PI: **DUERKSEN-HUGHES, PENELOPE J.**	Title: A novel combinatorial approach for treating HPV-associated malignancies	
Received: 01/04/2017	FOA: PA16-302	Council: 05/2017
Competition ID: FORMS-D	FOA Title: PHS 2016-02 OMNIBUS SOLICITATION OF THE NIH, CDC, FDA AND ACF FOR SMALL BUSINESS INNOVATION RESEARCH GRANT APPLICATIONS (PARENT SBIR [R43/R44])	
1 R43 CA221479-01	Dual: AI,HD	Accession Number: 4006098
IPF: 10044899	Organization: ISPIN, INC.	
Former Number:	Department:	
IRG/SRG: ZRG1 OTC-E (10)B	AIDS: N	Expedited: N
Subtotal Direct Costs (excludes consortium F&A) Year 1: 133,874	Animals: Y Humans: N Clinical Trial: N Current HS Code: 10 HESC: N	New Investigator: Early Stage Investigator:
Senior/Key Personnel:	*Organization:*	*Role Category:*
Maria Filippova	iSpin Inc.	PD/PI
Penelope Duerksen-Hughes	Loma Linda University	Other (Specify)-Subcontract PI

APPLICATION FOR FEDERAL ASSISTANCE
SF 424 (R&R)

3. DATE RECEIVED BY STATE	**State Application Identifier**

1. TYPE OF SUBMISSION*

❍ Pre-application ❍ Application ● Changed/Corrected Application

4.a. Federal Identifier

b. Agency Routing Number

2. DATE SUBMITTED | **Application Identifier**

c. Previous Grants.gov Tracking Number
GRANT12312381

5. APPLICANT INFORMATION

Organizational DUNS*: 0804577960000

Legal Name*: iSpin Inc.
Department:
Division:
[redacted]

Person to be contacted on matters involving this application
Prefix: Dr. First Name*: Penelope Middle Name: Last Name*: Duerksen-Hughes Suffix:
Position/Title: CEO
[redacted]
Phone Number*: [redacted] Fax Number: Email: [redacted]

6. EMPLOYER IDENTIFICATION NUMBER *(EIN) or (TIN)** [redacted]

7. TYPE OF APPLICANT* R: Small Business

Other (Specify):
Small Business Organization Type ● Women Owned ❍ Socially and Economically Disadvantaged

8. TYPE OF APPLICATION*

● New ❍ Resubmission
❍ Renewal ❍ Continuation ❍ Revision

If Revision, mark appropriate box(es).
❍ A. Increase Award ❍ B. Decrease Award ❍ C. Increase Duration
❍ D. Decrease Duration ❍ E. Other *(specify)* :

Is this application being submitted to other agencies?* ❍Yes ●No What other Agencies?

9. NAME OF FEDERAL AGENCY*
National Institutes of Health

10. CATALOG OF FEDERAL DOMESTIC ASSISTANCE NUMBER
TITLE:

11. DESCRIPTIVE TITLE OF APPLICANT'S PROJECT*
A novel combinatorial approach for treating HPV-associated malignancies

12. PROPOSED PROJECT
Start Date* Ending Date*
10/01/2017 09/30/2019

13. CONGRESSIONAL DISTRICTS OF APPLICANT
CA-31

SF 424 (R&R) APPLICATION FOR FEDERAL ASSISTANCE

14. PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR CONTACT INFORMATION

Prefix: Dr. First Name*: Maria Middle Name: Last Name*: Filippova Suffix:

Position/Title: Principal Investigator

Organization Name*: iSpin Inc.

Department:

Division:

[redacted]

Fax Number: Email*: [redacted]

15. ESTIMATED PROJECT FUNDING

a. Total Federal Funds Requested* [redacted]

b. Total Non-Federal Funds* [redacted]

c. Total Federal & Non-Federal Funds* [redacted]

d. Estimated Program Income* [redacted]

16.IS APPLICATION SUBJECT TO REVIEW BY STATE EXECUTIVE ORDER 12372 PROCESS?*

a. YES ❍ THIS PREAPPLICATION/APPLICATION WAS MADE AVAILABLE TO THE STATE EXECUTIVE ORDER 12372 PROCESS FOR REVIEW ON:

DATE:

b. NO ● PROGRAM IS NOT COVERED BY E.O. 12372; OR

❍ PROGRAM HAS NOT BEEN SELECTED BY STATE FOR REVIEW

17. By signing this application, I certify (1) to the statements contained in the list of certifications* and (2) that the statements herein are true, complete and accurate to the best of my knowledge. I also provide the required assurances * and agree to comply with any resulting terms if I accept an award. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. (U.S. Code, Title 18, Section 1001)

● I agree*

** The list of certifications and assurances, or an Internet site where you may obtain this list, is contained in the announcement or agency specific instructions.*

18. SFLLL or OTHER EXPLANATORY DOCUMENTATION File Name:

19. AUTHORIZED REPRESENTATIVE

Prefix: Dr. First Name*: Penelope Middle Name: Last Name*: Duerksen-Hughes Suffix:

Position/Title*: CEO

Organization Name*: iSpin Inc.

Department:

Division:

[redacted]

Fax Number: [redacted]

Signature of Authorized Representative*

[redacted]

Date Signed*

[redacted]

20. PRE-APPLICATION File Name:

21. COVER LETTER ATTACHMENT File Name:1234-Cover letter.pdf

424 R&R and PHS-398 Specific
Table Of Contents

OMB Number: 4040-0010
Expiration Date: 06/30/2016

Project/Performance Site Location(s)

Project/Performance Site Primary Location

❍ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: iSpin Inc.
Duns Number: [redacted]
Street1*: [redacted]
Street2:
City*: [redacted]
[redacted]
[redacted]
Province:
Country*: [redacted]
Zip / Postal Code*: [redacted]
Project/Performance Site Congressional District*: [redacted]

Project/Performance Site Location 1

❍ I am submitting an application as an individual, and not on behalf of a company, state, local or tribal government, academia, or other type of organization.

Organization Name: Loma Linda University
DUNS Number: [redacted]
Street1*: [redacted]
Street2:
City*: [redacted]
County: [redacted]
State*: [redacted]
Province:
Country*: [redacted]
Zip / Postal Code*: [redacted]
Project/Performance Site Congressional District*: [redacted]

Additional Location(s)

File Name:

Funding Opportunity Number: PA-16-302. Received Date: 2017-01-04T13:45:01.000-05:00

OMB Number: 4040-0001
Expiration Date: 06/30/2016

RESEARCH & RELATED Other Project Information

1. Are Human Subjects Involved?* ❍ **Yes** ● **No**

1.a. If YES to Human Subjects

Is the Project Exempt from Federal regulations? ❍ Yes ❍ No

If YES, check appropriate exemption number: _ 1 _ 2 _ 3 _ 4 _ 5 _ 6

If NO, is the IRB review Pending? ❍ **Yes** ❍ **No**

IRB Approval Date:

Human Subject Assurance Number

2. Are Vertebrate Animals Used?* ● **Yes** ❍ **No**

2.a. If YES to Vertebrate Animals

Is the IACUC review Pending? ● Yes ❍ No

IACUC Approval Date:

Animal Welfare Assurance Number [redacted]

3. Is proprietary/privileged information included in the application?* ● **Yes** ❍ **No**

4.a. Does this project have an actual or potential impact - positive or negative - on the environment?* ❍ **Yes** ● **No**

4.b. If yes, please explain:

4.c. If this project has an actual or potential impact on the environment, has an exemption been authorized or an environmental assessment (EA) or environmental impact statement (EIS) been performed? ❍ Yes ❍ No

4.d. If yes, please explain:

5. Is the research performance site designated, or eligible to be designated, as a historic place?* ❍ **Yes** ● **No**

5.a. If yes, please explain:

6. Does this project involve activities outside the United States or partnership with international collaborators?* ❍ **Yes** ● **No**

6.a. If yes, identify countries:

6.b. Optional Explanation:

	Filename
7. Project Summary/Abstract*	1238-SUMMARY.pdf
8. Project Narrative*	1239-NARRATIVE.pdf
9. Bibliography & References Cited	1240-NIH010517refs.pdf
10.Facilities & Other Resources	1241-Resources.pdf
11.Equipment	
12. Other Attachments	1242-SBC_001213287.pdf

Funding Opportunity Number: PA-16-302. Received Date: 2017-01-04T13:45:01.000-05:00

SUMMARY

High-risk types of human papillomaviruses (HPV) are responsible for virtually all cases of human cervical carcinoma, as well as an increasing number of other malignancies including those of the head and neck, anus and vulva. Unfortunately, good treatment options for late-stage HPV^+ malignancies are not currently available, in large part because the virus encodes a protein, E6, which disables cellular apoptotic pathways by accelerating the degradation of molecules such as FADD, caspase 8 and p53. This makes it difficult to eliminate HPV^+ cancer cells using conventional inducers of apoptosis. To overcome this obstacle, our laboratory has identified several small molecules that block the binding between E6 and partners such as caspase 8 and E6AP. According to our working model, the use of such molecules in a combinatorial manner will greatly increase the effectiveness of standard radio- and chemotherapeutic treatments. Both *in vitro* binding data and cellular data from our laboratory provide strong data in support of this working model, and it is now time to test our approach in an *in vivo* context. The overall objective of this current application, therefore, is to move our exciting *in vitro* and cellular observations into a mouse xenograft model. We will do this by combining spinacine, our best molecular candidate, with two potential therapeutic agents: TRAIL, a biologic, and cisplatin, a more conventional chemotherapeutic, asking whether either of these combinations can reduce or eliminate the growth of HPV^+ tumors, of either cervical or head and neck origin, in a nude mouse model. In particular, we will: **1) Determine the toxicity of spinacine in mice.** We will assess the toxicity of spinacine in mice, defining the maximum tolerated dose and identifying the optimum dose with which to carry out experiments designed to test its efficacy, and **2) Evaluate the ability of spinacine to synergize with TRAIL- and/or chemo-based therapies to reduce or eliminate HPV^+ tumor growth.** We will assess the ability of spinacine to synergize with hrTRAIL and/or the DNA damaging drug cisplatin to inhibit tumor growth in a xenograft model. At the conclusion of this work, we will have 1) Determined the toxicity of the E6-inhibiting molecule spinacine in mice, and 2) Evaluated the effectiveness of combining spinacine with TRAIL- and cisplatin-based treatments in an animal model. This work has the potential to save the lives of thousands of patients suffering from HPV-associated malignancies.

NARRATIVE

High-risk types of the human papillomavirus (HPV) are responsible for nearly all cancers of the cervix, as well as an increasing number of other cancers such as those of the head and neck, anus and vulva. It is difficult to effectively treat late-stage HPV^+ cancers, because the virus codes for a protein, E6, which makes cells resistant to most therapies. We have found a small molecule, spinacine, which prevents E6 from protecting these cells. We now want to ask if spinacine can be combined with either of two conventional therapies, TRAIL and cisplatin, to reduce or eliminate tumors in a mouse model. If successful, treatment for patients with HPV^+ tumors could be greatly improved.

FACILITIES AND OTHER RESOURCES

iSpin (Applicant)

Office Space: iSpin is a tenant of Loma Linda University Health's incubator, n3eight LLC. As part of its arrangement, iSpin has 1,000 sq ft of office space for business meetings and a place from which to run its operating activities out of the 11219 Anderson Street, Loma Linda, CA 92354.

Laboratory: Moreover, as part of its arrangement with n3eight LLC, iSpin also has access to 2,796 sq ft of laboratory space that comprise of bench space equipped for general molecular biology and cell culture work located at [redacted]. Common equipment areas contain a laminar flow hood, a CO_2 incubator, -80 and -20 freezers, multipurpose centrifuges, inverted and standard microscopes, and balances.

Loma Linda University (Subcontractor)

Intellectual environment (Loma Linda University): Loma Linda University provides a **rich interactive environment** that houses the Schools of Medicine, Dentistry, Pharmacy, Nursing, Public Health and Allied Health. The Basic Sciences of the School of Medicine host a weekly seminar series that attracts world class speakers from a variety of biomedical disciplines. LLU is in close proximity to both the LLU Medical Center (within 100 m) and the Jerry L. Pettis VA Memorial Medical Center (a few blocks), promoting cross-fertilization and exchange of ideas between clinicians, clinical researchers and basic science researchers. **The design and execution of our proposed and future studies have been and will continue to be facilitated by our productive, stimulating and frequent intellectual interactions with our network of present and potential collaborators in different areas**. **Should this work develop into a Phase I trial, this would be done in collaboration with individuals within this group.**

Animal Resources: Loma Linda investigators have available for their use the AALAC-approved (approved since 1967) University Animal Care Facility under the direction of David Wolf, DVM, PhD. This large and modern facility was very recently renovated, and includes approximately 20,000 square feet of space in the University Medical Center as well as 43,200 square feet in satellite animal facilities on the University campus. It has a separate ventilation system with temperature and humidity control, and includes a sterile surgical suite, two photoperiod and climate controlled rooms with light locks, operation theaters and areas for animal preparation and recovery. The facility also has a Specific Pathogen Free (SPF) area for housing immunodeficient mice as well as special areas for housing mice that have received virus vector treatments. To monitor the tumor growth and metastasis development facility is equipped with the IVIS Lumina II in Vivo Imaging System (PerkinElmer). **The tumor growth studies described in this application will be carried out within this facility**.

Laboratory: The subcontractor occupies two 600 square foot laboratories within Mortensen Hall equipped for general molecular biology and cell culture work. Within these laboratories is desk and bench space for seven researchers, as well as general molecular and cell biological equipment and materials including microcentrifuges, protein and DNA electrophoresis units, shakers, waterbaths, pipettors, pippettmen and a thermal cycler for PCR analysis. Adjacent to the subcontractor's laboratories are common equipment areas which contain laminar flow hoods, CO_2 incubators, -80^o and -20^o freezers, multipurpose centrifuges, inverted and standard microscopes, balances, and multiple liquid nitrogen tanks with space to store nearly 6000 cryopreservation vials. These facilities have been used to conduct the preliminary experiments described in this application, and will continue to be used for the proposed experiments. **Together, these individual and common-use facilities will enable the successful completion of the experiments described in this proposal**.

