New York State Department of Health-Funded Prostate Cancer Research Program
Call for Proposals

PROPOSAL COVER PAGE

Title of Project: pH-Low-Insertion-Peptide (pHLIP) as a Drug Carrier Targeting Acidic Prostate Tumors

Principal Investigators and Departments: Dr. Ming An, Assistant Professor, Department of Chemistry
Dr. Lan Yao, Research Assistant Professor, Department of Physics

Funding Requested: $33,480

Project involves: (check all that apply):
□ Human Subjects x Biosafety/rDNA □ Radiation Safety □ Stem Cells
□ Vertebrate Animals x Hazardous Waste □ Select Agents □ None of the above

Project Abstract (200 words or less):

Widely used prostate cancer chemotherapy drugs, such as taxanes, are also toxic to healthy tissues, leading to life-threatening side effects and unbearable pain. To improve chemotherapy, we wish to use pHLIP to deliver drugs selectively to cancer cells. Many prostate cancer tumors are slightly more acidic than healthy tissues, pHLIP can sense this pH difference and adhere to cancer cells more tightly because of low pH. There are two hypotheses: (1) Attaching drugs to pHLIP can reduce side effects by retaining drug selectively at the tumor; (2) Tumor acidity can impede the entry of drugs (e.g. doxorubicin) into cells. pHLIP can turn acidosis against cancer cells by delivering drugs to cytoplasm in response to lower extracellular pH. Since most solid tumors are acidic (not just prostate tumors), pHLIP-mediated drug delivery may overcome the heterogeneity of tumor cells and help broad patient populations. In this proposal, we aim to show that pHLIP-drug conjugates (choice of drug includes Taxol, doxorubicin, topotecan, and vinblastine) can kill PC-3 prostate cancer cells in a pH-dependent fashion in vitro (in cell culture). In the longer term, we hope to test promising pHLIP-drug formulations in mice and ultimately advance pHLIP-mediated drug delivery into clinical trials.
**Project Narrative**

**A. Project Summary**

**A.1. Background, Significance, and Hypotheses:**

Prostate cancer is the second leading cause of cancer death in American men (mortality rate over lifetime: ~ 1 in 40). Despite advances in surgery, radiation, hormone, and cell-based immuno-therapies, chemotherapy through taxanes (i.e. docetaxel, cabazitaxel) and mitoxantrone is still a mainstay in the management of hormone-refractory, metastatic, late stage prostate cancers. Unfortunately, these chemotherapy drugs are also toxic to certain healthy tissues (e.g. bone marrow, peripheral nerves), leading to unbearable pain and life-threatening conditions. Such off-target side effects dissuade patients from continuing with treatment, and are often dose limiting, making chemotherapy not as effective as it could be.

Due to fast growth and altered metabolism of cancer cells, many malignant tumors (including prostate tumors) have extracellular pH (pHe) in the range of 6.5-7.0, compared with pHe of 7.2-7.4 in healthy tissues. Acidosis and hypoxia are more prominent in aggressive tumors. They facilitate the selection of radiation and chemotherapy resistant cancer cells, which in turn lead to tumor recurrence and metastasis. Thus, targeting tumor acidosis offers a complementary approach to established therapies. Furthermore, such an approach also constitutes an important alternative to current targeting strategies based on binding to a single protein (e.g. kinase / growth receptor), which are (a) often met with rapid resistance and (b) applicable in relatively narrow patient populations.

We propose to target acidic cancer cells using the pH-Low Insertion Peptide (pHLIP). It inserts into membrane and forms a transmembrane α-helix in response to slight acidity (Figure 1). In a transgenic prostate cancer model in mice (Figure 2), fluorescantly labeled pHLIP selectively remained in prostate tumors (this work was done by Dr. Lan Yao, a co-investigator on this grant). Because of its unique membrane insertion properties, pHLIP can simultaneously serve as (a) a biosensor of tumor acidity, and (b) a drug carrier with a novel, build-in mechanism for trans-membrane cytoplasmic cargo delivery.

