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14. ABSTRACT

Mesothelioma therapy is a highly fatal disease that has poorly effective therapy with dose-limiting side-effects. Low expression of the CDK4/CDK6 inhibitor p16INK4a has been demonstrated in up to 90% of mesothelioma tumors. The objective of this application as a next step in the pursuit of this long term goal is to identify stabilized peptides that will mimic the interaction between p16INK4a and CDK4/6. The central hypothesis of this proposal is that protein-protein interactions can be replicated or disrupted by stabilized peptides that have been identified via the identification of pharmacophores of small peptides that interact with CDK4/6. The specific aims are as follows. (1) Determine structure-function relationships of overlapping peptides derived from p16INK4a that inhibit the activity of CDK4/6 and identify stabilized peptides that inhibit CDK4/6. (2) In vitro functional studies will be used to evaluate bioactivities of stabilized peptides. (3) In vitro ADME studies to evaluate the cell permeability, delivery, and efficacy of stabilized peptides.

15. SUBJECT TERMS
Mesothelioma, CDK4, CDK6, peptides, protein-protein interactions

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Table of Contents

Page

1. Introduction……. 4

2. Keywords……. 4

3. Overall Project Summary……... 4

4. Key Research Accomplishments…… 16

5. Conclusions……...…… 17

6. Publications, Abstractions, and Presentations…… 17

7. Inventions, Patents, and Licenses…….…….…..……. 18

8. Reportable Outcomes…….. 18

9. Other Achievements…… 18

10. References…… 18

11. Appendices…… 19

1. Introduction

Mesothelioma therapy is a highly fatal disease that has poorly effective therapy with dose-limiting side-effects. Low expression of the CDK4/CDK6 inhibitor $p16^{INK4a}$ has been demonstrated in up to 90% of mesothelioma tumors. Replacement of $p16^{INK4a}$ activity in laboratory models has demonstrated activity against CDK4 and CDK6, tumor response, and increase in survival in xenograft models. The long term goal of this laboratory is to identify key principles of drug discovery that will allow the appropriate and selective disruption or replacement of protein-protein interactions via stabilized peptides. The objective of this application as a next step in the pursuit of this long term goal is to identify stabilized peptides that will mimic the interaction between $p16^{INK4a}$ and CDK4/6. The central hypothesis of this proposal is that protein-protein interactions can be replicated or disrupted by stabilized peptides that have been identified via the identification of pharmacophores of small peptides that interact with CDK4/6. The specific aims are as follows. (1) Determine structure-function relationships of overlapping peptides derived from $p16^{INK4a}$ that inhibit the activity of CDK4/6 and identify stabilized peptides that inhibit CDK4/6. (2) *In vitro* functional studies will be used to evaluate bioactivities of stabilized peptides. (3) In vitro ADME studies to evaluate the cell permeability, delivery, and efficacy of stabilized peptides. We are using structural biology, computational modeling, *in vitro* efficacy, and *in vitro* ADME studies to design and evaluate anti-mesothelioma peptides. Work funded by this grant opportunity will lead to progress in targeted therapy with the potential for fewer side effects and higher efficacy.

2. Key words

peptide
mesothelioma
cell cycle
cyclin-dependent kinase (CDK)
protein-protein interaction
molecular dynamics
nuclear magnetic resonance (NMR)

3. Overall Project Summary

Major Goals of the Project (as defined in the Statement of Work)

Statement of Work

Specific Aim #1. Determine structure-function relationships of overlapping peptides derived from $p16^{INK4a}$ that inhibit the activity of CDK4/6 and identify stabilized peptides that inhibit CDK4/6. The pharmacophore(s) of mutated peptides from $p16^{INK4a}$ will be assessed via time-resolved fluorescent resonance energy transfer assays and nuclear magnetic resonance spectroscopy to elucidate which residues are important to bind CDK4 and/or CDK6. Stabilized peptides will be evaluated for inhibitory activity toward CDK4/6.

1. Task 1. Identification of the amino acid substitutions that enhance peptide. (Months 1-15)

a. Molecular dynamics analysis of proposed peptides. (Months 1-4) 80% accomplished
b. Analysis of stapled derivatives from peptides p16_10 and p16_20. (Months 3-6) 90% accomplished
c. Determination of the 3-D structure of the most active peptides via NMR. (Months 6-12) 40% accomplished
d. Enzyme kinetic analysis of the active peptides discovered. (Months 13-15) 0% accomplished

Milestone #1. Identification of all pharmacophores and peptides by the end of year 1.5. 80% accomplished

Specific Aim #2. *In vitro* functional studies will be used to evaluate bioactivities of peptidomimetics and stabilized peptides.

2. Task 2. Determination of the intracellular effects of peptides and compounds determined in Aim 1. (Months 13-24)
    a. Determination the ability of $p16^{INK4a}$-mimetic peptides to enter cells (Months 13-15) 10% accomplished (estimation on cell activity, will study further with more active peptides)
    b. Determination of the cell growth inhibitory activity of mimetics of $p16^{INK4a}$. (Months 16-18) 90% accomplished
    c. Evaluate how $p16^{INK4a}$ mimetics inhibit Rb phosphorylation in vitro. (Months 19-21) 90% accomplished
    d. Evaluation of efficacy of p16ink4a mimetics to prevent cell cycle progression into S-phase. (Months 22-24) 90% accomplished
    e. Determine if apoptosis is a mechanism of cell death after treatment with peptidomimetics. (Months 22-24). 90% accomplished

Milestone #2. Identification of the intracellular activity of small molecules and peptides by the end of year 2. 60% accomplished

Specific Aim #3. *In vitro* ADME studies to evaluate the cell permeability and potential delivery of peptidomimetics and stabilized peptides.

3. Task 3. ADME studies of compounds and peptides evaluated in aims 1 and 2. (Months 13-24) 0% accomplished
    a. Determine the metabolic stability of identified peptides and identification of responsible enzymes in metabolism of the peptides. (Months 16-18) 0% accomplished
    b. Cell permeability and transport studies of stapled peptides (Months 19-21) 0% accomplished
    c. Determination of plasma protein binding of identified peptides. (Months 22-24) 0% accomplished

What was accomplished under these goals?