Computational facilities: The office and laboratory of Dr. Duerksen-Hughes are equipped with several personal computers, printers and scanners that are connected to the campus network. Additional common use computers and printers (including color laser printers) are available. Office and laboratory computers are equipped with software for word processing, spreadsheet analysis, graphics imaging,

and reference management. **This equipment will facilitate the careful analysis of the findings from this study.**

Office: Drs. Duerksen-Hughes occupies approximately 100 square feet of office space near her laboratory space for her research, in addition to administrative office space supportive of her Associate Dean role. Secretarial assistance is available.

Clinical Resources: The **Loma Linda University Medical Center** is the largest teaching hospital serving the Inland Empire of Southern California with a state-of-the-art 627 bed facility. Of note, a brand new facility is currently being constructed, and is planned for opening in the year 2020. Our Medical Center serves as a resource for basic research and is a potential venue for future clinical studies. **These clinical facilities provides a wonderful opportunity for the implementation of findings from our studies.**

The U.S. Small Business Administration

SBIR.gov SBC Registration

SBC Control ID:	[redacted]		
Company Name:	iSpin Inc.		
Address:	[redacted]		
City:	[redacted]		
State:	[redacted]	**Zip:**	[redacted]
EIN (TIN):	[redacted]	**DUNS:**	[redacted]
Company URL:			

Number of Employees:	1
Is this SBC majority-owned by multiple venture capital operating companies, hedge funds, or private equity firms?	No
What percentage (%) of the SBC is majority-owned by multiple venture capital operating companies, hedge funds, or private equity firms?	0.00%

OMB Number: 4040-0001
Expiration Date: 06/30/2016

RESEARCH & RELATED Senior/Key Person Profile (Expanded)

PROFILE - Project Director/Principal Investigator

Prefix: Dr. First Name*: Maria Middle Name Last Name*: Filippova Suffix:

Position/Title*: Principal Investigator
Organization Name*: iSpin Inc.
Department:
Division:
Street1*: [redacted]
Street2:
City*: [redacted]
County:
State*:
Province:
Country*:
Zip / Postal Code*:

Phone Number*: [redacted] Fax Number:

E-Mail*: [redacted]

Credential, e.g., agency login: [redacted]

Project Role*: PD/PI Other Project Role Category:

Degree Type: Degree Year:

Attach Biographical Sketch*: File Name: 1236-BiosketchMF122216.pdf

Attach Current & Pending Support: File Name:

Funding Opportunity Number: PA-16-302 . Received Date: 2017-01-04T13:45:01.000-05:00

PROFILE - Senior/Key Person

Prefix: Dr. First Name*: Penelope Middle Name Last Name*: Duerksen-Hughes Suffix:

Position/Title*: Professor
Organization Name*: Loma Linda University
Department: Basic Sciences
Division:
Street1*: [redacted]
Street2:
City*: [redacted]
County: [redacted]
State*: [redacted]
Province:
Country*: [redacted]
Zip / Postal Code*: [redacted]

Phone Number*: [redacted] Fax Number:

E-Mail*: [redacted]

Credential, e.g., agency login: [redacted]

Project Role*: Other (Specify) Other Project Role Category: Subcontract PI

Degree Type: Degree Year:

Attach Biographical Sketch*: File Name: 1237-Biosketchpdh 010517.pdf

Attach Current & Pending Support: File Name:

OMB No. 0925-0001 and 0925-0002 (Rev. 10/15 Approved Through 10/31/2018)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Filippova, Maria

eRA COMMONS USER NAME (credential, e.g., agency login): [redacted]

POSITION TITLE: Research Associate Professor, Chief Scientific Officer of iSpin Inc

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Novosibirsk State University, Novosibirsk, Russia	M.S.	1982	Biology
Institute of Cytology and Genetics, Russian Academy of Science, Russia	Ph.D.	1990	Genetics

A. Personal statement

My research at Loma Linda University has focused on defining the molecular mechanisms of virus-induced tumorigenesis using a model of high-risk human papillomavirus (HPV) type 16-mediated carcinogenesis. The intended outcome of our research is to develop effective and novel approaches for the prevention and cure of tumors caused by HPV16, such as cervical carcinoma as well as head and neck cancer.

In particular, the primary focus of my studies has been the contribution of the E6 oncoprotein to the observed resistance of HPV^+ cells to apoptosis induced by ligands of the TNF-superfamily. We found that HPV16 E6 expression protects host cells from apoptosis induced by TNF-family ligands such as TNF-α, Fas ligand and TRAIL through binding to major apoptotic players including the TNF R1 receptor, FADD and procaspase 8. Once bound, E6 accelerates the degradation of FADD and caspase 8, thereby preventing the transmission of apoptotic signals from receptors of these TNF-family ligands to the terminal caspases. As a result of these E6 activities, HPV-infected cells become resistant to apoptotic signals and thus benefit from a survival advantage.

To find more effective treatments for HPV-mediated cervical and head and neck cancers, we have focused on inhibiting the ability of E6 to prevent apoptosis. We are accomplishing this goal by identifying and employing small molecules that prevent E6 from binding to its cellular partners. These molecules then sensitize infected cells to apoptosis induced both by TRAIL and chemotherapeutic agents. We have already proved the principle of this approach at the cellular level, identified a few small molecules as candidates for drug development, and continued to work on further large-scale screening. It is now time for us to move forward with this approach by testing our findings in an *in vivo* model, preparatory to initiating clinical trials.

To accelerate the transition to clinical trials and product development using our suggested combinatorial therapy combining spinacine with TRAIL and/or cisplatin, the company iSpin Inc was founded in 2016. At the initiation of the project described, I will be 51% employed in this company so as to move forward with this work in developing antitumor drugs.

In summary, my expertise and experience, as documented by my publications, provide evidence that I can successfully support completion of this project. I served as a primary investigator or co-investigator for these studies, and our major results have been published.

1. **Filippova, M.,** L. Parkhurst, and P.J. Duerksen-Hughes. The human papillomavirus 16 E6 protein binds to Fas-associated death domain and protects cells from Fas-triggered apoptosis. J Biol Chem, 2004. 279(24): p. 25729-44.
2. Garnett, T.O., **Filippova M**., and P.J. Duerksen-Hughes. Accelerated degradation of FADD and procaspase 8 in cells expressing human papilloma virus 16 E6 impairs TRAIL-mediated apoptosis. Cell Death Differ, 2006. 13(11): p. 1915-26.
3. **Filippova, M**., et al. The large and small isoforms of human papillomavirus type 16 E6 bind to and differentially affect procaspase 8 stability and activity. J Virol, 2007. 81(8): p. 4116-29.
4. Yuan, C.H., **Filippova, M**., Krstenansky, J.L. and Duerksen-Hughes, P.J. Flavonol and imidazole derivatives block HPV16 E6 activities and reactivate apoptotic pathways in HPV^{+} cells. Cell Death Dis. 2016. 21 p.2060.

B. Positions and Honors

1990-1996 Researcher, Institute of Cytology and Genetics, Novosibirsk, Russia
1993-1996 Researcher, Biological Chair, Southern Bohemian University, Ceske Budejovice, Czech Republic
1995-1996 Visiting Postgraduate Researcher, Developmental Biology Center, University of California, Irvine, Irvine, California.
1996-1998 Postgraduate Researcher, Department of Entomology/Toxicology, University of California, Riverside, Riverside, California
1998-2009 Research Associate, Research Specialist, Instructor, Center for Molecular Biology and Gene Therapy, Loma Linda University, Loma Linda, California
2009-present Research Associate Professor, Research Assistant Professor, Department of Basic Science, Center of Health Disparity and Molecular Medicine, Loma Linda University, Loma Linda, California

C. Contribution to Science

Molecular genetics and cytology: function of *drosha:* I was initially trained in the fields of Molecular Genetics and Cytology. Over the years, I have since become a multidisciplinary researcher in cellular biology and molecular biology with broad experience in additional interdisciplinary areas such as karyosystematics, population genetics, biochemistry and bioinformatics. As a postgraduate researcher, my interest focused on the organization of specific genomic regions, including the centromere (Drosophila), mobile elements and tissue-specific genes (Balbiani rings of Chironomidae) and on the function of certain genes including v-ATPase (mosquito), RNase H and *cul-1* (Drosophila). While working at the University of California at Riverside, I was involved in studies of two important genes involved in the regulation of RNAs, RNase H and a RNA III homolog which we named *drosha*. My responsibility in the RNase H project was to obtain a mutant of this gene and to investigate the function of this gene during Drosophila development and suggested that RNase H functioned primarily in morphogenesis. In another project related to the Drosophila RNase III homolog, I contributed by localizing the gene named *drosha* to the 2^{nd} chromosome in locus 43F2/F3 and by demonstrating the lethal effect of mutation of this gene on embryonic development. Nowadays, *drosha* is known to play a crucial role in global expression regulation during development, differentiation, cell death and tumorigenesis by way of the miRNAs that are processed by this enzyme.

1. **Filippova, M**., L.S. Ross, and S.S. Gill, Cloning of the V-ATPase B subunit cDNA from Culex quinquefasciatus and expression of the B and C subunits in mosquitoes. Insect Mol Biol, 1998. 7(3): p. 223-32.
2. Harvie, P.D., **Filippova M**., and Bryant P.J., Genes expressed in the ring gland, the major endocrine organ of Drosophila melanogaster. Genetics, 1998. 149(1): p. 217-31.
3. Filippov, V., **Filippova M**. et al., Temporal and spatial expression of the cell-cycle regulator cul-1 in Drosophila and its stimulation by radiation-induced apoptosis. J Exp Biol, 2000. 203(Pt 18): p. 2747-56.
4. Filippov, V., **Filippova M**. et al., A novel type of RNase III family proteins in eukaryotes. Gene, 2000. 245(1): p. 213-21.

In frame of HPV E6 field I also conducted the studies on another projects.

Project: Role of E6*. In this project I am studying the role, function, and protein structure of the E6 splice variant, referred to as E6*. E6* represents approximately the N-terminal third of E6 and is expressed together with full length E6. We found that E6* frequently functions in an opposite manner as does E6. For example, it negatively regulates the oncogenic activity of E6 by binding to the full length variant. In addition, we found that E6* reduces tumor development independently of E6 in a mouse xenograft model for cervical cancer. The major goal for this project is to provide information on the mechanisms of changes in networks that govern cell fate to be transform or not utilizing modern proteomic and transriptomic approaches together with classical cellular-molecular biological methods.
I served as the primary investigator of most of these studies.

1. **Filippova, M**., et al.The human papillomavirus 16 E6 protein can either protect or further sensitize cells to TNF: effect of dose. Cell Death Differ, 2005. 12(12): p. 1622-35.
2. **Filippova, M**., et al. Complexes of human papillomavirus type 16 E6 proteins form pseudo-death-inducing signaling complex structures during tumor necrosis factor-mediated apoptosis. J Virol, 2009. 83(1): p. 210-27.
3. Tungteakkhun, S.S., **Filippova M**. et al. The full-length isoform of human papillomavirus 16 E6 and its splice variant E6* bind to different sites on the procaspase 8 death effector domain. J Virol, 2010. 84(3): p. 1453-63.
4. **Filippova, M**., W. Evans, R. Aragon, V. Filippov, V. M. Williams, L. Hong, M. E. Reeves and P.Duerksen-Hughes. The small splice variant of HPV16 E6, E6*, reduces tumor formation in cervical carcinoma xenografts. Virology, 201. 450–451(0): 153-164.

Project: Role of oxidative stress induced by virus- and enviroment-derived factors in cancer development induced by HPV16. Establishing of the molecular mechanisms of OS induction by virus- and enviroment-mediated factors, with the overall goal of finding ways to prevent virus integration. If we could decrease cellular levels of OS, the frequency of HPV integration into the host genome would also decrease, and fewer cases of cancer would developed. In principle, OS could be reduced in several ways, including the use of dietary antioxidants as well as by targeting specific pathways that lead to chronic OS. This project is tightly connected to our studies on the etiology of cancer development. We mimic conditions of integration of the HPV genome into the host genome by experimentally inducing oxidative stress, then follow the processes of immortalization and transformation using methods related to network activation. We also focused on the health disparities observed for cervical cancer. To test our hypothesis that OS levels are related to the health disparity of cervical cancer incidence, we are collaborating with surgeons in the Department of Obstetrics and Gynecology to analyze the background levels of cellular oxidative stress in normal cervical tissue samples obtained from White, Black, and Hispanic patients.
I served as the primary investigator and co-investigator of these studies.

1. Chen Y, Williams V, **Filippova M**, Filippov V and Duerksen-Hughes P. Viral carcinogenesis: factors inducing DNA damage and virus integration. Cancers (Basel), 2014. 22;6(4):2155-86.
2. **Filippova M**., Filippov V.A., Zhang K., Williams V., Kokoza A., Bashkirova S., and P. Duerksen-Hughes. Cellular levels of oxidative stress affect the response of cervical cancer cells to chemotherapeutic drugs. Biomed Res Int, 2014. 2014: 574659, p.1-14.
3. Williams, V., **Filippova, M**., Filippov, V., K.J. Payne and P. Duerksen-Hughes. HPV16 E6* induces oxidative stress and DNA damage. J Virol. 2014. 88(12):6751-61.