For example, pHLIP-mediated delivery of the membrane-impermeable toxin phallodin inhibited the growth of cancer cells in a pH-dependent fashion (Figure 4a, this work was done by the PI). Furthermore, pHLIP-mediated delivery of an anti-sense PNA cargo silenced the target onco-miR (miR-155) in two mouse lymphoma models. In contrast, our main focus in this grant is on using pHLIP to improve chemotherapy drugs such as Taxol (the archetype taxane, microtubule-stabilizing), doxorubicin (a DNA-intercalating, topoisomerase-II inhibitor similar to mitoxantrone), topotecan (a topoisomerase-I inhibitor), and vinblastine (microtubule-dissolving).
We have two central hypotheses: (1) Conjugation of drugs to pHLIP can reduce side effects by retaining drug selectively at the tumor, thus improving biodistribution. (2) Tumor acidosis itself can reduce the potency of many drugs (e.g. doxorubicin (Dox), topotecan, and vinblastine), necessitating the use of high dose, which exacerbates off-target effects (e.g. cardio-toxicity for Dox). Many such acidosis-compromised drugs have amine group(s) that is more protonated, thus making the drug more membrane-impermeable at tumor pH. Formulation with pHLIP can turn acidosis against cancer by delivering acidosis-compromised drugs into cell cytoplasm in response to low pH. In the short term (i.e. the grant period), we aim to show that pHLIP-drug systems can preferentially kill PC3 prostate cancer cells at tumor acidity in vitro. In the long term (with external funding), we hope to test these pHLIP-drug conjugates in mice and ultimately advance pHLIP-mediated drug delivery into clinical trials.

A.2. Preliminary Data & Proposed Work:

First, we designed new pHLIP variants to improve pH response at tumor acidity (Figure 3). To close the gap between WT insertion pH50 of 6.2 (i.e. the pH at which 50% of pHLIP are inserted) and tumor average pH of 6.8 in patients, a structure-activity-relationship (SAR) study was carried out to search for desired pHLIP variants. We succeeded in that: (a) replacing Asp25 with the unnatural amino acid α-amino adipic acid (Aad) improves the pH50 to 6.74 (however, the sharpness of pH response suffered), (b) substituting Asp14 with γ-carboxyl glutamic acid (Gla) sharpens the pH-response (i.e. see black vs. green trace in Figure 3), and (c) these effects are additive — the Asp14Gla/Asp25Aad double variant shows a pH50 of 6.79 (red in Figure 3) with sharper transition than Asp25Aad.

Secondly, initial break-throughs have been achieved with pHLIP-Taxol and pHLIP-Dox. Taxol is conjugated to the C-terminus of pHLIP via a self-immolative, ‘traceless’ linker (i.e. free drug Taxol is released after disulfide cleavage in cytoplasm). Indeed Asp14Gla/Asp25Aad and P20G pHLIP-Taxol inhibited cancer cell growth in a pH-dependent fashion (pH 7.4 vs. 6.6) more effectively than WT pHLIP-Taxol. But the pH-dependence is not robust, and even WT pHLIP-Taxol is as potent as the free drug regardless of pH (Figure 4b). How do we make pHLIP-Taxol less potent at pH 7.4? We propose to use a pH-sensitive linker (maleamic acid) to attach a negatively charged modulator (e.g. 2-5 Asp/Glu) to pHLIP to discourage binding to cell surface at pH 7.4. When this polar modulator is selectively cleaved at lower pH, pHLIP-
Taxol is restored to deliver the cargo. In the case of Dox, since the free drug is more toxic at pH 7.4 than 6.6, even if pHLIP formulation gave mild (in favor of low pH) or no pH dependence in drug toxicity, it is still a gain vs. free drug, which we have already achieved with WT pHLIP-Dox (Figure 4c, PC-3 are prostate cancer cells). *The reversal of drug pH-profile is promising*. Next, we will explore if such an effect could be enhanced by using pHLIP variants. However, the potency of pHLIP-Dox is low compared to free drug. The challenge is that Dox targets DNA and topoisomerases-II, which are located in the nucleus, but the released Dox-linker compound does not reach the nucleus effectively. Therefore, as in the case for pHLIP-Taxol, we must develop a ‘traceless’ linker to release Dox exactly as the free drug (as proposed in Figure 5a).