Aims 1 and Aims 2 were pursued in parallel. The % accomplishment is listed after each goal. We have made significant discoveries and accomplishments under Aims 1 and 2.

Examination of Effects of Stabilized Peptides and Palbociclib.

We are currently evaluating iterations of TAT-peptides or stapled peptides (in comparison to palbociclib) in CDK4/6 enzymatic assays, cellular proliferation assays, for effects on Rb phosphorylation and cell cycle arrest. Stabilizing peptides were demonstrated to be a viable option for potential peptide therapeutics, and stabilized peptides are larger than small molecules. Larger sizes of peptides most likely will allow more effective targeting of protein-protein interactions. **Table 1** includes IC_{50} values encompassing multiple experiments – most of these results were obtained before our last annual report as a frame of reference for this report. The first 2 columns reflect the IC_{50} values derived from TR-FRET-based assays. These assays are used to evaluate the inhibition of CDK4 and CDK6 enzyme activity by the respective peptides or compound. It is a measure of the inhibition of phosphorylation of an Rb-based peptide substrate. The 3 final columns represent the IC_{50} values for the peptides or compound as tested in 3 separate mesothelioma cell lines. The Tat refers to a leader sequence (YGRKKRRQRRR) that is recognized to increase cell permeability of peptides. These sequences dramatically increased peptide efficacy in decreasing cell proliferation. In contrast to our previous experience, the stapled peptides did not enter into the cells well. These experiments were done with a new batch of peptides obtained from New England Peptide, Inc. There was significant precipitation at high concentrations (above 100 μM) that was not seen with previous batches of stapled peptide. As a reference for our peptides, we have included a previously obtained NMR model of the base 10mer peptide in A, the starting model of the 20mer peptide for molecular dynamics simulations (results listed later) in B, and the starting model for the stapled 10mer peptide molecular dynamics simulations in C. Results of molecular dynamics simulations are listed near the end.

Treatment of mesothelioma cell lines show a decrease in Rb phosphorylation when treated with palbociclib (**Figure 2** is an example) and both Tat peptides. Initial experiments have not shown the same degree of inhibition for native peptides or stapled peptides tested thus far, and we are further evaluating these experiments. Palbociclib was analyzed before the last annual report, and the peptides were analyzed after.

Palbociclib or peptides were evaluated for effects on the cell cycle. Treatment (with peptide or palbociclib) or control in mesothelioma cells was for a 24 hour incubation. Palbociclib distinctly inhibited the cell cycle at G1/S with a significant increase in the percent of cells in G1

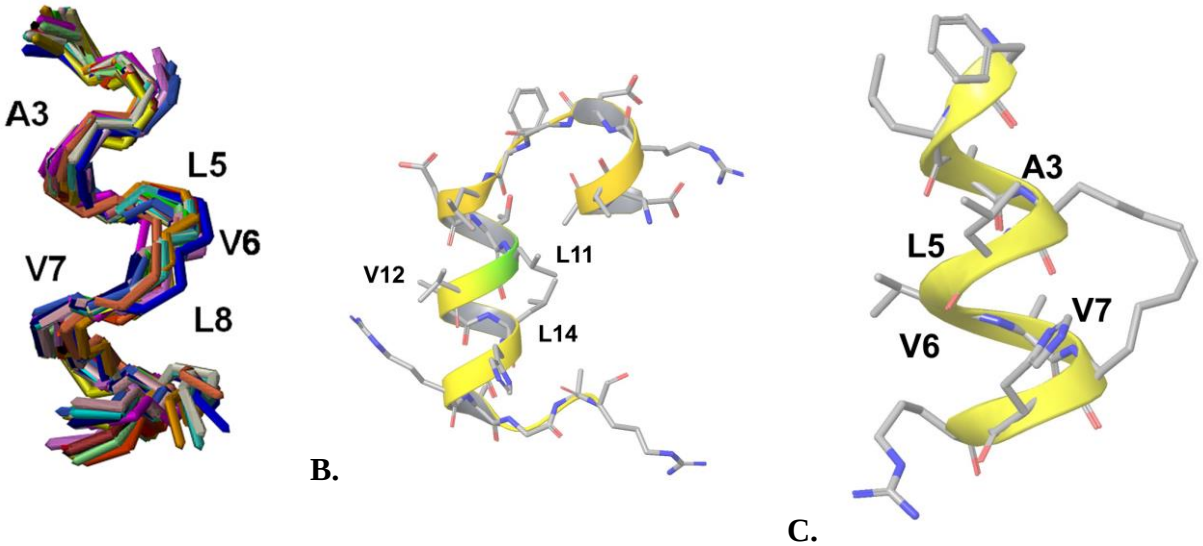

Figure 1. A. NMR-derived p16_10. B. Native 20mer C. Stapled 10mer

as evaluated by flow cytometry (**Table 2, Figure 3**). Similar results for native peptides (no TAT- or staples) demonstrated no effects on cell cycle as expected if they cannot enter cells (**Table 3**). TAT 10mers and 20mers were previously shown to inhibit cell cycle at G1/S at 50 μM. However, at 30 μM, two interesting findings occur (**Table 4**): 1) the S phase readings drop off halfway through S phase (not shown) and 2) the percent of cells of cells in G2 drops greatly. These findings suggest that either potential non-competitive inhibition of CDK4/6 or disruption of other protein-protein interactions may affect regulation of S phase and/or G2 phase. Data analysis also demonstrated that palbociclib induces apoptosis in mesothelioma cells (not shown).

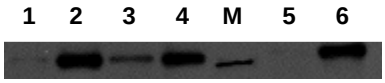

Figure 2. Immunoblotting results. Blots were probed with phospho-specific antibody to Rb. Lanes 1, 3 and 5 are treated with palbociclib, while lanes 2, 4, and 6 showed untreated cells. M, molecular weight marker. Lanes 1 and 2, 3 and 4, and 5 and 6 represent 3 separate mesothelioma cell lines. Lanes were evaluated qualitatively. Actin (not shown) was similar in all lanes.