Complete List of Published Work in My Bibliography and Google Scholar with h factor 19 as of 9/30/15:

http://www.ncbi.nlm.nih.gov/myncbi/collections/bibliography/48780557/

https://scholar.google.com/citations?user=Dozs5d8AAAAJ&hl=en&oi=ao

D. Research Support

Ongoing Research Support

Loma Linda University M.Filippova (Co-PI) and M.Reeves (Co-PI) 1/1/2015-12/31/2016

Grants to Promote Collaborative and Translational Research (GCAT) “A Novel Combinatorial Approach for Treating HPV-associated Malignancies”
Role: Co-PI

OMB No. 0925-0001 and 0925-0002 (Rev. 10/15 Approved Through 10/31/2018)

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Duerksen-Hughes, Penelope J.

eRA COMMONS USER NAME (credential, e.g., agency login): [redacted]

POSITION TITLE: Professor; Associate Dean for Basic Science and Translational Research

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Southern Adventist University, Collegedale, TN	B.S.	05/1982	Chemistry
Emory University, Atlanta, GA	Ph.D.	08/1987	Biochemistry
Emory University, Atlanta, GA	Postdoctoral	01/1992	Molecular Immunology/Virology

A. Personal Statement

HPV-induced malignancies, and in particular, cervical cancer, affect over half a million people each year. Unfortunately, when cancers are discovered at a late stage, available treatment options are limited and frequently ineffective. It has therefore been an ongoing goal of my laboratory, for the past two decades, to find better ways to treat these diseases. The E6 oncoprotein encoded by high-risk types of the virus is responsible for the remarkable resistance shown by HPV^+ tumors to conventional chemo- and radiotherapy, as E6 binds to and accelerates the degradation of multiple cellular partners that function in apoptotic pathways. In fact, it was our laboratory that found that E6 accelerated the degradation of both FADD and caspase 8, thereby inactivating apoptotic signaling through the receptor-mediated pathway. We mapped the sites on FADD and caspase 8 to which E6 bound, then demonstrated that peptides corresponding to that site could block E6 from interacting with its cellular partners. We then sought and found several small molecules which shared this ability and that were able to sensitize HPV^+ cells to apoptosis triggered by both biologic (TRAIL) and chemotherapeutic (doxorubicin and cisplatin) agents. We are now ready to take this work to the next level by asking if our best agent, spinacine, is also effective in an *in vivo* context. Our team is uniquely well positioned to undertake the work described, both because we made the initial observations on which this proposal is based, and because we have already developed the unique reagents and model systems essential for successful completion of this project. To the best of our knowledge, our team is the first to approach treatment of HPV^+ tumors with a combination of E6-blocking agents and conventional therapies that induce apoptosis. All necessary expertise, reagents and approaches are available in our laboratory, and we already have the momentum needed to make rapid progress towards our goal. As a Principle Investigator on several university-, NSF- and NIH-funded grants, I have experience in all aspects related to the successful completion of scientific projects, including planning, personnel, budgeting, collaboration and publication. In summary, my record of published discoveries in this area, combined with my expertise and experience provide evidence that I can successfully lead this project to completion.

- Garnett, T., M. Filippova, and **P. J. Duerksen-Hughes**. Accelerated Degradation of FADD and Pro-Caspase 8 in Cells Expressing Human Papillomavirus 16 E6 Impairs TRAIL-Mediated Apoptosis. *Cell Death and Differentiation*. **13**:1915-1926, 2006. PMID: 16528366
- Tungteakkhun, S., M. Filippova, J. W. Neidigh, N. Fodor and **P. J. Duerksen-Hughes**. The Interaction Between HPV 16 E6 and FADD is Mediated by a Novel E6 Binding Domain. *J. Virol*. 82:9600-9614, 2008. PMID: 18632871

- Yuan, C.-H., M. Filippova, S. S. Tungteakkhun, **P. J. Duerksen-Hughes** and J. L. Krstenansky. Small molecule inhibitors of the HPV16-E6 interaction with caspase 8. *Bioorganic and Medicinal Chemistry Letters*, **22**:2125-2129. 2012. PMID: 22300659
- Yuan, C.-H., M. Filippova, J. L. Krstenansky and **P. Duerksen-Hughes**. Flavonol and Imidazole Derivatives Block HPV16 E6 Activities and Reactive Apoptotic Pathways in HPV^{+} Cells. *Cell Death & Disease* **7**:2060-2072, 2016. PMID: 26794656.

B. Positions and Honors

Positions and Employment

1987-1992	Postdoctoral Fellow, Dept. of Microbiology and Immunology, Emory University, Atlanta, GA
1982-1993	Environmental Health Scientist, Division of Toxicology, Agency for Toxic Substance and Disease Registry, Atlanta, GA
1993-2000	Assistant Professor, Department of Biology, Georgia State University, Atlanta, GA
2000-2000	Associate Professor, Department of Biology, Georgia State University, Atlanta, GA
2000-2005	Associate Professor, Department of Basic Science, Loma Linda University, Loma Linda, CA
2005-present	Professor, Department of Basic Science, Loma Linda University, Loma Linda, CA
2010-2012	Interim Chair, Department of Basic Science, Loma Linda University, Loma Linda, CA
2012-present	Chair, Department of Basic Science, Loma Linda University, Loma Linda, CA
2010-present	Associate Dean for Basic Science and Translational Research, Loma Linda University, Loma Linda, CA

Other Experience and Professional Memberships

2003-present	*Ad Hoc* reviewer for NIH (VIRA, VIRB, MBRS, SCORE, NIDCR, NCI, and other Study Sections)
1996-present	*Ad Hoc* reviewer for NSF
1993-present	*Ad Hoc* reviewer for several journals including Oncogene, Cancer Research, Journal of Virology, Journal of Biological Chemistry, Carcinogenesis, Journal of Cellular Biochemistry, Archives of Virology, Virus Research, etc.

Honors

1978	National Merit Scholarship
1982, 1985	Who's Who Among Students in American Universities and Colleges
1981	National Science Foundation Undergraduate Research Participant, University of Tennessee at Chattanooga, Chattanooga, Tennessee
1988-1991	National Institutes of Health Postdoctoral Fellowship (NRSA), Emory University School of Medicine, Atlanta, Georgia
1992	Diplomat of the American Board of Toxicology (DABT)

C. Contributions to Science

1. ***p53 as an indicator of genotoxic damage:*** My PhD studies regarding ubiquitin-mediated proteolysis, as well as my postdoctoral studies focused on virus evasion of the immune response, catalyzed my interest in the role and function of p53. I was intrigued by the fact that p53 levels increase following genotoxic damage, and recognized that we might be able to exploit this response to develop a human-based, rapid and inexpensive screening assay for genotoxicity. Current protocols utilize various versions of the bacteria-based Ames assay, so the idea of moving toward a human-based alternative, more likely to yield results relevant to human health, was appealing. Briefly, the idea was that human cells would respond to genotoxic damage by up-regulating their levels of p53, and that this up-regulation could be easily detected through the use of a reporter system. I found that this approach was indeed a viable alternative to the Ames assay, with an ability to accurately detect genotoxic substances that was equal to or better than that reported for the Ames.
   - Yang, J. and **P. J. Duerksen-Hughes.** A New Approach to Identifying Genotoxic Carcinogens: p53-Induction as an Indicator of Genotoxic Damage. *Carcinogenesis* **19**(6):1117-1125, 1998. PMID: 9667752

- **Duerksen-Hughes, P. J.,** J. Yang and 0. Ozcan. p53-Induction as a Genotoxic Test for Twenty-five Chemicals Undergoing *in vivo* Carcinogenicity Testing. *Env. Health Perspectives* **107**:805-812, 1999. PMID: 10504146
- Filippova, M. and **P. J. Duerksen-Hughes**. Inorganic and Dimethylated Arsenic Species Induce Cellular p53. *Chemical Research in Toxicology* **16**:423-431, 2003. PMID: 12641444
- Applied for a United States Letters Patent on "Method of Determining Carcinogenicity of Substances" on 4/2/96, and a Provisional US Patent on “Cells and Assays for Genotoxicity” on 7/26/01.

2. ***p53-independent pathways activated by DNA damage:*** An unexpected observation that certain cells lacking p53 responded to chemotherapeutic agents in a very similar manner as did matching cells expressing p53 led us to seek p53-independent apoptotic pathways that had the potential to serve as targets for therapy. This endeavor has led us in a number of exciting directions; we have found that alternative splicing and ceramide metablism are involved in these pathways, that specific variants of IL-24 may serve as excellent therapeutic anti-cancer agents, and that ceramide-mediated apoptosis may play a role in Alzheimer’s and other neurological diseases.
   - Filippov, V., M. Filippova and **P. J. Duerksen-Hughes**. The Early Response to DNA Damage Can Lead to Activation of Alternative Splicing Activity Resulting in CD44 Splice Pattern Changes. *Cancer Research*. **67**:7621-7630, 2007. PMID: 17699766
   - Whitaker, E. L., V. Filippov, M. Filippova, C. F. Guerrero-Juarez, **and P. J. Duerksen-Hughes**. Splice variants of mda-7/IL-24 differentially affect survival and induce apoptosis in U2OS cells. *Cytokine,* 56:272-281, 2011. PMID: 21843952
   - Filippov, V., M.A. Song, K. Zhang, H. V. Vinters, S. Tung, W. M. Kirsch, J. Yang, and **P. J. Duerksen-Hughes**. Increased Ceramide in brains with Alzheimer’s and other neurodegenerative diseases. *Journal of Alzheimer’s Disease,* **29**:537-547*,* 2012. PMID: 22258513
   - Haynes, T.-A. S., V. Filippov, M. Filippova, J. Yang, K. Zhang, and **P. J. Duerksen-Hughes**. DNA damage induces down-regulation of UDP-Glucose Ceramide Glucosyltransferase, increases ceramide levels and triggers apoptosis in p53-deficient cancer cells. *BBA – Molecular and Cell Biology of Lipids,* **1821**:943-953, 2012. PMID: 22349266

3. ***E6-mediated inhibition of receptor-mediated apoptosis:*** My postdoctoral work initiated an ongoing fascination with the layers of measures and countermeasures found in virus/host relationships. In particular, I was quite interested in the ability of the high-risk HPV E6 protein to block apoptotic signaling pathways. Its ability to interfere with the p53 mediated pathway was already well-known, but my laboratory made the discovery that it could also interfere with apoptosis triggered by interactions between members of the TNF family and their receptors by binding to and (in some cases) accelerating the degradation of several of the molecular players, including TNF R1, FADD and Caspase 8. It turns out, therefore, that E6 is able to sabotage both the internal and the extrinsic apoptotic pathways so as to maintain host cell survival and maximize viral replication. Our body of work has led to a much better appreciation of the capacity of E6 to interfere with host-mediated apoptotic pathways, significantly advancing the fields of HPV biology and host/virus interactions, and identifying promising targets for thereapeutic intervention.
   - Filippova, M., H. Song, J. L. Connolly, T. S. Dermody and **P. J. Duerksen-Hughes.** The Human Papillomavirus 16 E6 Protein Binds to TNF R1 and Protects Cells from TNF-Triggered Apoptosis. *J. Biol. Chem*. **277**:21730-21739, 2002. PMID: 11934887
   - Filippova, M., Parkhurst, L. and **P. J. Duerksen-Hughes**. HPV 16 E6 Binds to FADD and Modulates Fas-Triggered Apoptosis*. J. Biol. Chem*. **279**:25729-25744, 2004. PMID: 15073179
   - Garnett, T., M. Filippova, and **P. J. Duerksen-Hughes**. Accelerated Degradation of FADD and Pro-Caspase 8 in Cells Expressing Human Papillomavirus 16 E6 Impairs TRAIL-Mediated Apoptosis. *Cell Death and Differentiation*. **13**:1915-1926, 2006. PMID: 16528366
   - Filippova, M., V. A. Filippov, M. Kagoda, T. Garnett, N. Fodor and **P. J. Duerksen-Hughes**. Complexes of Human Papillomavirus 16 E6 Proteins Form Pseudo-DISC Structures During TNF-Medicated Apoptosis. *J. Virol*. **83**:210-227, 2009. PMID: 18842714

4. ***Role of E6*:*** The E6 oncoprotein from high-risk HPV is produced as several splice variants; the full-length version, as well as several truncated versions known collectively as E6*. Even though epidemiological evidence points toward a role of E6* in pathogenesis, very little was previously known regarding the shorter isoforms other than their existence. My laboratory has addressed this gap, developing and optimizing ways to reliably detect, isolate and characterize this relatively uncooperative and disordered protein, and demonstrating its roles in increasing cellular oxidative stress while inhibiting tumor growth. These activities

make E6* a logical focus in our efforts to decrease conversion to cancer in HPV-infected individuals, as well as our efforts to develop better therapeutic approaches for HPV-associated cancers.

- Tungteakkhun, S. S., M. Filippova, N. Fodor and **P. J. Duerksen-Hughes**. The Full Length Isoform of HPV 16 E6 and its Splice Variant E6* Bind to Different Sites on Procaspase 8 DED. *J. Virology*. **84**:1453-1463, 2010. PMID: 19906919
- Filippova, M., W. Evans, R. Aragon, V. Filippov, V. Williams, L. Hong, M. E. Reeves, and **P. J. Duerksen-Hughes**. The Small splice variant of HPV16 E6, E6*, reduces tumor formation in cervical carcinoma xenografts. *Virology*. **450-451**:153-164, 2014. PMID: 24503078
- Williams, V. M., M. Filippova, V. Filippov, K. J. Payne and **P. J. Duerksen-Hughes**. HPV 16 E6* induces oxidative stress and DNA damage. *J. Virology* **88**:6751-6761, 2014. PMID: 2469478. **Selected for a JVI Spotlight Feature.**
- Evans, W., M. Filippova, V. Filippov, S. Vashkirova, G. Zhang, M. E. Reeves and **P. J. Duerksen-Hughes.** Overexpression of HPV16 E6* Alters β-Integrin and Mitochondrial Dysfunction Pathways in Cervical Cancer Cells. *Cancer Genomics & Proteomics* **13**:259-73, 2016. PMID: 27365376.