Lastly, topotecan and vinblastine are drugs (with complementary mechanisms of action) that suffer the same fate as Dox (i.e. less toxic at low pH due to reduced membrane permeability), which make them attractive candidates for pHLIP formulation. We will do initial studies of pHLIP-vinblastine and pHLIP-topotecan conjugates if time allows (Figure 5b/c).

**B. Team Strengths and Member Contributions.** Our research team consists of Dr. Ming An (PI), Dr. Lan Yao (co-PI), Lukas Klees (2nd yr. graduate student), and Anqi Zhang (2nd yr. graduate student). Dr. An and Dr. Yao have studied pHLIP for the past 8-9 yrs. We have published 8 papers on pHLIP since 2009 (plus 15 meeting presentations and 6 invited colloquium talks), including 3 high quality papers from our independent An-Yao lab (2015 *Angewandte Chemie*, impact factor 11.3; 2015 *Nature Communications*, i.f. 11.5; and 2014 *ACS Chemical Biology*, i.f. 5.3). Dr. An and Dr. Yao will jointly lead overall research efforts (see budget justification for more details). Mr. Klees and Ms. Zhang will carry out chemical synthesis, biophysical studies, and cellular assays of pHLIP-drug conjugates, as well as manuscript writing. In summary, we have the theoretical knowledge and the experimental know-how to lead this project to success.

**C. Benefits to Binghamton University and Strategy for External Funding.** The proposed project, if funded, can bring benefits to our university through external funding and enhanced reputation. We believe pHLIP-mediated drug delivery has direct application in improving cancer chemotherapy. Thus, various aspects of the pHLIP project are fundable with a number of agencies. In Fall 2015 we have submitted pHLIP proposals to NIH (NIGMS R35, $1,803k total cost), NSF (MCB-Molecular Biophysics, $815k t.c.), and American Cancer Society (ACS-RSG, $792k t.c.). In the April to July period of 2016, we plan to submit additional pHLIP-based applications to NIH (NCI: R21/R01, NIGMS: R01) and NSF (CAREER award). Furthermore, we obtained promising preliminary data using the Start-up fund (now 90% gone). As we continue to publish results in high impact factor journals, the visibility of our university in cancer research and the chance of funding will be improved. So far we reached ‘pay-if’ status with ACS-RSG in March of 2015 (9-13 percentile, passed review, requires donor pick-up). We may be on the cusp of being funded. However, as of yet, we have not received external funding. The PI is likely to go up for tenure review during 2017-18. Thus, this grant is a crucial bridging fund to sustain our on-going efforts in this limbo period.


# Proposal Budget

## A. Personnel
(Indicate percent effort, salary, and names of personnel)

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<thead>
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<th>Personnel</th>
<th>One Year</th>
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<tbody>
<tr>
<td>Grad Student 1&amp;2 @ 100% summer effort (3 months)</td>
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<tr>
<td>Fringe Benefits Total</td>
<td>1,440</td>
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<tr>
<td>CATEGORY TOTAL</td>
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## B. Permanent Equipment (Itemize)

<table>
<thead>
<tr>
<th>CATEGORY TOTAL</th>
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## C. Supplies (Group into major categories)
- Buy or synthesize peptides | 5,000 |
- Synthetic chemistry efforts / biophysics supplies | 5,000 |
- Cell culture work | 7,000 |
- Equipment or facility rental/user fees | 1,000 |
- Other lab supplies | 2,040 |
| CATEGORY TOTAL | 20,040 |

## D. Travel (Domestic only)
- PI / key personnel / graduate student | 3,000 |
| CATEGORY TOTAL | 3,000 |

## E. Miscellaneous
(List specific amounts for each item)
- Publication fees | 1,000 |
| CATEGORY TOTAL | 1,000 |

## F. Subcontracts (Categorize on continuation page)

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## G. Indirect Costs: ____% (Excluding permanent equipment)
- TOTAL INDIRECT COSTS
- TOTAL ANNUAL COSTS | 33,480 |

## H. Total Amount Requested
(Sum of all years including indirect costs; transfer this amount to the budget section of the on-line form) | $33,480 |
JUSTIFICATION OF BUDGET

Personnel:
**Principal Investigator, Dr. Ming An**, Assistant Professor. No salary support is requested. PI is on 9-month academic-year salary. He will lead research efforts on the optimization of pHLIP-mediated drug delivery, including experimental design, data interpretation, and manuscript preparations. He will direct efforts of all participants in the lab. In addition, PI will contribute to lab work requiring particular expertise in organic synthesis (> 20 years of experience). Dr. An has studied pHLIP-mediated cargo delivery for 9 years.