In addition, we have molecular dynamics studies underway. Dr. Yuk Sham has assisted with these utilizing the Minnesota Supercomputing Institute. Even though we have NMR-derived structural evidence that a peptide forms a helical structure, a peptide can be quite mobile in solution, potentially sampling multiple conformations. Molecular dynamics can simulate this motion, to see if the propensity is to stay in a particular conformation, or multiple conformations. There is evidence that **increased helical propensity of a peptide may correlate with increased protein binding**[38]. If a smaller proportion of the peptide population changes from less structured conformation (i.e. closer to coil) to a more structured conformation (i.e. helix or beta sheet), then a smaller entropic penalty is "paid."

The PI has conducted several molecular dynamics simulations of several variants of the native 10mer and 20mer peptide. For the 10mer, the NMR-derived structure of the peptide with sequence FLATLVVLHR was used as a starting structure. The program DESMOND was used to conduct molecular dynamics simulations for 10 nanoseconds[39]. Alanines were subsequently substituted at each site except A3, where the original aspartate was substituted. **Figures 3 and 4** shows the percent helicity, beta sheet, or coil per peptide. There was a significant decrease in helicity in V7A, suggesting that helix formation at this residue. For the other residues, the beta sheet population increased, suggesting that a switch from helix to beta-sheet conformation may pull the V6, L8, H9, and R10 to a less favorable conformation. This is the percent conformation (denoted percent helicity on the Y axis) for beta, helical, or coil

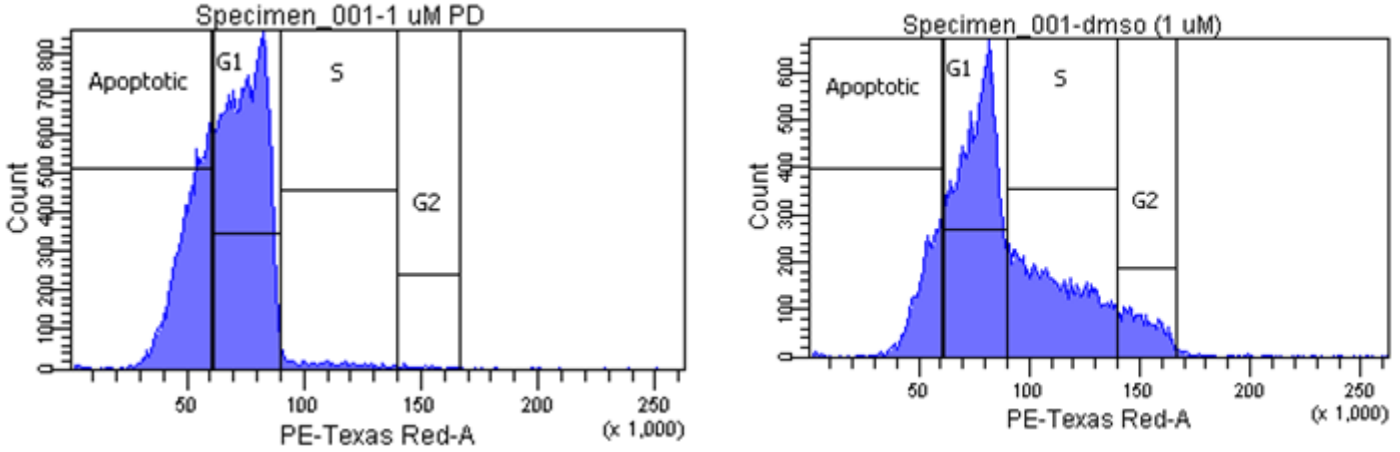

Figure 3. Flow cytometry analysis. Left panel, control mesothelioma cells. Right, cells treated with 1 μM palbociclib. The proportion of cells in G1 phase vs. S and G2 significantly increases after treatment with palbociclib.

conformation. We are analyzing CDK4/6 assays to determine if the IC_{50} values correlate to predict structure (nearly complete). The PI has also conducted simulations of the 20mer. The starting structure is the native helix-turn-helix from the $p16^{INK4a}$-CDK6 crystal structure coordinates. As this peptide is larger, similar substitutions showed a slightly different effect on predicted conformation population. Substitution of alanine for leucine at position 11 in the 20mer (corresponding to the leucine at position 5 in the 10mer) shows a greater decrease in helix propensity than in the 10mer. In addition, the substitution of the aspartate with alanine shows a larger change in formation in the 20mer compared to the 10mer. We are working on a manuscript to publish these findings. Simulations are underway for the stapled peptides and peptides with substitutions at several positions (D3, L5, V6, V7, and V8, especially in the 10mer and corresponding positions in the 20mer). Figure 5 shows the RMSD of select peptides. The larger RMSD corresponds with lower structural stability.

We have also begun molecular dynamics of these peptide lengths in the context of the larger p16 protein.

Table 1.	**IC_{50} values: 1st 2 columns: Peptide or compound tested in enzymatic assay against CDK4 or CDK6 (Last 3 columns are peptide or compound tested against mesothelioma cell lines)**				
Peptide or Compound	**CDK4**	**CDK6**	**2373**	**2461**	**2596**
10mer	21.2 μM	20.8 μM	> 1 mM	> 1 mM	> 1 mM
20mer	19 μM	14.6 μM	> 1 mM	> 1 mM	> 1 mM
Tat 10mer	2.47 μM	Analyzing	33.3 μM	37.6 μM	44.2 μM
Tat 20mer	860 nM	257 μM	42.7 μM	88.2 μM	43.2 μM
Stapled 10mer	55.3 μM	105 μM	> 1mM	> 1mM	> 1mM
Stapled 20mer	49 μM	87.4 μM	> 1mM	> 1mM	> 1mM
palbociclib	9.48 nM	97.5 nM	1.6 μM	Analyzing	26.9 μM

Table 2. Cell Cycle Analysis of Palbociclib in Mesothelioma Cells				
	%G1	**%S**	**%G2**	**Apoptotic**
Control DMSO for 1 μM	**54.45**	**30.65**	**7.1**	**7.75**
Palbociclib 1 μM	**71.35**	**8.1**	**1.3**	**19.85**
Control DMSO for 10 μM	**50.13**	**32.23**	**8.07**	**8.6**
Palbociclib 10 μM	**72.63**	**8.67**	**3.77**	**14.83**