5. ***Approaches to decrease the burden of HPV-mediated cancers and the associated health disparity:*** Our findings regarding the roles of E6* and E6 in initiating and promoting cancer have suggested several opportunities for developing better therapeutic approaches. One approach is to look for small molecules that interfere with the ability of E6 to bind to its cellular partners; two papers have been published, others are in progress, disclosures have been made, a patent has been applied for, and screening and characterization of lead compounds is active and ongoing. A second approach is to ask whether the abilities of E6* to decrease tumor growth can be mimicked in some way; and a third approach asks whether interventions that decrease overall oxidative stress in cells and in individuals can decrease the conversion rate.
   - Yuan, C.-H., M. Filippova, S. S. Tungteakkhun, **P. J. Duerksen-Hughes** and J. L. Krstenansky. Small molecule inhibitors of the HPV16-E6 interaction with caspase 8. *Bioorganic and Medicinal Chemistry Letters*, **22**:2125-2129. 2012. PMID: 22300659
   - Filippova, M., W. Evans, R. Aragon, V. Filippov, V. Williams, L. Hong, M. E. Reeves, and **P. J. Duerksen-Hughes**. The Small splice variant of HPV16 E6, E6*, reduces tumor formation in cervical carcinoma xenografts. *Virology*. **450-451**:153-164, 2014. PMID: 24503078
   - Yuan, C.-H., M. Filippova, J. L. Krstenansky and **P. Duerksen-Hughes**. Flavonol and Imidazole Derivatives Block HPV16 E6 Activities and Reactive Apoptotic Pathways in HPV^{+} Cells. *Cell Death & Disease* **7**:2060-2072, 2016. PMID: 26794656.
   - Chen Wongworawat, Y., M. Filippova, V. M. Williams, V. Filippov**, P. J. Duerksen-Hughes**. Chronic oxidative stress increases the integration frequency of foreign DNA and human papillomavirus 16 in human keratinocytes. *Am. J. Cancer Research* **6**:684-680, 2016. PMID: 27186429.

A complete list of my published work is available in My Bibliography, which represents an **h factor of 28** as of 10/31/16:
http://www.ncbi.nlm.nih.gov/sites/myncbi/18AeNxgYrvY/bibliograpahy/40441248/public/?sort=date&direction=ascending
https://scholar.google.com/citations?user=evNsI4EAAAAJ&hl=en

D. Research Support

Ongoing Research Support

[redacted]

Loma Linda University **Knutsen (Co-PI) and Duerksen-Hughes (Co-PI)** 1/1/15 – 12/31/16
Grants for Research and School Partnerships (GRASP)
The Association between Adverse Life Events and Biological Aging
The overall goal is to study the association between adverse life events, the DNA methylome and telomere length.
Role: Co-PI

Completed Research Support

R21 NS073059 **Duerksen-Hughes (Principle Investigator)** 9/27/10 – 8/31/13
HTS Screening for Inhibitors of HPV 16 E6/Caspase 8 Binding

The goal of this successful project was to optimize a high-throughput screening assay that can be used to identify small molecules that can block E6/caspase 8 binding. Such molecules have the potential to be developed into clinically useful therapies for individuals suffering from HPV-associated malignancies.

OMB Number: 4040-0001
Expiration Date: 06/30/2016

RESEARCH & RELATED BUDGET - SECTION A & B, Budget Period 1

ORGANIZATIONAL DUNS*: [redacted]

Budget Type*: ● Project ❍ Subaward/Consortium

Enter name of Organization: iSpin Inc.

Start Date*: 10-01-2017 **End Date*:** 03-31-2018 **Budget Period: 1**

A. Senior/Key Person

	Prefix	First Name*	Middle Name	Last Name*	Suffix	Project Role*	Base Salary ($)	Calendar Months	Academic Months	Summer Months	Requested Salary ($)*	Fringe Benefits ($)*	Funds Requested ($)*
1.	[redacted]	[redacted]		[redacted]		PD/PI	[redacted]	[redacted]			[redacted]	[redacted]	[redacted]

Total Funds Requested for all Senior Key Persons in the attached file

Additional Senior Key Persons: File Name:

Total Senior/Key Person [redacted]

B. Other Personnel

Number of Personnel*	Project Role*	Calendar Months	Academic Months	Summer Months	Requested Salary ($)*	Fringe Benefits*	Funds Requested ($)*
	Post Doctoral Associates						
	Graduate Students						
	Undergraduate Students						
	Secretarial/Clerical						
1	Laboratory Assistant	1.20			[redacted]	[redacted]	[redacted]
1	**Total Number Other Personnel**					**Total Other Personnel**	[redacted]
						Total Salary, Wages and Fringe Benefits (A+B)	[redacted]

RESEARCH & RELATED Budget {A-B} (Funds Requested)

Funding Opportunity Number: PA-16-302 . Received Date: 2017-01-04T13:45:01.000-05:00

RESEARCH & RELATED BUDGET - SECTION C, D, & E, Budget Period 1

ORGANIZATIONAL DUNS*: [redacted]

Budget Type*: ● Project ❍ Subaward/Consortium

Organization: iSpin Inc.

Start Date*: 10-01-2017 **End Date*:** 03-31-2018 **Budget Period: 1**

C. Equipment Description

List items and dollar amount for each item exceeding $5,000

Equipment Item	**Funds Requested ($)***
Total funds requested for all equipment listed in the attached file	
Total Equipment	

Additional Equipment: File Name:

D. Travel	**Funds Requested ($)***
1. Domestic Travel Costs (Incl. Canada, Mexico, and U.S. Possessions)	[redacted]
2. Foreign Travel Costs	
Total Travel Cost	[redacted]

E. Participant/Trainee Support Costs		**Funds Requested ($)***
1. Tuition/Fees/Health Insurance		
2. Stipends		
3. Travel		
4. Subsistence		
5. Other:		
Number of Participants/Trainees	**Total Participant Trainee Support Costs**	

RESEARCH & RELATED Budget {C-E} (Funds Requested)

Tracking Number: GRANT12312421 Funding Opportunity Number: PA-16-302 . Received Date: 2017-01-04T13:45:01.000-05:00

RESEARCH & RELATED BUDGET - SECTIONS F-K, Budget Period 1

ORGANIZATIONAL DUNS*: [redacted]

Budget Type*: ● Project ❍ Subaward/Consortium

Organization: iSpin Inc.

Start Date*: 10-01-2017 **End Date*:** 03-31-2018 **Budget Period: 1**

F. Other Direct Costs	Funds Requested ($)*
1. Materials and Supplies	[redacted]
2. Publication Costs	[redacted]
3. Consultant Services	[redacted]
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	[redacted]
6. Equipment or Facility Rental/User Fees	
7. Alterations and Renovations	
Total Other Direct Costs	[redacted]

G. Direct Costs	Funds Requested ($)*
Total Direct Costs (A thru F)	[redacted]

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)*
1 . Modified Total Direct Costs	10.00	[redacted]	[redacted]0
		Total Indirect Costs	[redacted]

Cognizant Federal Agency DHHS
(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	Funds Requested ($)*
Total Direct and Indirect Institutional Costs (G + H)	[redacted]

J. Fee	Funds Requested ($)*

K. Budget Justification* File Name: 1235-iSpinjustification.pdf
(Only attach one file.)

RESEARCH & RELATED Budget {F-K} (Funds Requested)

BUDGET JUSTIFICATION

iSpin, Inc.

Senior/Key Personnel

Maria Filippova, Ph.D. (51% effort, 3.06 calendar months) will be the **Principle Investigator** on this grant. She has extensive experience in cell culture and molecular biology, and either carried out or supervised the majority of the work leading to this application. She will be responsible for the overall conduct of the work, and work closely with the subcontractor in data analysis and interpretation, as well as the publication of results.

Other Personnel

[redacted] a **Laboratory Assistant** who will provide general technical support to this project.

Other Expenses

Travel: Funds are budgeted for one trip during the award period to a national meeting to present results.

Animal Costs: Funds are budgeted for the purchase and *per diem* care of animals as described in the proposal.

Laboratory supplies: Funds are budgeted for the purchase of basic laboratory supplies such as cell culture media, disposable cell culture plastic ware, chemicals, syringes, etc.

Histopathology: Funds are budgeted for the purchase of appropriate antibodies and staining material and visualization as described in this application.

Publication Costs: Funds are budgeted for the publication of one manuscript resulting from the work described in this proposal.

Consultant Services: Funds are budgeted for toxicology services related to Aim 1.

Indirect Costs

In the absence of a federally negotiated F&A rate, 10% indirect is requested using a modified total direct costs base.

RESEARCH & RELATED BUDGET - Cumulative Budget

	Totals ($)	
Section A, Senior/Key Person		[redacted]
Section B, Other Personnel		[redacted]
Total Number Other Personnel	1	
Total Salary, Wages and Fringe Benefits (A+B)		[redacted]
Section C, Equipment		
Section D, Travel		[redacted]
1. Domestic	[redacted]	
2. Foreign		
Section E, Participant/Trainee Support Costs		
1. Tuition/Fees/Health Insurance		
2. Stipends		
3. Travel		
4. Subsistence		
5. Other		
6. Number of Participants/Trainees		
Section F, Other Direct Costs		[redacted]
1. Materials and Supplies	[redacted]	
2. Publication Costs	[redacted]	
3. Consultant Services	[redacted]	
4. ADP/Computer Services		
5. Subawards/Consortium/Contractual Costs	[redacted]	
6. Equipment or Facility Rental/User Fees		
7. Alterations and Renovations		
8. Other 1		
9. Other 2		
10. Other 3		
Section G, Direct Costs (A thru F)		[redacted]
Section H, Indirect Costs		[redacted]
Section I, Total Direct and Indirect Costs (G + H)		[redacted]
Section J, Fee		

OMB Number: 4040-0001
Expiration Date: 06/30/2016

RESEARCH & RELATED BUDGET - SECTION A & B, Budget Period 1

ORGANIZATIONAL DUNS*: [redacted]

Budget Type*: ❍ Project ● Subaward/Consortium

Enter name of Organization: Loma Linda University

Start Date*: 10-01-2017 **End Date*:** 03-31-2018 **Budget Period: 1**

A. Senior/Key Person

	Prefix	First Name*	Middle Name	Last Name*	Suffix	Project Role*	Base Salary ($)	Calendar Months	Academic Months	Summer Months	Requested Salary ($)*	Fringe Benefits ($)*	Funds Requested ($)*
1 .		Penelope		Duerksen-Hughes	PhD	PD/PI	[redacted]	[redacted]			[redacted]	[redacted]	[redacted]

Total Funds Requested for all Senior Key Persons in the attached file

Additional Senior Key Persons: File Name:

Total Senior/Key Person [redacted]

B. Other Personnel

Number of Personnel*	Project Role*	Calendar Months	Academic Months	Summer Months	Requested Salary ($)*	Fringe Benefits*	Funds Requested ($)*
	Post Doctoral Associates						
1	Graduate Students	6.00			[redacted]	0.00	[redacted]
	Undergraduate Students						
	Secretarial/Clerical						
1	**Total Number Other Personnel**					**Total Other Personnel**	[redacted]
						Total Salary, Wages and Fringe Benefits (A+B)	[redacted]

RESEARCH & RELATED Budget {A-B} (Funds Requested)

Funding Opportunity Number: PA-16-302 . Received Date: 2017-01-04T13:45:01.000-05:00

RESEARCH & RELATED BUDGET - SECTION C, D, & E, Budget Period 1

ORGANIZATIONAL DUNS*: [redacted]

Budget Type*: ❍ Project ● Subaward/Consortium

Organization: Loma Linda University

Start Date*: 10-01-2017 **End Date*:** 03-31-2018 **Budget Period: 1**

C. Equipment Description	
List items and dollar amount for each item exceeding $5,000	
Equipment Item	**Funds Requested ($)***
Total funds requested for all equipment listed in the attached file	
Total Equipment	
Additional Equipment: File Name:	

D. Travel	**Funds Requested ($)***
1. Domestic Travel Costs (Incl. Canada, Mexico, and U.S. Possessions)	
2. Foreign Travel Costs	
Total Travel Cost	

E. Participant/Trainee Support Costs		**Funds Requested ($)***
1. Tuition/Fees/Health Insurance		
2. Stipends		
3. Travel		
4. Subsistence		
5. Other:		
Number of Participants/Trainees	**Total Participant Trainee Support Costs**	

RESEARCH & RELATED Budget {C-E} (Funds Requested)

Tracking Number: GRANT12312421 Funding Opportunity Number: PA-16-302 . Received Date: 2017-01-04T13:45:01.000-05:00

RESEARCH & RELATED BUDGET - SECTIONS F-K, Budget Period 1

ORGANIZATIONAL DUNS*: [redacted]

Budget Type*: ❍ Project ● Subaward/Consortium

Organization: Loma Linda University

Start Date*: 10-01-2017 **End Date*:** 03-31-2018 **Budget Period: 1**

F. Other Direct Costs	Funds Requested ($)*
Total Other Direct Costs	

G. Direct Costs	Funds Requested ($)*
Total Direct Costs (A thru F)	[redacted]

H. Indirect Costs

Indirect Cost Type	Indirect Cost Rate (%)	Indirect Cost Base ($)	Funds Requested ($)*
1 . Modified Total Direct Costs	10.00	[redacted]	[redacted]
		Total Indirect Costs	[redacted]

Cognizant Federal Agency DHHS

(Agency Name, POC Name, and POC Phone Number)

I. Total Direct and Indirect Costs	Funds Requested ($)*
Total Direct and Indirect Institutional Costs (G + H)	[redacted]

J. Fee	Funds Requested ($)*

K. Budget Justification*	File Name: 1248-LLUjustification.pdf
	(Only attach one file.)

RESEARCH & RELATED Budget {F-K} (Funds Requested)

SUBAWARD BUDGET JUSTIFICATION

Loma Linda University

Senior/Key Personnel

Penelope Duerksen-Hughes, Ph.D. (10% effort, 0.60 calendar months) will be the **Subaward leader**. She is an expert in HPV biology, and it was within her laboratory that the work leading up to this application was conducted. She will be responsible for the work carried out by Loma Linda University (and in particular, by the graduate student), and will work closely with Dr. Filippova in data analysis and in the interpretation and publications of results.

Other Personnel

[redacted] (100% effort, 6.00 calendar months) is a 4th year **Graduate Student** who will be working on this project. She participated in generating much of the preliminary data presented in this application. Funds are requested to support her salary.

Indirect Costs

Loma Linda University's negotiated F&A rate is 58%, however is requesting 10% indirect using a modified total direct costs base.