**Co-Investigator, Dr. Lan Yao**, Research Assistant Professor. No salary support is requested. Dr. Yao will train and oversee efforts of all students from the An-Yao lab, and meet with them on daily basis to review results and to plan experimental strategies. Dr. Yao will also be responsible for biophysical characterization and cell culture evaluations of pHLIP-drug systems. Dr. Yao has 8 years of experience in various studies of pHLIP.

**Graduate Research Assistants, Lukas Klees and Anqi Zhang**, (100% summer effort/year for both students): Funds are requested to support 2 graduate students (Mr. Lukas Klees and Ms. Anqi Zhang) during summer only. Mr. Klees and Ms. Zhang will carry out chemical synthesis, biophysical studies, and cellular assays of the proposed variant pHLIP-drug conjugates, as well as manuscript preparations.

Fringe Benefits:
The Research Foundation’s Fringe Benefit Rate (FBR) for graduate research assistants is 18% in year 1.

Supplies:
Funds are requested for the purchase or syntheses of crude pHLIP peptides ($5,000).
Funds are requested for HPLC supplies (including solvents, columns and service), lipids and other supplies for biophysical studies and chemical reagents for pHLIP-drug syntheses ($5,000).
Funds are requested for cancer cell lines, cell culture supplies and bio-reagents and MTS assay reagents ($ 7,000).
Funds are requested for service and instrument / equipment / facilities user fees (Mass Spectroscopy send out, confocal microscope time, ect. ($1,000)
Funds are requested for other lab supplies including small equipment, computer, software, glassware, general chemicals, gases and disposable lab supplies, etc. ($2,040)

Travel:
Funds are requested for the PIs and/or graduate students to attend domestic conferences in order to share and disseminate research findings.

Publication Expenses:
Funds are requested for publications activities, including editorial service (manuscript preparation, open access fees, page charges, fees for color illustration, reprint orders, etc.).
Biographical Sketch

Ming An, Ph.D.
Assistant Professor
Department of Chemistry, SUNY-Binghamton University
4400 Vestal Pkwy East, Binghamton, NY 13902
607-777-3224
aming@binghamton.edu

A. Professional Preparation
University of Michigan, Ann Arbor / Chemistry & Molecular Cellular Biology / BS (Honors) 1996
University of California, Berkeley / Organic Chemistry & Mechanistic Enzymology / PhD 2003
University of California, San Francisco / Chemical Biology & Neuroscience / Postdoc 2003-05
Yale University / Membrane Biophysics / Postdoc 2007-11

B. Appointments
September 2011 — present: Assistant Professor, Dept. of Chemistry, State University of New York - Binghamton University

C. Products (names of undergraduate co-authors are underlined)

Most closely related to proposed project:

Other significant products:
D. Synergistic Activities

1. **Mentoring Undergraduate Researchers:** Since Fall 2011, the PI has mentored 15 undergraduates, including seven female students and one student from under-represented groups (see Activity 2 below). Three undergraduates have (and in addition, 11 will) become co-authors to peer-reviewed publications: Rachel Langenbacher (2 papers out (PO), 1 under preparation (UP)), Michael S. Chung (2PO, 1UP, stayed for 1 yr. after graduation to obtain MS degree), Chee-Huat Eng (1PO, 2UP), Emma A. Gordon (2UP), Syris Winge-Barnes (1-2UP), Rebecca Chandler (1UP), Raemer J. Lapid (1UP), Elizer Lichter (2UP), Meghan Bell (1UP), Vladyslav Nazarenko (1-2UP), Ashley Fancher (1UP), Jason Ng (1UP), Christie Murray (1UP), Ilana Bandler (1UP).