Table 3. Cell Cycle Analysis of Native Peptides and one Stapled Peptide in Mesothelioma Cells			
	%G1	**%S**	**%G2**
Control DMSO	**63.33**	**27.53**	**8.03**
Stapled 10mer 30 μM	**61.2**	**31.5**	**5.9**
Native 10mer 30 μM	**65.55**	**29.35**	**3.7**
Native 20mer 30 μM	**67.9**	**27.25**	**3.45**

Table 4. Cell Cycle Analysis of TAT-peptides in Mesothelioma Cells			
	%G1	**%S**	**%G2**
Control DMSO	**65.5**	**26.6**	**6.75**
TAT 10mer 10 μM	**63.55**	**27.8**	**0.85**
TAT 10mer 30 μM	**64.1**	**31.75**	**1.95**
TAT 20mer 10 μM	**60.05**	**34.35**	**4.35**
TAT 20mer 30 μM	**65.3**	**25.75**	**0.15**

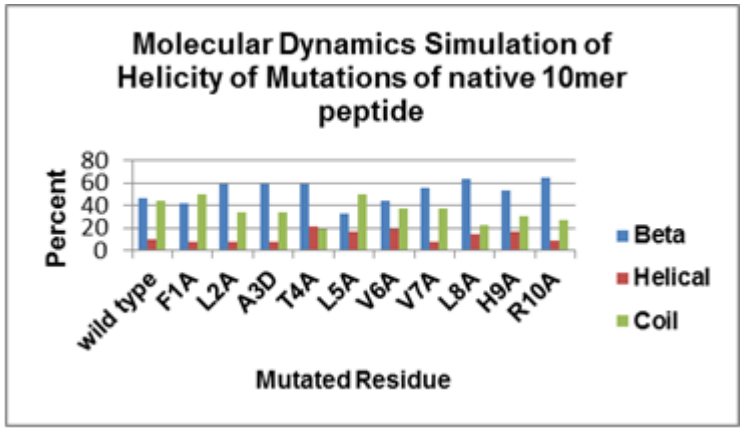

Figure 3. Percent conformation determined via molecular dynamics simulations for 10mer and alanine-scanned derivatives. Y axis, Percent of all computational snapshots in beta, helical, or coil conformation (color coded). X axis, 11 separate peptides, each represented by a single alanine substitution designation.

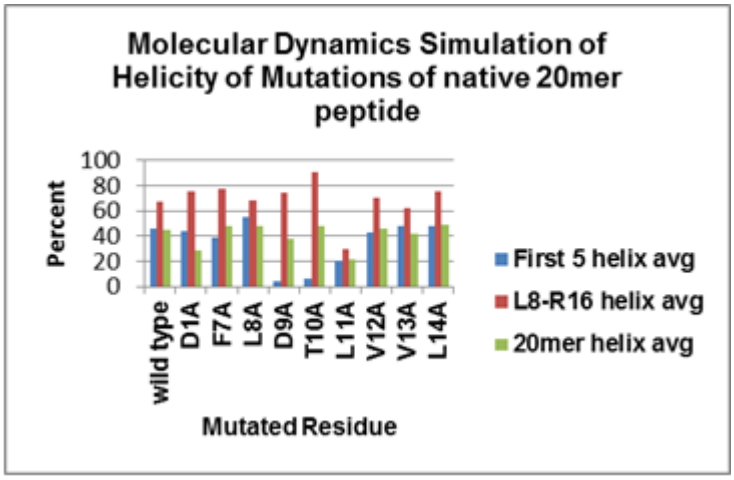

Figure 4 . Percent helical conformation determined via molecular dynamics simulations for 10mer and alanine-scanned derivatives. Y axis, Percent of all computational snapshots in helical conformation as determined for either the 1st 5 peptide residues, residues 8-16, or entire peptide (color coded). X axis, 11 separate peptides, each represented by a single alanine substitution designation.