RESEARCH & RELATED BUDGET - Cumulative Budget

	Totals ($)
Section A, Senior/Key Person	[redacted]
Section B, Other Personnel	[redacted]
Total Number Other Personnel	1
Total Salary, Wages and Fringe Benefits (A+B)	[redacted]
Section C, Equipment	
Section D, Travel	
1. Domestic	
2. Foreign	
Section E, Participant/Trainee Support Costs	
1. Tuition/Fees/Health Insurance	
2. Stipends	
3. Travel	
4. Subsistence	
5. Other	
6. Number of Participants/Trainees	
Section F, Other Direct Costs	
1. Materials and Supplies	
2. Publication Costs	
3. Consultant Services	
4. ADP/Computer Services	
5. Subawards/Consortium/Contractual Costs	
6. Equipment or Facility Rental/User Fees	
7. Alterations and Renovations	
8. Other 1	
9. Other 2	
10. Other 3	
Section G, Direct Costs (A thru F)	[redacted]
Section H, Indirect Costs	[redacted]
Section I, Total Direct and Indirect Costs (G + H)	[redacted]
Section J, Fee	

Total Direct Costs less Consortium F&A

NIH policy (NOT-OD-05-004) allows applicants to exclude consortium/contractual F&A costs when determining if an application falls at or beneath any applicable direct cost limit. When a direct cost limit is specified in an FOA, the following table can be used to determine if your application falls within that limit.

Category	Budget Period 1	Budget Period 2	Budget Period 3	Budget Period 4	Budget Period 5	TOTALS
Total Direct Costs less Consortium F&A	[redacted]	0	0	0	0	[redacted]

OMB Number: 4040-0001
Expiration date: 06/30/2016

SBIR/STTR Information

Program Type (select only one)*

● SBIR ❍ STTR ❍ Both (See agency-specific instructions to determine whether a particular agency allows a single submission for both SBIR and STTR)

SBIR/STTR Type (select only one)*

● Phase I ❍ Phase II ❍ Fast-Track (See agency-specific instructions to determine whether a particular agency participates in Fast-Track)

Questions 1-7 must be completed by all SBIR and STTR Applicants:

1a. Do you certify that at the time of award your organization will meet the eligibility criteria for a small business as defined in the funding opportunity announcement?* ● Yes ❍ No

1b. Anticipated Number of personnel to be employed at your organization at the time of award.* 1

2. Does this application include subcontracts with Federal laboratories or any other Federal Government agencies?* ❍ Yes ● No

If yes, insert the names of the Federal laboratories/agencies:*

3. Are you located in a HUBZone? To find out if your business is in a HUBZone, use the mapping utility provided by the Small Business Administration at its web site: http://www.sba.gov * ❍ Yes ● No

4. Will all research and development on the project be performed in its entirety in the United States?* ● Yes ❍ No

If no, provide an explanation in an attached file. Explanation:*

5. Has the applicant and/or Program Director/Principal Investigator submitted proposals for essentially equivalent work under other Federal program solicitations or received other Federal awards for essentially equivalent work?* ❍ Yes ● No

If yes, insert the names of the other Federal agencies:*

6. Disclosure Permission Statement: If this application does not result in an award, is the Government permitted to disclose the title of your proposed project, and the name, address, telephone number and e-mail address of the official signing for the applicant organization, to organizations that may be interested in contacting you for further information (e.g., possible collaborations, investment)?* ● Yes ❍ No

7. Commercialization Plan: If you are submitting a Phase II or Phase I/Phase II Fast-Track Application, include a Commercialization Plan in accordance with the agency announcement and/or agency-specific instructions.*

Attach File:*

OMB Number: 4040-0001
Expiration date: 06/30/2016

SBIR/STTR Information

SBIR-Specific Questions:

Questions 8 and 9 apply only to SBIR applications. If you are submitting ONLY an STTR application, leave questions 8 and 9 blank and proceed to question 10.

8. Have you received SBIR Phase II awards from the Federal Government? If yes, provide a company commercialization history in accordance with agency-specific instructions using this attachment.* ❍ Yes ● No

Attach File:*

9. Will the Project Director/Principal Investigator have his/her primary employment with the small business at the time of award?* ● Yes ❍ No

STTR-Specific Questions:

Questions 10 and 11 apply only to STTR applications. If you are submitting ONLY an SBIR application, leave questions 10 and 11 blank.

10. Please indicate whether the answer to BOTH of the following questions is TRUE:* ❍ Yes ❍ No

(1) Does the Project Director/Principal Investigator have a formal appointment or commitment either with the small business directly (as an employee or a contractor) OR as an employee of the Research Institution, which in turn has made a commitment to the small business through the STTR application process; AND

(2) Will the Project Director/Principal Investigator devote at least 10% effort to the proposed project?

11. In the joint research and development proposed in this project, does the small business perform at least 40% of the work and the research institution named in the application perform at least 30% of the work?* ❍ Yes ❍ No

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OMB Number: 0925-0001

Expiration Date: 10/31/2018

1. Human Subjects Section

Clinical Trial? ○ Yes ● No

*Agency-Defined Phase III Clinical Trial? ○ Yes ○ No

2. Vertebrate Animals Section

Are vertebrate animals euthanized? ● Yes ○ No

If "Yes" to euthanasia

Is the method consistent with American Veterinary Medical Association (AVMA) guidelines?

● Yes ○ No

If "No" to AVMA guidelines, describe method and proved scientific justification

3. *Program Income Section

*Is program income anticipated during the periods for which the grant support is requested?

○ Yes ● No

If you checked "yes" above (indicating that program income is anticipated), then use the format below to reflect the amount and source(s). Otherwise, leave this section blank.

*Budget Period	*Anticipated Amount ($)	*Source(s)

Tracking Number: GRANT12312421 Funding Opportunity Number: PA-16-302 . Received Date: 2017-01-04T13:45:01.000-05:00

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4. Human Embryonic Stem Cells Section

*Does the proposed project involve human embryonic stem cells? ○ Yes ● No

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://grants.nih.gov/stem_cells/registry/current.htm. Or, if a specific stem cell line cannot be referenced at this time, please check the box indicating that one from the registry will be used:

☐ Specific stem cell line cannot be referenced at this time. One from the registry will be used.

Cell Line(s) (Example: 0004):

5. Inventions and Patents Section (RENEWAL)

*Inventions and Patents: ○ Yes ○ No

If the answer is "Yes" then please answer the following:

*Previously Reported: ○ Yes ○ No

6. Change of Investigator / Change of Institution Section

☐ Change of Project Director / Principal Investigator

Name of former Project Director / Principal Investigator

Prefix:

*First Name:

Middle Name:

*Last Name:

Suffix:

☐ Change of Grantee Institution

*Name of former institution:

PHS 398 Research Plan

OMB Number: 0925-0001
Expiration Date: 10/31/2018

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3. Research Strategy*	1244-NIH010517strategy.pdf
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SPECIFIC AIMS

High-risk types of human papillomaviruses (HPV) are responsible for virtually all cases of human cervical carcinoma, as well as an increasing number of other HPV-associated malignancies, including those of the head and neck, anus and vulva. One growing group of patients particularly affected by HPV includes those with compromised immune systems resulting from HIV infection, other diseases or medical treatments. Perhaps the most noteworthy advance in recent years has been the development of safe and effective vaccines targeted against HPV. However, these vaccines are not beneficial for patients who are already infected, appropriate for use in patients with compromised immune systems, or readily available in all developing countries. Once cancer has developed, current treatment options are relatively limited and focus on physically removing the cancer through surgery. Unfortunately, tumors frequently return, particularly following late-stage diagnosis and/or if the patient is immunocompromised. Chemo- and radio-therapies that rely on the induction of apoptosis in HPV^+ tumor cells are relatively ineffective, primarily due to the actions of a virus-encoded oncoprotein, E6, that subverts both intrinsic and extrinsic apoptotic pathways by accelerating the degradation of key molecular players. Therefore, new approaches that can eliminate HPV-containing cells, even in the absence of a functional adaptive immune system, must be developed.

To meet this need, we propose to combine spinacine, a small, naturally occurring molecule whose E6-inhibiting abilities were recently discovered by our laboratory, with existing therapeutic approaches that function by inducing apoptosis. Our laboratory and others have shown that high-risk versions of the HPV E6 oncoprotein induce resistance to both intrinsic and extrinsic apoptosis by mediating the rapid degradation of p53, caspase 8 and FADD [1-6]. The absence of these molecules in turn leads to the protection of infected cells from agents that would otherwise induce programmed cell death. To counter this, we searched for molecules that would inhibit the ability of E6 to bind to its cellular apoptotic partners by screening over 3000 compounds. Spinacine was selected as our lead candidate, because it is able to block the binding of E6 to both caspase 8 and E6AP, thereby sensitizing HPV^+ cells to apoptosis triggered by agents such as TRAIL (a ligand that selectively induces apoptosis in cancer cells) and chemotherapeutic drugs such as cisplatin and doxorubicin. As predicted by our model, spinacine restores cellular levels of caspase 8, FADD and p53. Together, these observations support the *scientific premise* of our proposal.

The *long-term goal* of our laboratory is to develop novel, effective therapies for patients suffering from HPV-associated malignancies, and the *overall objective* of this current application is to move our exciting *in vitro* and cellular observations into a mouse xenograft model. In this model, we will 1) Assess the toxicity of spinacine, and 2) Determine the *in vivo* effectiveness of a combinational therapy that pairs spinacine with an agent that induces apoptosis (TRAIL or cisplatin). The *conceptual framework* supporting this proposal states that by targeting the E6/apoptotic protein interactions with small, drug-like molecules such as spinacine, we will be able to increase cellular levels of p53, caspase 8 and FADD. This will sensitize cells to apoptosis, thereby enabling agents that induce apoptosis to eliminate or decrease the growth of HPV^+ tumors *in vivo*. All necessary assays, reagents and expertise are available to us, and for these reasons, we are well positioned to immediately undertake the work described in the following specific aims:

> **Specific Aim 1: Determine the toxicity of spinacine in mice.** We will assess the toxicity of spinacine in mice, defining the maximum tolerated dose and identifying the optimum dose with which to carry out experiments designed to test its efficacy.
>
> **Specific Aim 2: Evaluate the ability of spinacine to synergize with TRAIL- and/or chemo-based therapies to reduce or eliminate HPV^+ tumor growth.** We will assess the ability of spinacine to synergize with hrTRAIL and/or the DNA damaging drug cisplatin to inhibit tumor growth in a xenograft model.

At the conclusion of this work, we will have 1) Determined the toxicity of the E6-inhibiting molecule spinacine in mice, and 2) Evaluated the effectiveness of combining spinacine with TRAIL- and cisplatin-based treatments in an animal model. This work has the potential to save the lives of patients suffering from HPV-associated malignancies.

SIGNIFICANCE

HPV is responsible for nearly all cervical cancers and the majority of head and neck cancers. High-risk types of human papillomavirus (HPV) are responsible for virtually all cases of human cervical carcinoma. Worldwide, this is the second most common cancer in women; over 400,000 women are newly diagnosed with this disease each year, of which approximately half will die [7]. Additional deaths occur due to other HPV-associated malignancies, such as those of the head and neck, anus and vulva. Head and neck (HN) cancers are of particular interest, because the incidence of HPV-mediated HN cancer in the US has dramatically risen [8-10].

Current treatment options for HPV-associated tumors are limited and frequently ineffective. Public health policies and initiatives claim responsibility for significant progress against HPV-mediated cancers in the contexts of infection prevention and early detection. Currently available prophylactic vaccines appear to be both safe and effective [11-13]. Importantly, however, these vaccines are not effective interventions for individuals already infected with HPV, nor are they appropriate for immunodeficient individuals [14]. Further concerns include recent reports regarding side effects of vaccination [15], as well as challenges inherent in making the vaccine available in developing countries. Early detection of cervical cancer is frequently possible through the use of population-wide Pap screening, though an equivalent approach for identifying HN and other HPV-associated tumors is not yet available. Current treatment options focus on removing the tumor, which can be effective when the tumor is detected in time. However, options for tumors detected later are much less effective. Survival is low, due in large part to the activities of the E6 oncoprotein (see below), and patients, especially those who are immunocompromised, frequently relapse [14, 16]. In fact, cervical cancer is highly resistant to chemotherapy, with only 15 – 20% of tumors responding to treatment [17]. The best results thus far have been obtained using combined chemo-radiotherapies, and approaches based on cisplatin, carboplatin, paclitaxel, topotecan, 5-fluorouracil and radiotherapy are frequently combined into regimens drawing on two or even three agents. However, even these combinatorial treatments have limited efficacy and relatively serious side effects [18-20]. Together, these issues point toward the compelling and urgent need to develop better treatment options for patients with cervical cancer and other HPV-associated malignancies.

The E6 oncoprotein encoded by high-risk HPV limits the effectiveness of chemo- and radiotherapies by compromising cellular apoptotic pathways. Most chemo- and radiotherapies are based on the idea that they can damage DNA, and that that damage will then trigger apoptotic death of the cancer cells. Unfortunately, the E6 oncoprotein produced by high-risk types of the virus, such as HPV16 and 18, subverts both the intrinsic and extrinsic apoptotic pathways by accelerating the degradation of key molecular players. This means that apoptosis-inducing treatments are much less effective than they would be in the absence of E6. The first target to be documented was E6AP; the binding of E6 to E6AP leads to rapid degradation of the p53 tumor suppressor, thereby inhibiting the induction of p53-mediated intrinsic apoptosis in HPV-infected cells [1-3]. Since then, our laboratory has demonstrated that TNF R1 [21], FADD [5] and caspase 8 [4], all molecules involved in receptor-mediated apoptosis, are also targets of high-risk E6 oncoproteins.

Treatments based on the TRAIL-mediated apoptosis pathway have the potential to be effective against HPV-mediated malignancies. TRAIL-based therapies, which activate the extrinsic apoptotic pathway, have elicited significant interest, largely due to their ability to kill tumor cells while sparing most normal cells. TRAIL receptors 1 and 2 are highly expressed on a large number of solid and hematologic cancers, making these tumors sensitive to apoptosis induced both by TRAIL itself and by antibodies to the receptor [22-27]. However, TRAIL therapy can be limited in that cancer cells can develop resistance toward TRAIL [28, 29]. In the case of HPV-mediated malignancies, the only cancer-mediated agent is high risk HPV. Our laboratory found that in the case of HPV^+-mediated malignancies, E6 is the major factor responsible for resistance to TRAIL [6, 30].

