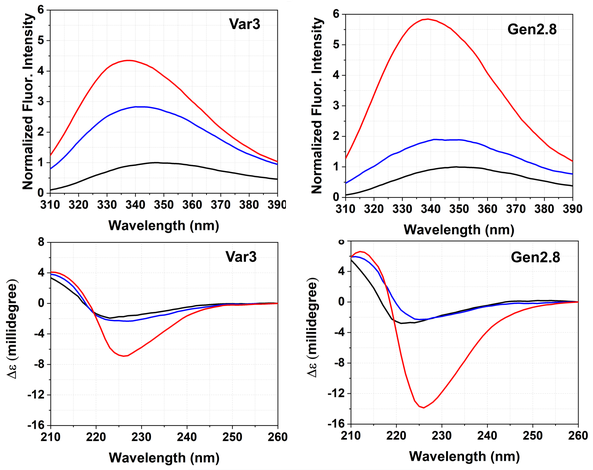


- Mice with CT26 tumors were treated using Gen2.0-ICG. At 24 h and 48 h timepoints, mice were euthanized and tumors and organs were collected. The fluorescence intensity of ICG dye was then recorded using a Stryker 1588 AIM endoscopic system.
- Biodistribution shows that Gen2.0 peptides target tumors in mice and remain at the tumor for at least 48 h. The second generation 2.8 peptide showed an increase in signal over the original Var3 peptide. ICG signal drops in organs after 24 h indicating in is not inserting and is being flushed out.



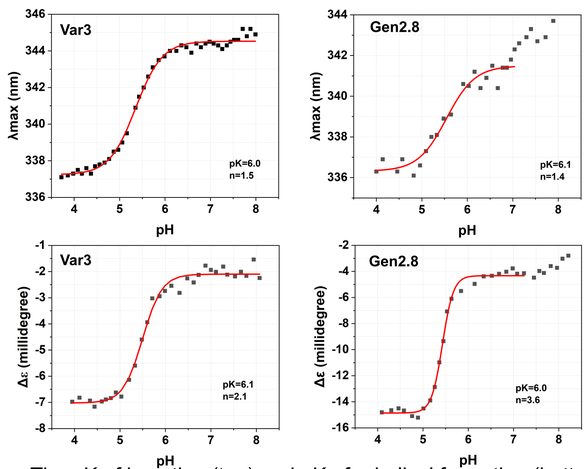


Steady State



- Fluorescence and CD spectroscopy measurements were recorded using a spectrofluorometer and spectrometer.
- Both an increase of fluorescence intensity shift in fluorescence peak position indicates both constructs insert into the lipid membrane. Circular dichroism spectroscopy shows the formation of an α-helix peak at lower pH and a random coil signal at higher pH.
- pHLIP Gen2.8 shows an approximate1.3x increase in fluorescence intensity and 2x increase in α-helical signal over pHLIP Var3.

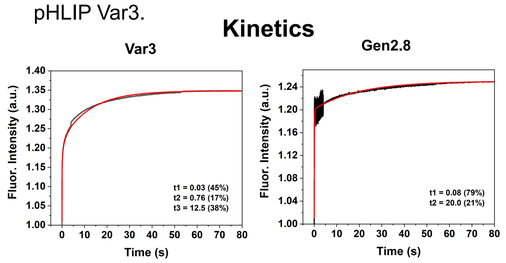
pH Dependence



- The pK of insertion (top) and pK of α-helical formation (bottom) show that that both constructs will insert at the pH of the TME.
- The pK is shifted up 0.6 to account for the lack of a pH gradient across a liposome bilayer.
- The cooperativity (n) allows us to calculate the percent of inserted pHLIP at any pH.

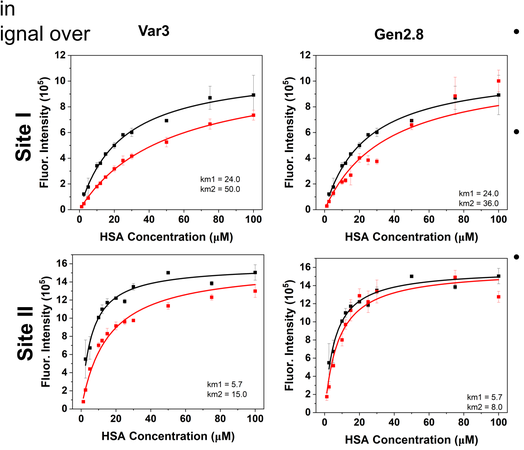
$$pH\ Dep. = S_{II} + \frac{S_{III} - S_{II}}{1 + 10^{n(pH-pK)}}$$

Kinetics



- Quick mixing pHLIP to drop the pH and recording fluorescence intensity over time allows us to find the speed of insertion and equilibration. Kinetics data shows fast insertion initial insertion of pHLIP into a lipid bilayer with slower time components of pHLIP equilibrating within the membrane.

HSA Binding



- A competitive fluorescence binding assay allows us to measure pHLIP's binding strength to HSA.
- Dansylamide (DNSA) and Dansylglycine (DNSG) are fluorescent markers that bind to specific sites on Albumin.
- Gen2.8 peptides have a weaker binding affinity to HSA than Var3.

0 μM pHLIP
5 μM pHLIP

$$y = \frac{(\mathbf{Vmax} \times x)}{(\mathbf{K_m} + x)}$$

$$\mathbf{K_m} = \frac{\mathbf{Vmax}}{2}$$

Main conclusions

- pHLIP Var3 has shown promising results for applications in tumor targeting for both imaging and therapy and pHLIP Gen2.8 shows comparable biophysical results with greater tumor targeting potential.
- With pHLIP's versatility to deliver cargo both intra and extracellularly. It has a wide variety of applications in both tumor therapy and tumor imaging.
- By improving tumor targeting potential, pHLIP can deliver cytotoxic molecules to diseased tissue while sparing healthy cells. pHLIP can also deliver immuno-modulators both intra and extracellularly to trigger an immune response to cancer. Additionally, it can deliver radiosensitizers to tumor sites to aid in radiotherapy.
- For imaging, delivering radiotracers for PET imagining has also been explored using Zirconium-89. Metallic nanoparticle have also been studied in the past for possible use for MRI imaging.
- Finally, pHLIP Var3-ICG is currently undergoing phase II clinical trials at Memorial Sloan Kettering Cancer Center for fluorescence guided surgery.

References

1. Wyatt, L. C., Lewis, J. S., Andreev, O. A., Reshetnyak, Y. K., Engelman, D. M. (2017, July). Applications of pHLIP Technology for Cancer Imaging and Therapy. *Trends in Biotechnology, 35* (7), 653-664.
2. Reshetnyak, Y. K., Moshnikova, A., Andreev, O. A., & Engelman, D. M. (2020). Targeting Acidic Diseased Tissues by pH-Triggered Membrane-Associated Peptide Folding. *Frontiers in bioengineering and biotechnology*, *8*, 335.