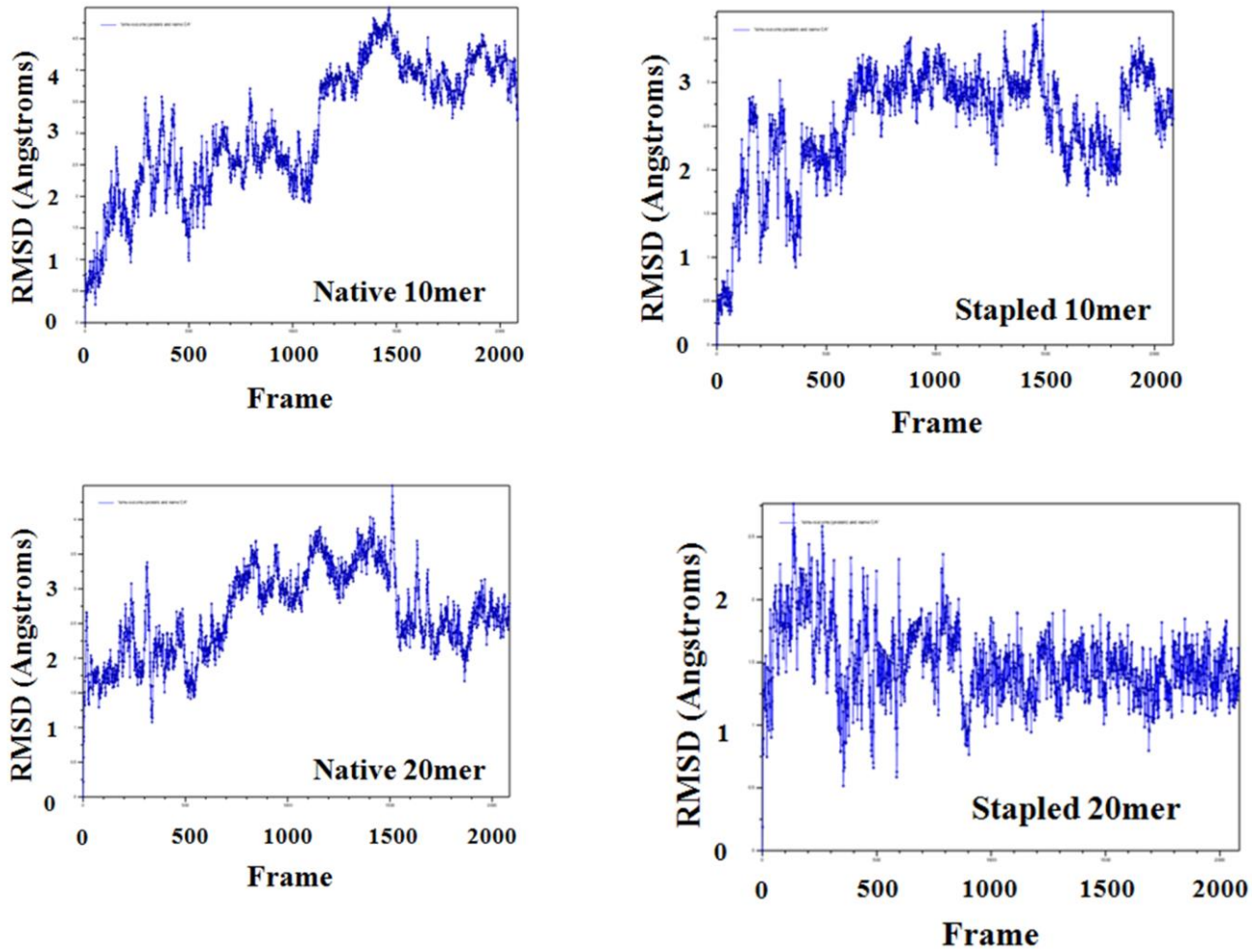

Figure 5. RMSD of native and stapled 10mer and 20mer peptides.

As part of molecular dynamics studies, we discovered that linking the helix-turn-helix (see Fig 1B) increases the helical propensity greatly of the entire peptide. However, when tested in CDK4/6 enzyme assays and against mesothelioma cell lines, there was not as much activity. We suspect this is from precipitation at concentrations > 100 μM, so this skews the IC50 curves. However, there is activity of the TAT-linked peptide with a disulfide bond up to 100 μM which shows additive activity when added to palbociclib in mesothelioma cell cultures. We will modify the disulfide bond placement after further molecular dynamics simulations.

Finally, we have started evaluations of palbociclib plus peptides. Figure 6 shows the effects of palbociclib in combination with the TAT-20mer peptide (3665), stapled 10mer peptide (1204), or TAT-20mer peptide with a disulfide bond bridging the helix-turn-helix motif. MD studies not show demonstrated the 20mer with a disulfide bond had the highest stability of all peptides. However, a complete IC50 curve in cells has not been finished as it likely precipitates at concentrations above 100 μM. Overall, we have demonstrated that the addition of likely indirect CDK4/6 inhibition via peptides is additive to direct CDK4/6 inhibition via palbociclib.

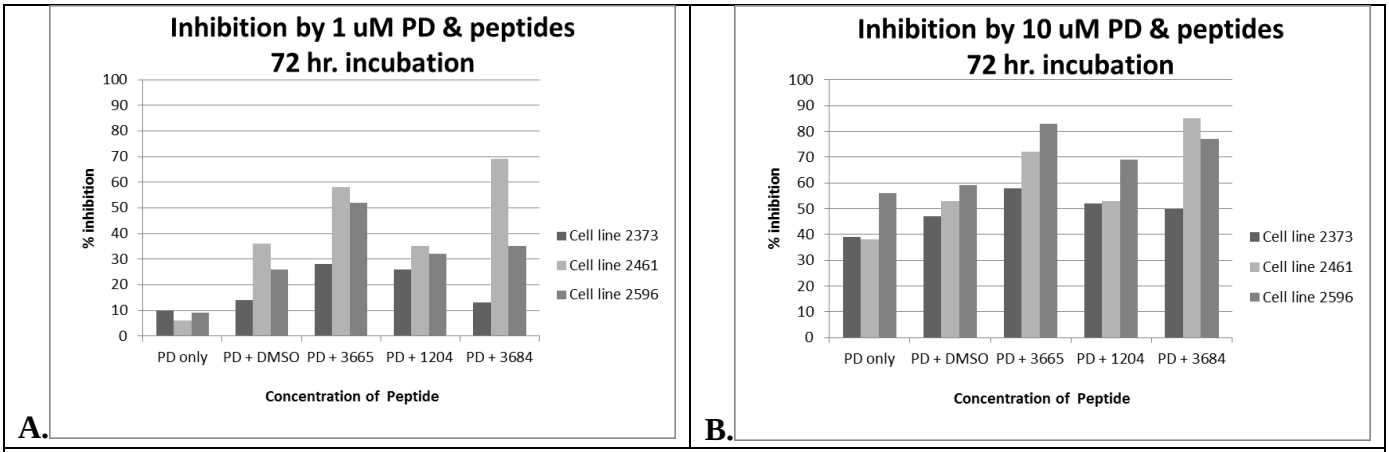

Figure 6. Effects of combination of palbociclib + peptide (3665 = TAT-20mer, 1204 = stapled 10mer, 3684 = TAT-20mer (with disulfide bond). A. 1 μM palbociclib + peptide. B. 10 μM palbociclib + peptide. Concentration of peptides 20 μM peptide 3665, 100 μM peptide 1204, 100 μM peptide 3684

Methods:

Mesothelioma Cell lines. The mesothelioma cell lines used in this study (H 2373, H2461 and H2596) were obtained from either the ATCC (American Type Culture Collection) or in collaboration with Dr. Frederick Kaye (National Cancer Institute)[1]. All mesothelioma cell lines express wild-type pRb and lack functional $p16^{INK4a}$. The cells were grown in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA), 10 mM HEPES (Corning, Manassas VA), 1 mM sodium pyruvate(Sigma, St. Louis, MO, USA) , 1.5% sodium bicarbonate (Sigma, St. Louis, MO, USA), and 1x concentration of antibiotic/antimycotic reagent (Gibco BRL, Grand Island, NY, USA) at 37^{o} C and 5% CO_2.