Restoring the level of E6-targeted apoptotic molecules can sensitize HPV^+ cells to apoptosis. Our efforts over the past 10-15 years [4-6,30], along with those of others, have implicated E6 as an excellent target for therapeutic intervention. This is because disrupting the binding between E6 and its target proteins can result in restoration of these apoptotic proteins, leading to a re-sensitization of HPV^+ cells to apoptotic triggers [4-6, 21, 30-32]. Some progress has been made in identifying small molecules that can interfere with E6 activities [33-38], but no studies have yet combined E6 inhibitors with apoptosis-inducing agents. Our laboratory has identified and tested several small molecules that block E6/caspase 8 interactions, and our preliminary studies provide proof-of principle evidence that molecules that block E6/caspase 8 interactions can, indeed, re-sensitize HPV^+ cells to apoptotic triggers [30, 31, 39]. In particular, we found that both myricetin and spinacine, small molecules selected on the basis of their ability to block E6/caspase 8 binding, sensitize E6-expressing cells to death mediated by both TRAIL and chemotherapeutic agents such as cisplatin and doxorubicin [30, 40]. As predicted, these small molecules restore cellular levels of p53 and procaspase 8, thus validating our working model.

The marketability for E6 inhibitors such as spinacine is potentially high. The overall incidence cost of HPV-associated cancers in 2010 was $958 million in the US alone (for a total of $8 billion), with approximately $1

billion directed toward direct cancer treatment [41]. Unfortunately, these large outlays do not produce good results for late-stage cancers. We propose to change this situation by employing spinacine, a relatively cheap, naturally occurring compound that carries within its structure multiple options for optimization. Importantly, we do not anticipate late-stage trial failure resulting from spinacine treatment, both because it is a naturally occurring compound already consumed by the public and because our preliminary results indicate very low toxicity profiles in both cells and mice. Preliminary estimates suggest possible sales revenue of $48.8B during the first 10 years of commercialization, assuming that iSpin will receive FDA approval to sell the compound in 2015, the total number of patients with HPV-associated in cancer in 2025 will be similar to that reported in 2012, a growth rate of 4% is achieved, and that the costs of spinacine are $10,000 per patient per year.

INNOVATION

HPV-associated malignancies remain a significant clinical challenge. We propose to meet this challenge by re-establishing the ability of HPV^+ cells to respond to apoptotic signals by employing small molecules that prevent E6 from binding to its cellular partners. Our success thus far has been based on an innovative combination of our discoveries regarding how HPV E6 blocks apoptosis with state-of-the-art, high-throughput screening approaches. To the best of our knowledge, this is a novel concept for which we have already established proof-of principle. We believe that the next steps in this project are also highly innovative for several reasons.

- We are the first to ask whether "rescuing" molecules such as FADD and caspase 8 can **restore the responsiveness** of HPV^+ cells to apoptotic triggers.
- Spinacine, unlike other potential E6-inhibitory molecules, **inhibits both E6/caspase 8 and E6/E6AP interactions**. This unique property will allow us to simultaneously restore both the intrinsic and extrinsic apoptotic pathways, potentially amplifying treatment effectiveness.
- We anticipate that combinatorial treatment plans utilizing spinacine are likely to demonstrate **low toxicity,** a critical innovation in the context of HPV-associated malignancies. Spinacine itself has low toxicity, as does TRAIL. Furthermore, the anticipated effectiveness of the combinatorial approach may enable a reduction in the dosage, and hence, the side-effects of agents such as cisplatin.
- This study will **directly compare** the effectiveness of spinacine in partnering with TRAIL and with cisplatin, a relatively novel biologic and a traditional chemotherapeutic. These results will direct future work by enabling us to concentrate on developing the most effective approach to therapy.

APPROACH

Rationale and Overview: High-risk versions of the HPV E6 protein block transmittal of apoptotic signals through both the intrinsic and extrinsic pathways by accelerating the rapid degradation of apoptotic mediator molecules. Because most anticancer treatments work by triggering cell death, these actions of E6 present a substantial hurdle to effective clinical treatment. Therefore, eliminating the ability of E6 to protect its host cells from apoptosis is a logical and promising approach to improve clinical options for these patients. To do this, we propose to enlist small molecules that bind to E6 and prevent it from interacting with its cellular partners such as caspase 8 and E6AP. The overall logic for our approach is outlined in **Figure 1**, where **1A** shows the normal flow of apoptotic signaling, **1B** the interference with these signaling pathways caused by E6, and **1C** how our approach can restore apoptotic signaling in these HPV+ cells.

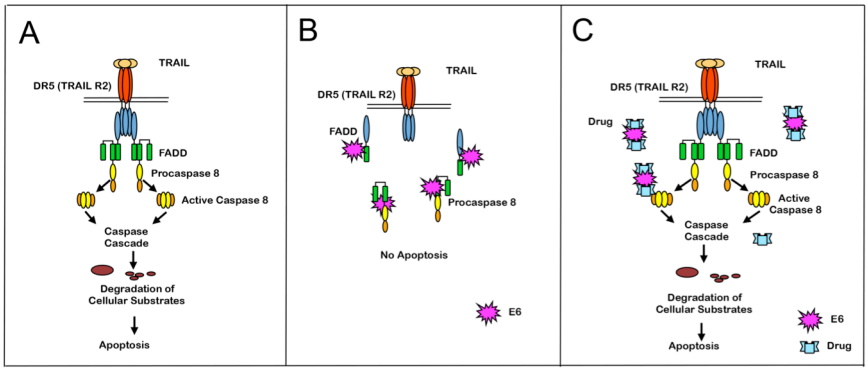

Figure 1: Working Model

In our previous work, we developed a bead-based assay based on PerkinElmer's AlphaScreen® technology, then used it to query both a ~1,000-compound flavonol library and a 2,000-compound diverse library (Actiprobe-2K), both obtained from TimTec, for small molecules capable of inhibiting E6/caspase 8 binding. Hit identification, lead identification and optimization were evaluated in a multi-step process including primary library screening, dose-response analysis, counter-screening, optimization based on SAR analysis, and finally, an isothermal scanning calorimetric (ITC) assay, considered the "gold standard" for kinetic measurements. At the conclusion of this process, we had identified one candidate from each library queried, the flavonol myricetin and the imidazole amino acid derivative of histidine, spinacine [30, 40].

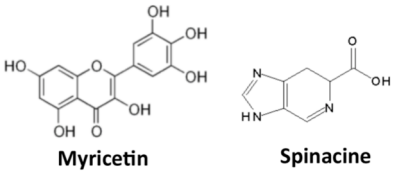

Figure 2: Myricetin and Spinacine

The structures of the two compounds are shown in **Figure 2**. Myricetin is a

natural product, ingested by humans, with a number of reported activities [42-44]. Unfortunately, myricetin has a fairly broad range of activities against kinases, and may be difficult to chemically optimize. Spinacine, a derivative of the amino acid histidine, is also a natural product found in certain food items including dairy products and ginseng [45, 46]. There is little available information on the biological activity of spinacine, with the exception of toxicological data in rats showing no toxic effect at a dose of 300 mg/kg of body weight per day [47]. The molecule is also Rule Of Five (R05) compliant according to The Human Metabolome Database, indicating that it is likely to be orally active. Spinacine was therefore selected as the compound of greatest interest. As noted below, we have already obtained cellular data pointing to its ability to selectively sensitize HPV$^+$ cells to apoptotic triggers. We will now take this molecule into animal studies by 1) Assessing its *in vivo* toxicity, and 2) Assessing its ability to partner with TRAIL and cisplatin to reduce or eliminate HPV$^+$ tumor growth *in vivo*.

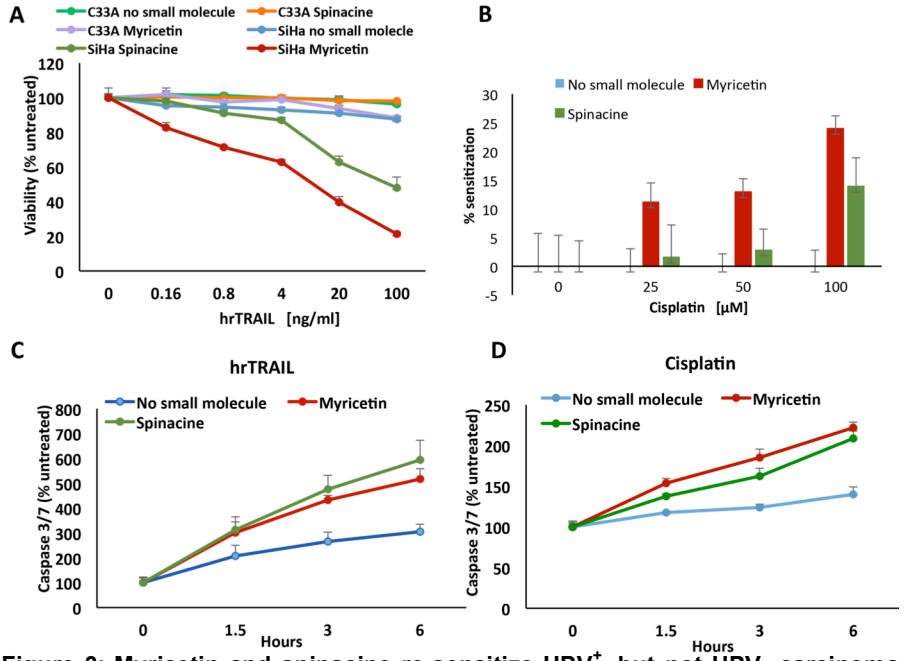

Figure 3: Myricetin and spinacine re-sensitize HPV$^+$, but not HPV- carcinoma cells to treatment with TRAIL (A) and cisplatin (B). A) 2x10^4 SiHa or 1x10^4 C33A cells were incubated with 100 μM of myricetin or 50 μM of spinacine for 4 h before adding the indicated concentrations of TRAIL in the presence of cycloheximide (5 μg/ml) and incubated for 16 h. **B)** 2x10^4 SiHa cells plated on a 96-well plate were incubated with myricetin (100 μM), spinacine (50 μM) or vehicle 4 h before adding the indicated concentrations of cisplatin for 16 h. Sensitization was calculated by subtraction of % of viable cells in the presence of small molecules from values for untreated cells. For A and B, cell viability was monitored using the MTT assay. **C and D)** 2x10^4 SiHa cells were pretreated with 100 μM of myricetin or 50 μM of spinacine for 4 h prior to the addition of TRAIL (100ng/ml) in the presence of cycloheximide (5 μg/ml) or 50 μM of cisplatin. Caspase 3/7 activity was measured at the indicated times using the Caspase-Glo® 3/7 Assay (Promega). Activity at 0 h of treatment was set at 100%.

Preliminary Data:

Myricetin and spinacine bind to E6 and prevent the binding of E6 to E6AP and caspase 8: The ability of myricetin and spinacine to prevent the binding of E6 to E6AP and caspase 8 was demonstrated using a bead-based binding assay, complemented by a secondary counter-screen [30, 40]. In the case of spinacine, we confirmed and quantified this activity with isothermal scanning calorimetry (ITC). In particular, we found that the affinity of binding between E6 and E6AP (Kd = 2.4x10^{-7}) was higher than between E6 and caspase 8 (Kd = 3.6x10^{-7}), and that the addition of spinacine to either reaction mixture decreased the measured affinity between E6 and its binding partner (E6-spinacine + E6AP: Kd=3.6x10^{-7} and E6-spinacine + caspase 8: Kd=9.3x10^{-7}).

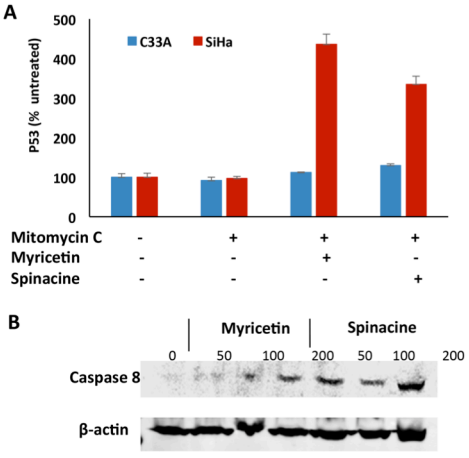

Figure 4: Myricetin and spinacine restore p53 (A) and caspase 8 (B). A) 1x10^4 SiHa or C33A cells were treated with 200 μM of Myricetin or 100 μM of Spinacine together with 4 μg/ml of Mytomycin C for 24 h. p53 levels were measured by ELISA, where the level of p53 in untreated cells was set at 100%. **B)** 1x10^6 SiHa cells seeded onto 6 well plates were incubated with the indicated concentration of Myricetin and Spinacine for 24 h. Lysates were subjected to PAGE, transferred to an Immobilon FL membrane, then caspase 8 and β-actin were detected by Western blot analysis.

Myricetin and spinacine sensitize HPV$^+$, but not HPV$^-$, cells to apoptosis: To carry out these experiments, we selected the SiHa cell line as a cellular model for cervical cancer, and several lines derived from head and neck (HN) carcinomas as cellular models for HN cancer (UM-SCC47 (47), UM-SCC104 (104), 93UV-147-Up-Clone 6 (93), UM-SCC47-TC-Clone 3 (47c3), UPCI-SCC90-Up-Clone 35 (90), and UD-SCC-2TC-Clone 5 (2TC)) [48]. Each of these lines express E6, and are therefore resistant to both receptor-mediated apoptotic signals and to chemotherapeutic drugs [40]. As predicted, we were indeed able to demonstrate that both myricetin and spinacine could sensitize HPV$^+$ cervical and HN cells to cell death mediated by TRAIL, cisplatin and doxorubicin [40]. To ask whether the ability of spinacine and myricetin to sensitize cells to apoptosis was due to an interaction of these molecules with the HPV E6 protein, we compared this sensitizing ability in cells expressing or not expressing E6. Results from cervical cells are shown in **Figures 3 and 4**; similar results were obtained using HN cells [40]. Our results indicated that while spinacine and myricetin were able to sensi-

tize HPV$^+$ cells (SiHa) to TRAIL **(Figure 3A)** and to cisplatin **(Figure 3B**), this activity was lost in cells lacking E6 (C33A), indicating that these actions are indeed specific for E6 (**Figure 3A).** Furthermore, we found that cell death proceeds through the apoptotic pathway, as demonstrated by the increase in caspase 3/7 activity in SiHa cells treated with myricetin/spinacine plus TRAIL and cisplatin (**Figure 3C and 3D**). The mechanism for this sensitization was found to be due to an increase in steady state levels of pro-apoptotic molecules made possible by the inactivation of E6, as shown for p53 in **Figure 4A**, and for caspase 8 in **Figure 4B**.