2. **Faculty Advisor for Minority Students:** Mentored Anthony Awad, a MA Biology graduate student from under-represented minorities (African American). The PI also participated in HHMI-Binghamton University Interdisciplinary Undergraduate Research Program (2011-2014): mentored one minority (African American) undergraduate researcher: Syris Winge-Barnes (Summer 2013 to May 2014), who has at least one paper under preparation.

3. **Peer Reviewing:** Since Fall 2011, this PI reviewed several manuscripts for *Organic Letters, ACS Chemical Neuroscience, Neuropharmacology, Biochemistry, and Bioorganic & Medicinal Chemistry Letters.*

E. Collaborators and Other Affiliations

1. **Collaborators**
   - Christof Grewer: SUNY, Binghamton University
   - Juntao Luo: SUNY Upstate Medical University
   - Gretchen Mahler: SUNY, Binghamton University
   - Joong-ho Moon: Florida International University
   - Wei Qiang: SUNY, Binghamton University
   - Lan Yao: SUNY, Binghamton University

Total: 6

2. **Graduate and Postdoctoral Advisors**
   - Donald M. Engelman: Yale University (Postdoctoral Advisor, 2007-2011)
   - Pamela M. England: University of California, San Francisco (Postdoctoral Advisor, 2003-05)

3. **Graduate Students and Postdocs**
   - **Graduate Students:**
     - Joab O. Onyango (PhD, 2011-14, currently Assistant Prof. at Technical University of Kenya)
     - Michael S. Chung (MS, 2013-14, currently in MD-PhD at U. of Connecticut School of Medicine)
     - Anthony Awad (MA, 2013-15, American University of the Caribbean School of Medicine)
     - Lukas Klees (second yr. PhD student, 2014-current)
     - Anqi Zhang (second yr. PhD student, 2014-current)

Total: 5

**Postdoc:** None so far.
Biographical Sketch

Lan Yao, Ph.D.
Research Assistant Professor
Department of Physics, Binghamton University, SUNY
4400 Vestal Pkwy East, Binghamton, NY 13902
607-777-3224
lan>yao@binghamton.edu

A. Professional Preparation.

<table>
<thead>
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<th>Institutes</th>
<th>Location</th>
<th>Major</th>
<th>Degree &amp; Years</th>
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<td>Wuhan University of Technology</td>
<td>Wuhan, China</td>
<td>Mechanical Engineering</td>
<td>B.S., 1998</td>
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<tr>
<td>Wuhan University of Technology</td>
<td>Wuhan, China</td>
<td>Mechanical and Electrical Engineering</td>
<td>M.S., 2001</td>
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<tr>
<td>University of Rhode Island</td>
<td>Kingston, RI</td>
<td>Physics</td>
<td>Ph.D., 2008</td>
</tr>
</tbody>
</table>

b. Appointments

2011-present Research Assistant Professor, Department of Physics, Applied Physics and Astronomy, Binghamton University, State University of New York, Binghamton, NY

c. Products (the names of undergraduate co-authors are underlined)

Most Closely-related Products


Other Significant Products


d. Synergistic activities

Mentoring undergraduate research

- As a postdoctoral fellow, I mentored one undergraduate student (Jennifer Danniels) for one year, who became a co-author for two peer-review papers.
- Since Fall 2011, I have mentored 10 undergraduates (Rachel Langenbacher, Michael Chung, Daniel Plavin, Reamer Lapid, Emma Gordon, Syris Winge-Barnes, Rebecca Chandler, Chee Huat Eng, Meghan Bell, Vlad Nazarenko). Three of them have become co-authors to peer-reviewed publications.

e. Collaborators and other affiliations

1. Collaborators:

   Ming An          SUNY, Binghamton University
   Christof Grewer  SUNY, Binghamton University
   Juntao Luo       SUNY Upstate Medical University
   Gretchen Mahler  SUNY, Binghamton University
   Joong-ho Moon   Florida International University
   Wei Qiang        SUNY, Binghamton University
   Total: 6

2. Graduate and Postdoctoral Advisors:

   Oleg A. Andreev  University of Rhode Island (Ph.D. Advisor)
   Yana Reshetnyak  University of Rhode Island (Postdoctoral Advisor)
   Total: 2

3. Graduate Students and Postdocs

   None