$p16^{INK4a}$ Peptides and PD 0332991. Several peptides containing amino acid residues from $p16^{INK4a}$, which constitute the Cdk 4/6 inhibitory sequence, were used. These included the native 10 residue sequence (FLATLVVLHR-NH_2) and the native 20 residue sequence (DAAREGFLATLVVLHRAGAR-NH_2). In addition, the native sequences were each linked to a protein transduction domain (TAT) of the HIV-1 virus (YGRKKRRQRRRGFLATLVVLHR-NH_2 and YGRKKRRQRRRGDAAREGFLATLVVLHRAGAR-NH_2). These four peptides were synthesized at the BioMedical Genomics Center at the University of Minnesota (Minneapolis, MN, USA). Two stabilized (stapled) versions of the native peptides ([Cyc(4,8)] H_2N-FLA(S5)LVV (S5)HR-0H and [Cyc(10,14)] Ac-DAAREGFLA(S5)LVV(S5)HRAGAR-OH) were manufactured by New England Peptide (Gardner, MA, USA). All peptides were dissolved in DMSO and then diluted to a standard stock solution of 10mM peptide in 5% DMSO in dH_20 and stored at -70^o C. The Cdk 4/6 inhibitor, PD 0332991 isethionate, was obtained from Sigma, St. Louis, MO, USA.

Antibodies. Various antibodies were used including anti-Rb , anti-Rb (phospho S795), anti-Rb (phospho T821), anti-Rb (phospho T826), horse radish peroxidase-conjugated goat/anti-rabbit (Abcam, Cambridge, MA, USA); anti Rb, horse radish peroxidase-conjugated goat/anti- mouse (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and anti-actin (Sigma, St. Louis, MO, USA).

Cell proliferation assays. Live cells, as determined by Trypan blue dye exclusion assay, were counted on a hemocyometer and plated on 96-well plates (3000 cells/100ul/well) in the RPMI media (including supplements)[1]. After 24 hours, 10 ul of the various $p16^{INK4a}$ peptides in varying concentrations were added to the wells and gently vortexed to mix. After a 72 hr incubation at 37^o C and 5% CO_2, 10 ul of solution from Cell Counting Kit – 8 (Dojindo Laboratories, Kunamoto, Japan) was added to each well and the plates were incubated for 2 hr. The plates were read at 450 nm using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Because addition of the peptides created a precipitate in the higher concentrations (i.e. 1 mM and 100 uM), which interfered with the optical readings, the contents of the wells containing a precipitate were placed in a microfuge tube and spun for 15 seconds in a microcentrifuge at 14,000 rpm. The supernatant was removed and read again in the microplate reader. The reduction in optical density represented the reduction in mitochondrial succinate dehydrogenase activity, hence the reduction in surviving cell numbers.

Kinase assay. The effect of the peptides on the kinase activity of Cdk4 and Cdk6 was evaluated using a commercially available time-resolved fluorescent resonance energy transfer (TR-FRET) assay[2]. The Adapta Universal Kinase Assay (Invitrogen Corporation, Carlsbad, CA 92008) measures kinase activity by correlating ADP formation with substrate phosphorylation. In the first phase, a kinase reaction is created by placing a kinase, the substrate (Rb), ATP, and various concentrations of a peptide into wells of a 396 well plate for 60 minutes. After the reaction, a detection solution consisting of a europium labeled anti-ADP antibody, an Alexa Fluor 647-labeled ADP tracer, and EDTA is added to the well. In the absence of an inhibitor, ADP formed by the kinase reaction will displace the labeled ADP tracer from the antibody, resulting in a decrease in the TR-FRET signal. In the presence of an inhibitor, the amount of ADP formed is reduced and the resulting intact antibody-tracer interaction results in a higher TR-FRET signal. After a 30 minute equilibration period, the signal was read using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA).

Effect of Peptides on cellular proteins. Live cells, as tested and counted above, were plated on 6 well plates (1.0 x10^6 cells/well for 24 hour assays and 0.15x10^6cells/well for 72 hour assays). On day 2, the cells were aspirated and usual medium with the addition of 1% DMSO and various concentrations of the different peptides were placed in the wells. The cells were incubated for either 24 or 72 hours at 37^o C and 5% CO_2, then harvested and processed for Western blot analysis.

Immunoblotting. Cells were removed from the plates by trypsinization, washed in phosphate-buffered saline (PBS) and lysed in cold lysis buffer (50 mM Tris, pH 7.4 and 1% Triton X-100 containing 1x protease inhibitor mixture (Sigma, St. Louis, MO), 1x phosphatase inhibitor mixture (Sigma, St. Louis, MO) and 1 mM PMSF) for 10 minutes[1]. Cellular debris was pelleted by centrifugation in a microcentrifuge at 14,000 rpm at 4^o C for 10 minutes. The supernatant (lysate) was stored at -70^o C. Protein content was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). For Rb Westerns, twenty to thirty micrograms of protein was mixed with equal volumes of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) and placed in boiling water for 2 minutes. Samples were subjected to electrophoresis on 7.5% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. For actin, 3 micrograms of protein was subjected to electrophoresis on 12% gels and transferred to PVDF membranes. Membranes were rinsed in Tris buffered saline with 0.05% Tween 20 (TBS-T) and incubated in blocking buffer (5% bovine serum albumin (BSA)in TBS-T) for 1 hour at 4^o C (Rb blots) or overnight (actin blots). Rb blots were then incubated in primary antibodies diluted in TBS-T containing 0.5% BSA, overnight at 4^o C. Actin blots were incubated in primary antibody for 1 hour at room temperature. The blots were washed 6x with TBS-T and then incubated with an appropriate secondary antibody, diluted in TBS-T for 1 hour at room temperature. Following 6 washes in TBS-T, blots were developed with a chemiluminescence reagent (Pierce ECL Western Blotting Substrate, Thermo Scientific, Rockford, IL, USA or Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare, Buckinghamshire, UK) and exposed to x-ray films. X-ray film was processed on an Agfa-CP1000 developer.