Myricetin reduced tumor size in a mouse xenograft model: To ask whether agents that inhibit E6 can also reduce the growth of HPV$^+$ tumors, we developed a xenograft model in which we injected 1 x 10^7 HPV$^+$ SiHa cells into nude mice and then followed tumor growth. In a pilot experiment examining the ability of myricetin and human recombinant TRAIL (hrTRAIL), separately or in combination, to inhibit SiHa-induced tumor growth, myricetin treatment (10 mg/kg) was performed for 3 days, followed by 3 days of hrTRAIL treatment by intratumoral injection. We found a reduction in tumor growth of more than 35% at our end-point of 35 days when the two compounds were applied together, providing proof-of-principle evidence for our working model **(Figure 5A).** Because we will also be assessing the effectiveness of our compounds in the context of head and neck tumors, we proceeded to develop a HPV$^+$ HN model by comparing tumor growth of our six HN cell lines in nude mice (**Figure 5B**). The growth curve clearly demonstrates that UM-SCC47 (47) cells produce the fastest growing tumors. Hence, these cells were selected for the creation of a HN xenograft model for the *in vivo* studies described below. Of note, these cells have previously been used for the creation of a xenograft model [49].

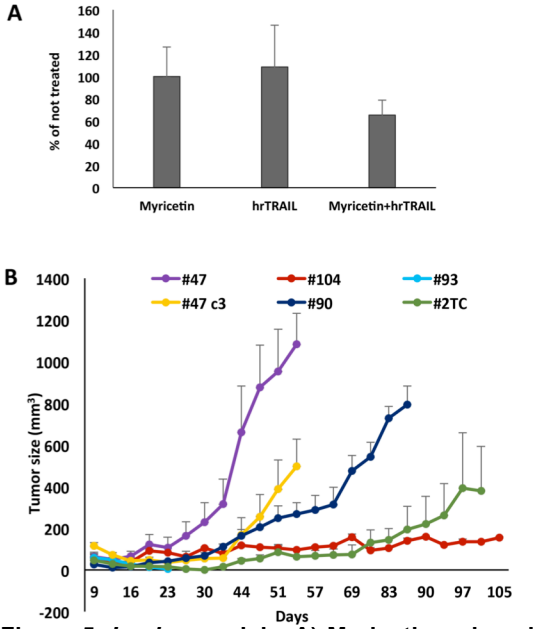

Figure 5: *In vivo* models: **A) Myricetin reduced tumor size by 35%.** Tumors were produced by injecting 1 x 10^7 SiHa cells into nude mice s.c..At day 25, myricetin treatment was performed for 3 days, followed by 3 days of hrTRAIL treatment by intratumoral injection. Tumor size at the end-point on day 35 after commencement of treatment is presented as % of tumor size at day 5 after cell implantation. **B) Six HPV$^+$ head and neck cell lines produced tumors in a mouse xenograft model at different rates.** 1 x 10^7 head and neck cells were injected into nude mice s.c. and tumors allowed to grow. Every 3-4 days, tumor size was monitored using calipers.

Spinacine may possess a favorable safety profile: We performed a pilot dose-finding experiment using spinacine in preparation for the tumor growth inhibition experiments described below. We selected our initial *in vivo* doses of spinacine to be 0 mg/kg, 5 mg/kg, 10 mg/kg and 20 mg/kg. Four groups of CD1 mice, with 3 mice per group, were injected i.p. with the indicated concentrations of spinacine on days 1 and 2, with rest on the third day. This cycle was repeated 5 times for a total treatment time of 2 weeks. Animals were weighted every 3-4 days and observed for changes indicative of declines in health, such as hunched posture, weight loss, or lack of grooming. None of these clinical signs of decline in health or mortality were observed. Moreover, these observations were consistent with hematological results as well as post-mortem evaluation of major organs in the control and high dose groups, none of which showed signs of toxicity. This data indicates that spinacine is not toxic to mice at doses up to 20 mg/kg. In summary, we are now ready to take these preliminary studies with spinacine to the next level, assessing its toxicity and efficacy in an *in vivo* mouse xenograft model.

Experimental Approach and Methods

Rationale and overview

In preparation for assessing the use of spinacine in further clinical trials, we will determine its toxicity and antitumor efficacy in mice. **Specific Aim 1** is therefore focused on the toxicity profile of spinacine. In particular, we will determine its maximum tolerated dose (MTD) and toxicity profile in mice. These experiments will allow us to determine the range of tolerable doses to be used for the determination of antitumor efficacy. In **Specific Aim 2**, we will determine the antitumor effects of spinacine in synergy with TRAIL and with the chemotherapeutic drug cisplatin. We will utilize mouse xenograft models, initiating the growth of HPV$^+$ cervical and HN tumors, then determining the extent to which any of the treatment plans reduce or eliminate tumor growth. These experiments will enable us to evaluate the antitumor effect of spinacine in this combined therapy, determine its efficacy on the growth of cervical and HN-derived tumors, and compare the relative efficacy of two different types of partners – those that work through the intrinsic apoptotic pathway such as cisplatin, and those, like TRAIL, that work by triggering surface receptors. The proposed experiments will also serve as an *in vivo* model for preclinical lead validation for potentially more E6 inhibitors to be identified in the future.

Specific Aim 1: Determine the toxicity of spinacine in mice. We will assess the toxicity of spinacine in mice, defining the maximum tolerated dose and identifying the optimum dose with which to carry out experiments designed to test its efficacy.

1.1 Determination of maximum tolerated dose (MTD): The MTD will be defined as the highest dose administered by a single injection that will be tolerated and not produce major life threatening toxicity. Twenty-four (12 naïve male and 12 naïve female) 5-6 week-old CD-1 mice will be purchased from Charles River Laboratories (San Diego, CA) and housed in the LLU Animal Care Facility. Animals will be randomly assigned to one of four dose groups: 0 (control), 200, 500 and 1000 mg/kg. All experimental procedures will be performed under 3% inhaled isoflurane anesthesia. Spinacine or vehicle (PBS) will be injected into the lower left abdominal quadrant of each mouse as a single intraperitoneal (i.p.) administration. The starting dose of 200 mg/kg per single injection was selected based on our pilot toxicity experiment that demonstrating that a 20 mg/kg dose administered 10 times over a 2-week period (total amount 200 mg/kg) was not toxic to the mice. Consistent with our findings, the oral administration of spinacine at 300 mg/kg on rats as a part of their diet for 13 weeks showed no signs of toxicity [47]. Animals will be weighed every three to four days and observed for changes indicative of declines in health, such as hunched posture, weight loss, or lack of grooming. If animal exhibits signs of pain or distress, they will be euthanized immediately. Ten days after the initial injection, mice will be sacrificed by CO_2 asphyxiation. The following end point parameters will then be evaluated: mortality, clinical signs, body weight, hematology and serum biochemistry, post mortem organ evaluation (all animals), and histopathological examination of major organs (control and high dose groups).

1.2 Dose-finding studies: Twelve naïve male and 12 naïve female nude mice (4-5 weeks old) will be purchased from Charles River Laboratories (San Diego, CA), then housed under pathogen-free conditions in the LLU Animal Care Facility in accordance with all applicable guidelines. Animals will be randomly assigned to 4 groups based on body weight (3 males and 3 females in each group). Mice (6 per group) will be treated with vehicle (PBS), or with low, medium or high doses of spinacine. The highest dose per injection will be calculated as a 1/10 of the dose determined in the MTD studies (**1.1**). Spinacine will be administered by i.p. injections on days 1, 2 and resting on day 3, and this cycle will be repeated 5 times. The planned end point of this experiment is Day 10 after the last injection. Animals will be evaluated as described above.

1.3 Expected results, data interpretation, possible pitfalls and alternative strategies: We expect to determine a dose range for spinacine that will not affect the animal's health. If we cannot find MTD due to the low toxicity of spinacine even after the administration of the highest dose, we will consider that spinacine is not toxic within the tested range. If all tested doses will display some signs of toxicity, for example, weight loss, a follow-up experiment using lower doses will be performed. For the dose-finding studies, a one-way analysis of variance (ANOVA) will be used to analyze clinical pathology values and body weight data. Dunnett's t-test will be used for pairwise comparisons between treated and untreated control groups. Group comparisons will be evaluated at the 5.0%, two-tailed probability level. The highest dose that demonstrates minimal toxicity will be used in experiments assessing tumor growth inhibition. We do not expect any insurmountable difficulties with obtaining results for this part of study, as standard protocols are being followed.

Specific Aim 2: Evaluate the ability of spinacine to synergize with TRAIL- and chemo-based therapies to reduce or eliminate HPV^+ tumor growth. We will assess the ability of spinacine to synergize with hrTRAIL and/or the DNA damaging drug cisplatin to inhibit tumor growth in a xenograft model.

2.1 Experimental model: To evaluate the ability of spinacine to synergize with TRAIL and cisplatin *in vivo*, we will utilize a xenograft model, already established in our lab, in which HPV^+ cells are injected into athymic nude mice. SiHa cells injected into female mice will serve as a model for cervical cancer (patients with cervical cancer are female), and UM SCC47 cells injected into male mice will serve as a model for HN cancer (males suffer from HN cancer more frequently than do females) [50]. We will utilize bioluminescence imaging to follow tumor size and metastasis in the same animal over time. We have already created both SiHa pLuc and UM-SCC47 (47) pLuc cells that express luciferase at a high level. 10^7 cells will be injected subcutaneously on both sides of each athymic mouse. After a few weeks, or when tumors reach approximately 100 mm^3 (6 mm x 6 mm) in size, treatment regimens will begin. As treatments progress, measurements of tumor growth and metastasis development will be performed after i.p. injection of the luciferase substrate, GoldBio luciferin potassium salts (GoldBio) (2 mg in 200 μl per injection). Images will be obtained using the IVIS Lumina II In Vivo Imaging System (PerkinElmer), and tumor sizes will be calculated using the Living Image software (PerkinElmer).

2.2 Experimental design and treatment plan: For each model, SiHa (cervical) and 47 (HN), spinacine will be paired with both hrTRAIL and cisplatin. Six groups of 4-5 week old Crl-CD1-Foxn1 nude mice, with nine animals per group, will be employed. The groups will be as follows: 1) Vehicle 2) Spinacine 3) hrTRAIL 4) Cisplatin 5) Spinacine + hrTRAIL and 6) Spinacine + Cisplatin. The number of mice per group was calculated, as in our preliminary experiments with SiHa cells, so as to obtain a statistically significant difference in tumor growth

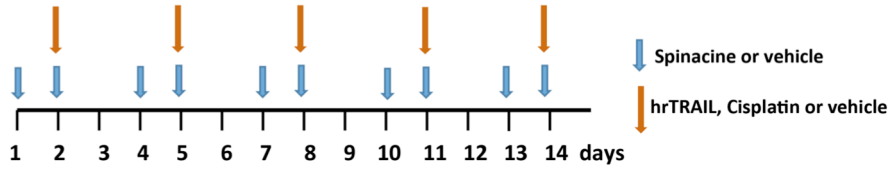

Figure 6: Experimental scheme.

between control and tested groups. Power calculations indicate that in order to detect a difference of more than 35% in mean tumor volume when administering the therapeutic agent, with a power of 90% and an alpha (Type I) value of 0.05, 9 mice will be necessary for each group (9 mice x 6 groups = 54 mice/experiment). Animals bearing tumors will be randomly assigned into the various treatment groups based on body weight and tumor size, and treatments will be started once tumors reach approximately 100 mm^3 (or 6 mm x 6 mm) in size. Before treatments begin, the tumor size will be measured using calipers, and the volume calculated using the following equation: tumor volume (mm^3) = length x width1 x width2 x 0.5. Treatment will be as outlined in the scheme shown in **Figure 6**. On day 1, spinacine or vehicle will be administered, and on day 2, that compound will be applied in combination with hrTRAIL (10 mg/kg) or cisplatin (5 mg/kg) [51]. hrTRAIL and cisplatin will be administered i.p., and spinacine or vehicle will be injected directly into the tumor. After one day of rest, the same series of treatments will be repeated 4 more times for 2 weeks (for a total of 5 treatments) after the initial treatment. Tumor growth will be determined every fourth day, using biochemiluminescent tumor imaging after injection of the luciferase substrate. The end-point of the experiments will be the point at which tumors in control groups will reach 2 cc, or earlier, if signs of pain and suffering are observed. Mice will be sacrificed by CO_2 asphyxiation, after which the tumors will be harvested and analyzed.

2.3. Data analysis: Data on tumor growth will be calculated as a group average in mm^3 at each time point +/- standard error (SE). Within each group, tumor size at a certain time point will be expressed as a percentage of tumor size at the treatment start point. The t-test will be used to determine statistical significance of any observed differences between groups at each measurement, including the end-point. Tumors will be dissected in cold PBS, photographed near a ruler (for size estimation) and weighed. Half of each tumor sample will be embedded in paraffin, sectioned, and examined for morphology. Selected sections will then be examined for evidence of apoptosis and subjected to analysis by immunohistochemistry. We will estimate the level of apoptosis occurring within the tumors using the TUNEL apoptosis detection kit (Millipore) along with immunohistochemical detection of other apoptotic markers, such as the activation of executor caspases (caspases 3/7). We will also use immunohistochemistry to ask whether differences can be seen in the levels of VEGFR1, a marker for tumor-related angiogenesis. The remaining half sample will be frozen in liquid nitrogen, and then analyzed for the level of expression of FADD, caspase 8, p53 and E6 by immunoblot and/or qRT-PCR.