NMR. Two-dimensional NMR spectra of the most active peptide derived from peptides identified in Aims 1a and 1b are being obtained[3-8]. TOCSY (total correlational spectroscopy) spectra (to assign peaks to specific amino acids) and NOESY (nuclear Overhauser Effect Spectroscopy) spectra (to determine through-space interactions < 5 Å) will be obtained and used to computationally model the peptide structure.The program NMRPipe will be used to process data, and the program Sparky will be used to evaluate spectra and assign peaks. The program X-PLOR will then be used to perform structural calculations and refinements based on NOE internuclear distant constraints, as mentioned above. The result of this subaim will yield molecular models of the three-dimensional structure of the peptides based on the NMR-derived data.

Molecular Dynamics Simulations

For peptides: The program DESMOND was used to conduct molecular dynamics simulations of the peptides listed in tables 1 and 2. Either the lowest energy NMR-derived structure of the 10mer peptide (or substituted peptide) or the crystal structure of the relevant sequence from p16INK4 (PDB 1BI7) was used as the starting structure. Model structures were solvated in an

orthorhombic box. Simulations were carried out for 10 ns. Visual Molecular Dynamics was used to identify secondary structure. A script (developed by Jacob Smith and Yuk Sham) was used to calculate the percent secondary structure. VMD was also used to calculate root-mean-squared deviation from the starting structure over 2086 frames.

For longer-length p16-based peptides: The above approach was used, but simulations were carried up for up to 100 ns.

Changes in approach or reasons for change.

We have not changed any of the aims significantly. Due to some difficulty with solubility and also with developing the necessary programs for molecular dynamics studies, we chose to also pursue parts of aim 2 in parallel with the goal of still having the vast majority of aim 1 finished by the 1.5 year mark or so. We have delayed some NMR and ADME studies to identify more potent peptides. We requested a change to study palbociclib with pemetrexed in preparation for a concept for a clinic trial in mesothelioma.

Actual or anticipated problems or delays and actions or plans to resolve them.
Please see below.

Changes that had a significant impact on expenditures.

Regarding the Statement of Work, we have made significant progress on Aims 1 and 2 since the last annual report. At that time, we were working on analyzing several peptides. We have made significant progress in understanding the biology of the peptides and understanding concepts about the biology of the peptides in molecular dynamics terms (via molecular dynamics simulations done at the University of Minnesota Supercomputing Institute, in collaboration with Dr. Yuk Sham). However, we have not done ADME studies yet and requested a no-cost extension.

There are three main reasons we requested the no-cost extension.

1. The company we had make the stapled peptides, AAPTec (Louisville, KY), ran into unexpected technical difficulties making peptides. This was not anticipated, as one of the lead chemists at the company has extensive experience in the origins of this technology. However, they were able to get past that difficulty, and we just received the first peptide requested. Therefore, we have not pursued the Aim 3 ADME studies proposed, so as not to prematurely utilize funds on peptides that are suboptimal.

   Of note, we just had one of the peptides shipped (it took the company 5 months longer to make than anticipated. This is a 20mer peptide with an i,i+7 linker.

2. Through molecular dynamics studies, we have discovered that stapling peptides is context-dependent. As opposed to the literature available on stapled peptide dynamics, we have discovered that stapling in some circumstances may decrease stapled peptide helical propensity (not expected in the literature, but supported by our dynamics studies and

biological activity studies of certain stapled peptides). Through molecular dynamics studies, we discovered that a different stabilizing modification of a larger peptide may have a very high degree of structure stabilization by stabilizing the helix-turn-helix with a disulfide bridge staple. We have that peptide and derivatives for evaluation.

3. Through using palbociclib (a CDK4/6 inhibitor commercially available and recently approved by the FDA for use in breast cancer) as part of controls, we discovered that it may be also be useful as mesothelioma treatment. It is not known if peptides will turn out to be better than palbociclib, or complementary, so we propose to add a few experiments to determine if pemetrexed in combination with the peptides or palbociclib may enhance the activity. If so, a clinical trial could be developed quickly for palbociclib alone in the refractory setting or for palbociclib plus pemetrexed in the first-line setting. In the last week, we have discovered that our peptides have an additive effect to palbociclib.

Based on the above, we request extending the grant/contract to September 14, 2016 to allow for the maximum amount of knowledge and benefit to be gain from this project. We plan to make the following changes.

1. We will evaluate the new stapled 20mer peptide with a disulfide cross-linker in addition to the stapled peptides ordered. We will also continue molecular dynamics studies to further refine our understanding.

2. We will pare down the ADME studies to just evaluating CaCO2-cell permeation and plasma protein binding experiments in only those peptides deemed to have good in vitro activity.

3. We will evaluate palbociclib and active peptides in combination with pemetrexed. This may yield a clinical study that could start within 1-2 years.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.
Not applicable and nothing to report.

What opportunities for training and professional development has the project provided?

Seminars attended:
I have regularly attended the weekly seminar series offered by the Medicinal Chemistry Department at the University of Minnesota.

Poster presented at the Annual Protein Society Meeting, July 2014:
Some of the work accomplished on this grant was presented at the Annual Meeting of the Protein Society, July 22-25, 2014.

How were the results disseminated to communities of interest?

I presented a talk at our local VA Primary Care Service line on this research January 29, 2015 and to the Division of Hematology, Oncology, and Transplantation, Department of Medicine, University of Minnesota, Minneapolis, MN on February 6, 2015.

I presented a poster at the Protein and Antibody Engineering Summit, Therapeutics Stream, May 6-7, 2015, Boston, MA

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on this project?

Name:	Mark Klein, M.D.
Project Role:	Principal Investigator
Research identifier:	
Nearest person month worked:	6
Contribution to project	Principal Investigator and conduct molecular dynamics studies and NMR studies
Funding Support	This grant.

Name:	Marian Kratzke, Ph.D.
Project Role:	Biological research scientist
Research identifier:	
Nearest person month worked:	9
Contribution to project	Conducted *in vitro* laboratory experiments
Funding Support	This grant.

Name:	Robert Kratzke, M.D.
Project Role:	Mentor
Research identifier:	
Nearest person month worked:	1
Contribution to project	Mentor
Funding Support	None from this grant

Name:	Yuk Sham, Ph.D.
Project Role:	Collaborator
Research identifier:	
Nearest person month worked:	1
Contribution to project	Collaborator on molecular dynamics studies
Funding Support	None from this grant.

Name:	Garrett McClean, Bachelor's Degree
Project Role:	Collaborator
Research identifier:	
Nearest person month worked:	1
Contribution to project	Collaborator on molecular dynamics studies

Funding Support	None from this grant.

Has there been a change in the active other support of the PD/PI or senior/key personnel since the last reporting period?

Mark Klein, M.D., PI
No changes

Robert Kratzke, M.D., Mentor
No changes

Yuk Sham, Ph.D., Collaborator
No changes.

What other organizations were involved as partners?

University of Minnesota
Minneapolis, Minnesota

Facilities: use of the Supercomputing Institute and NMR facility
Collaboration: Dr. Yuk Sham, Center for Drug Design

4. Key Research Accomplishments

What was the impact on the development of the principal disciplines of the project?

- We have identified that TAT-p16 peptides have good potency against CDK4. These were nearly as potent against mesothelioma cell lines as PD0332991 (palbociclib). Stapled peptides had less efficacy against CDK4/6 than anticipated. We suspect this may be due to the olefin tether interfering with the binding of the peptide to CDK4/6. In addition, the peptides did not enter into the cells as they previously have. That has led us to hypothesize that we need to identify more properties with mesothelioma cell biology before designing several new peptides. We are continuing to analyze peptide mutations now.
- We have demonstrated that the TAT-p16 peptides decrease Rb phosphorylation in vitro and affect the cell cycle in vitro.
- In addition, we have shown that inhibition is additive when adding various peptides to palbociclib in vitro.
- We are quite encouraged by our findings in the last year. These findings are encouraging in 2 ways. First, we have demonstrated that palbociclib has activity against mesothelioma – we are in the midst of planning a concept for a clinical trial in mesothelioma utilizing this reagent. Also, the peptide activity is encouraging for further development based on the single agent and additive activity in addition to palbociclib. I am presenting a concept for a clinical trial to the Alliance for Clinical Trials Oncology fall group meeting November 7, 2015.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report

5. Conclusion

We have made significant strides in understanding how peptides can be designed to inhibit the cell cycle in mesothelioma. Peptides evaluated thus far are either promising leads or have led to changes in our understanding of optimal peptide design against CDK4/6, the cell cycle, and mesothelioma. We have demonstrated that specific changes affect peptide activity in vitro. Also, we have now have a wealth of data regarding molecular dynamics studies of p16-related peptides. Our data involving palbociclib is promising enough that we are discussing a concept for a clinical trial with the Alliance in Clinical Trials in Oncology at the November meeting in Chicago, where I will present a concept involving palbociclib treatment of mesothelioma. We also have demonstrated that the combination of palbociclib and peptides can result in additive effects against mesothelioma cell lines. We have already submitted a grant for a Merit Award based on this data to the VA Research Service and to the Mesothelioma Research Foundation. It is our intention to also apply for an NIH R01 regarding peptide design as well.

6. Publications, Abstracts, and Presentations

The PI produced one poster at a major meeting, and two oral presentations since beginning the grant. They are listed below. Two manuscripts are under preparation with a third planned.

Poster presentation at the Protein and Antibody Engineering Summit, Therapeutics Stream, May 6-7, 2015, Boston, MA

Title: Evaluation of p16INK4A-Derived Peptides in Mesothelioma

Introduction: Mesothelioma is a highly fatal disease with limited therapeutic options. Low expression of p16INK4A, an endogenous tumor suppressor acting as CDK4/CDK6 inhibitor, has been demonstrated in up to 50-90% of mesothelioma tumors. Replacement of p16INK4A activity via gene therapy has demonstrated activity against CDK4 and CDK6, antitumor response, and an increase in survival in xenograft models. Truncated peptides (FLDTLVVLHR, FLATLVVLHR, and DAAREGFLDTLVVLHRAGAR) derived from the 3rd anykyrin repeat of p16INK4A have been shown to exhibit similar activity to the full-length protein. Subsequently, the isolated 10mer peptide derivative has been shown to maintain its native helical structure from its full length protein.

Hypothesis: Amino-acid substitution will affect the structure and function of p16INK4A-derived peptides. Molecular dynamics simulation will provide the molecular basis for designing stabilized p16INK4A-derived peptides with improved antitumor activity against mesothelioma

Results and Discussion: We have conducted molecular dynamics simulations of alanine-substituted p16INK4A-derived peptides based on the FLATLVVLHR (10mer) and DAAREGFLDTLVVLHRAGAR (20mer) sequence to explore its structure and function. Each modeled NMR-derived 10mer peptide or native helix-turn-helix 20mer peptide from the p16INK4a-CDK6 crystal structure was simulated for 10ns using DESMOND. For the 10mer peptide, alanine substitution has significant effects on the alpha helicity of the native solution structure. A significant decrease in alpha helicity was observed for V7A mutant while the beta sheet propensity increases for alanine substitution at V6, L8, H9, and R10 positions. For the larger 20mer peptide, similar substitutions showed a slightly different effect on predicted conformation population. V11A exhibited a greater decrease in alpha helix propensity as compared to its corresponding V7A 10mer peptide. There was significant conformational difference between the D9A 20mer as compared to its corresponding D3A 10mer peptide. We are conducting CDK4/6 assays with substituted 10mer peptides to examine the correlation between the IC50 values with its predicted tertiary structural propensity. We have evaluated several p16INK4A-derived peptides described above for activity against CDK4/CDK6 and for anti-mesothelioma cell activity. We anticipate the molecular dynamics studies will inform the design of further stabilized peptide candidates.

Research talk at the Minneapolis VA Medical Center January 29, 2015
Research talk at the University of Minnesota, Garibaldi Research Conference, February 6, 2015

Websites or other Internet sites:
Nothing to report.

Technologies or techniques
Nothing to report

7. Inventions, patent, and licenses
Nothing to report

8. Reportable Outcomes
Nothing to report

9. Other Achievements
Nothing to report

10. References

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11. Appendices

Nothing to report.