2.4. Expected results, potential pitfalls and alternative strategies: We expect to find that spinacine in combination with hrTRAIL and/or cisplatin will inhibit the growth of HPV^+ tumors. It is possible that spinacine will show less activity *in vivo* than anticipated based on the cellular results. One option would be to modify the treatment protocol by increasing the number of spinacine injections and/or to increase the concentrations of TRAIL/cisplatin. If cisplatin is not able to reduce tumor size, we will replace it with another, more aggressive chemotherapeutic drug, doxorubicin. We also note that if the issue is the ability of hrTRAIL to induce cell death following i.p. injection, we have the option of changing the mode of administration by either injecting the agent i.v. or directly into the tumor. It has been shown that the toxicity of spinacine in rats is low [47], but if issues arise due to problems related to adsorption, distribution, metabolism or excretion, we will modify and optimize spinacine so as to address these concerns, or choose a different active structure to pursue.

FUTURE DIRECTIONS

The work proposed in this application is designed to translate our discoveries in the laboratory into approaches that can be tested in the clinic. By the end of this project period, we will have obtained *in vivo* data regarding the ability and extent of spinacine to inhibit tumor growth. In addition, we will have further developed our *in vivo* model for the testing of possible future small molecule candidates. This is important, because in addition to the two small libraries from which myricetin and spinacine were identified, we also have *in vitro* binding data from a 5,000 compound screen performed in collaboration with Kansas University, as well as an agreement with NCATS to screen almost 500,000 small molecules using our assay. Therefore, the further optimization of our *in vivo* model, as described in this application, will not only enable us to evaluate spinacine for potential therapeutic use (following optimization), but will also put us in a good position to evaluate the new leads we anticipate from these additional queries. We also note that the work described in this application will provide strong preliminary data supporting the submission of an SBIR phase II grant focused on the next steps of the drug discovery sequence – Product Development and Clinical Trials.

VERTEBRATE ANIMALS

1. **Spectrum of animal use and description of procedures:**
   a. **Maximum Tolerated Dose (MTD) experiments**: We will use 24 5-6 week old Crl-CD1 mice (022 from Charles River, San Diego, CA), 24 males and 24 females. Mice will be randomly assigned to one of 4 groups, then receive either 0 (Vehicle control), 200, 500 or 100 mg/kg of spinacine by a single intraperitoneal (i.p.) injection into the lower left abdominal quadrant of each mouse.
   b. **Dose-finding studies:** Twelve naïve male and 12 naïve female 4-5 week old Crl-CD1-Foxn1 nude mice (086 from Charles River, San Diego, CA) will be randomly assigned to one of 4 groups based on body weight (3 males and 3 females per group). Mice with each group will be treated with vehicle (PBS), or with low, medium or high doses of spinacine. The highest dose per injection will be calculated as 1/10 of the dose determined in the MTD studies. Spinacine will be administered by i.p. injections on days 1 and 2, resting on day 3, and this cycle will be repeated 5 times. The planned end point is Day 10 after the last injection.
   c. **Anti-tumor activity**: To evaluate the ability of spinacine to synergize with TRAIL and cisplatin *in vivo,* we will use 108 4-5 week old Crl-CD1-Foxn1 nude mice (086 from Charles River, San Diego, CA). 54 female mice (6 groups of 9 mice) will be used for experiments utilizing the cervical cancer-derived SiHa cell line, and 54 male mice (6 groups of 9 mice) will be used for experiments utilizing the HN-derived cell line UM-SCC47.
      i. 10^7 SiHa cervical cells or UM-SCC47 head and neck cells will be injected subcutaneously into the flanks of athymic mice. Subcutaneous tumors will be allowed to develop for approximately 1-3 weeks or until they reach ~80 mm^3. Tumor growth will be determined by daily measurements using calipers, and the volume calculated using the following equation: Tumor volume (mm^3) = length x width1 x width2 x 0.5.
      ii. When tumor size reaches 100 mm^3, the appropriate treatments will be applied to each group using sterile techniques. Six groups of mice, with nine mice per group, will be employed for the SiHa experiment; another six groups of mice, with nine mice per group will be employed for the UM-SCC47 experiment. The groups will be as follows: 1) Vehicle 2) Spinacine 3) hrTRAIL 4) Cisplatin 5) Spinacine + hrTRAIL and 6) Spinacine + Cisplatin. On day 1, spinacine or vehicle will be administered, and on day 2, that compound will be applied in combination with hrTRAIL (10 mg/kg) or cisplatin (5 mg/kg). hrTRAIL and cisplatin will be administered i.p., and spinacine or vehicle will be injected directly into the tumor. After one day of rest, the same series of treatments will be repeated 4 more times for 2 weeks (for a total of 5 treatments) after the initial treatment. Tumor growth will be determined every fourth day, using biochemiluminescent tumor imaging after injection of the luciferase substrate.
      iii. Power calculations indicate that in order to detect a difference of more than 35% in mean tumor volume when administering the therapeutic agent, with a power of 90% and an alpha (Type I) value of 0.05, 9 mice will be necessary for each group (9 mice x 6 groups = 54 mice for each of the two experiments).

2. **Justification of animal model:**
   a. Our ultimate goal is to identify and optimize lead molecules, such as spinacine, that can be carried forward into the clinic. Mammalian species provide the best correlary for human studies, and neither computer models nor *in vitro* techniques can replace the proposed animal procedures. We have chosen the smallest possible mammal for this study, and smaller, less sentient species cannot be substituted for these experiments.
   b. Nude mice are an appropriate model for these experiments, as xenografts of human cancer cells can be injected into flanks of the animals without the risk of immune rejection. We will be able to monitor tumor growth both by using calipers and by visualizing tumors by imaging after i.p. injection of the luciferase substrate, GoldBio luciferin potassium salts (GoldBio) (2 mg in 200 ml per injection). Images will be obtained using the IVIS Lumina II In Vivo Imaging System (PerkinElmer), and tumor sizes will be calculated using the Living Image software (PerkinElmer).

3. **Veterinary medical care:**
   Animals within the LLU animal care facility have continuous access to veterinary medical care.

4. **Minimization of pain and distress:**
   a. In the dose finding and tumor growth studies, the animals will be injected with the tumor cells, and later, with solutions containing hrTRAIL, cisplatin and/or spinacine. These procedures are expected to result in minimal to no pain and/or discomfort. We will be using 3% isoflurane for 3-5 min when doing any injection procedures to minimize any pain sensed by the mice. All surgical procedures will be performed after euthanasia.
   b. Animals will be weighed every three to four days and observed for changes indicative of declines in health. If animal exhibits signs of pain or distress, they will be euthanized immediately.
   c. The following are post-procedural indicators of distress that could contribute to a decision of euthanasia:
      1. Systemic Physical
         i. Severe weight loss (>15%) or weight loss that is not recovered within an acceptable period of time after the procedure(s)
         ii. Dehydration, loss of skin elasticity after procedure(s) that is not remedied by hydration intervention
      2. Local Physical
         i. Edema, inflammation, abnormal discharge or severe bleeding at the injection area that does not resolve within an acceptable post-injection period.
         ii. Tumor size greater than 2cc, or exceeding 10% of body weight
      3. General Behavior
         i. Unconscious or completely unresponsive to stimuli
         ii. Severe lethargy in response to stimuli
         iii. Ataxia (wobbly gait) during movement
         iv. Abnormal, hunched posture combined with unwillingness to move and not relieved by analgesia
         v. Lack of grooming, as evidenced by a matted appearance of the fur that produces a "scruffy" appearance
         vi. Overly aggressive behavior beyond what would be considered normal defensive tendencies to handling.
         vii. Abnormal vocalizations (i.e., “squawking”).

5. **Euthanasia:**
   Animals will be sacrificed for analysis at the indicated times, when toxicity becomes excessive, or when tumors reach 2.5 cc in size. CO_2 asphyxiation is the preferred method for euthanasia and will therefore be employed. Asphyxiation will be followed by cardiac puncture or exsanguinization as a secondary. All procedures will be carried out as approved by the Loma Linda IACUC committee, and as consistent with the AVMA Guidelines for the Euthanasia of Animals.

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RESOURCE SHARING PLAN

Data generated from this study will be shared with the scientific community by publication in peer-reviewed journals. In addition, we will continue our practice of making available to the research community reagents and resources (such as constructs) presented in publications, once the papers have been published or accepted for publication. This will be done at no cost using standard Material Transfer Agreements.

AUTHENTICATION OF KEY BIOLOGICAL AND CHEMICAL RESOURCES

The proposed research will utilize the following key biological resources:

1. **SiHa cervical carcinoma cell line and UM-SCC47 head and neck carcinoma cell line**
   a) **Preparation and Use:** SiHa and UM-SCC47 cells will be used to induce tumor growth in a mouse xenograft model. SiHa cells will be grown in MEM media while UMSCC-47 will be grown in DMEM media. Both media will be supplemented with 10% FBS and penicillin/streptomycin.
   b) **Validation:** The authenticity of the SiHa cells used in our laboratory was verified by the ATCC using STR Profiling following ISO 9001:2008 and ISO/IEC 17025:2005 quality standards. Their report from November 7, 2012 indicated that the profile of SiHa cells submitted by our laboratory is an exact match for the HTB-35 (SiHa) human cell line(s) in the ATCC STR database.
   UMSCC-47 cell line were purchased in 2012 from the Head and Neck Cancer Biology lab, Department of Otolaryngology/Head and Neck Surgery University of Michigan Cancer Center where this cell line was established. This cell line was maintained in conditions suggested by this lab.
   c) **Quantification:** Cells will be counted using a hemocytometer.
   d) **Expiration/Loss of activity:** Aliquots of cells will be thawed from frozen stocks, maintained and grown in culture for the length of time necessary to prepare for injection into mice. This procedure can be repeated indefinitely.

2. **CD1 and Crl-CD1-Foxn1 nude mice**
   Preparation and Use: All mice will be purchased from Charles River Laboratories (San Diego, CA), which ensures the quality of service. CD-1 mice will be used to determine the MTD (1.1), while Crl-CD1-Foxn1 nude mice will be used in the dose-finding experiment (1.2) and to determine the extent with which spinacine synergizes with hrTRAIL or with cisplatin to inhibit tumor growth (2.2).

3. **Cisplatin** (Sigma-Aldrich)
   a) **Preparation and Use**: Cisplatin will be dissolved in DMSO at a concentration of 5 mg/ml and stored according to the manufacturer's protocol. 25 µl of cisplatin solution will be injected i.p. into mice (5 mg/kg) for each treatment.
   b) **Validation:** The ability of cisplatin to kill SiHa and UMSCC-47 cells will be tested using a standard viability assay. The ability of each new batch of cisplatin to induce cell death will be compared against the activity of previously used cisplatin purchased from Sigma-Aldrich.
   c) **Expiration/Loss of Activity:** When stored as a powder, cisplatin does not have an expiration date. As a solution, it will be used at the same date at which it was dissolved into DMSO.

4. **Spinacine** (TimTec)
   a) **Preparation and Use**: Spinacine will be dissolved in PBS and stored at -20^{0}C. Selected concentrations and volumes of spinacine solution will be injected in mice intratumorally. The concentrations of spinacine used for each injection will be determined by the dose-finding experiment (1.2).
   b) **Validation:** The biological activity of spinacine to sensitize cells to TRAIL or cisplatin-induced apoptosis will be tested by applying the indicated combinations to SiHa and UMSCC-47 cells, then carrying out a viability assay. The ability of spinacine from each new batch to sensitize cells to apoptosis will be compared with the activity of cisplation previously purchased from TimTec.

c) **Expiration/Loss of Activity:** As a powder, spinacine does not have an expiration date. As a solution, it will be used at the same date at which it was dissolved. Unused solution will be discarded.

5. GoldBio luciferin potassium salts (GoldBio)

a) **Preparation and Use**: Luciferin will be dissolved and stored according to the manufacturer's protocol. Luciferin solution will be injected i.p. into mice.
b) **Validation:** The ability of luciferin to produce a luminescent image if the tumor expresses Firefly luciferase will be monitored using the IVIS Lumina II In Vivo Imaging System (PerkinElmer).
c) **Expiration/Loss of Activity:** Luciferin solution will be stored according to manufacture recommendation.

6. hrTRAIL

a) **Preparation and Use:** The extracellular portion (aa 95-281) of the human TRAIL was previously cloned in frame with 6His into the pTriEx-4 plasmid (Novogen). Following introduction into BL21 pLys cells (Novogen), expression of His-TRAIL (hrTRAIL) will induced. Protein purification will be performed as described (Seok-Hyun Kim, Kunhong Kim, Jae G. Kwagh, David T. Dicker, Meenhard Herlyn, Anil K. Rustgi, Youhai Chen, and Wafik S. El-Deiry. The Death Induction by Recombinant Native TRAIL and Its Prevention by a Caspase 9 Inhibitor in Primary Human Esophageal Epithelial Cells. JBC, Vol. 279, No. 38, pp. 40044–40052, 2004). 100 µl of 2.5 mg/ml of hrTRAIL solution will be injected i.p.into each mouse (10 mg/kg).
b) **Validation:** Verification that our isolated protein is indeed the extracellular domain of TRAIL will be obtained by Western blot using anti-TRAIL antibodies (Abcam (ab42243), raised against a peptide from the extracellular domain of human TRAIL). hrTRAIL from Peprotech will serve as a positive control. The biological activity of recombinant TRAIL will be assessed by inducing apoptosis of U2OS cells. The activity of TRAIL purified in-house will be compared with activity with hrTRAIL produced and purchased from Peprotech in the cell viability assay.
c) **Expiration/Loss of Activity:** hrTRAIL solution will be aliquoted and stored at -80^{0}C. Under these conditions, the protein does not lose activity for at least 1 month. Thawed aliquot will be used the same day.