IF14-002

COVER SHEET FOR GRANT PROPOSALS

State Board of Education

SBOE PROPOSAL NUMBER: (to be assigned by SBOE)	AMOUNT REQUESTED: $50,000

TITLE OF PROPOSED PROJECT:
Liposome Delivery of Cancer Killing Nullomer Peptides

SPECIFIC PROJECT FOCUS: Combining 198 BSU-developed anticancer peptides called Nullomers (patent pending) with BSU liposomal delivery vehicles to increase efficacy and make animal testing possible.

We have received $100,000 grant for one year (of a requested $150,000) for part of this project. But the Pardee Foundation will not fund: the required materials for liposome delivery or the commercialization efforts.

PROJECT START DATE: 07/01/13	PROJECT END DATE: 06/30/14
NAME OF INSTITUTION: Boise State University	DEPARTMENT: Office of Sponsored Programs

ADDRESS: 1910 University Drive, Boise, ID 83725

E-MAIL ADDRESS: osp@boisestate.edu	PHONE NUMBER: 208-426-4420

	NAME:	TITLE:	SIGNATURE:
PROJECT DIRECTOR/PRINCIPAL INVESTIGATOR	Greg Hampikian	Professor	
CO-PRINCIPAL INVESTIGATOR			

NAME OF PARTNERING COMPANY: WestVet	COMPANY REPRESENTATIVE NAME: Jeff Brourman, President

NAME: Jeff Brourman, President SIGNATURE:

Authorized Organizational Representative		
	Lisa Jordan, CRA	

SUMMARY PROPOSAL BUDGET

Name of Institution:

Name of Project Director:

A. PERSONNEL COST (Faculty, Staff, Visiting Professors, Post-Doctoral Associates, Graduate/Undergraduate Students, Other)

Name/ Title	Salary/Rate of Pay	Fringe	Dollar Amount Requested
Greg Hampikian, Ph.D., principal investigator	$8,001/ 1 month	$2,720	10,721
Student Assistant	$5,888	$412	$6,300

% OF TOTAL BUDGET:	34	SUBTOTAL:	$17, 021

B. EQUIPMENT: (List each item with a cost in excess of $1000.00.)

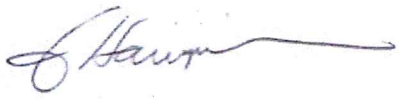
Item/Description	Dollar Amount Requested
None	
SUBTOTAL:	

G. TRAVEL:

Dates of Travel (from/to)	No. of Persons	Total Days	Transportation	Lodging	Per Diem	Dollar Amount Requested
	1	3	~400	~420	80	900
	1	3	~400	~420	80	900
	1	3	~400	~420	80	900
SUBTOTAL:						2,700

H. Participant Support Costs:

	Dollar Amount Requested
1. Stipends	
4. Other	
SUBTOTAL:	

I. Other Direct Costs:	Dollar Amount Requested
1. Materials and Supplies	$30,279
2. Publication Costs/Page Charges	
3. Consultant Services (Include Travel Expenses)	
4. Computer Services	
5. Subcontracts	
6. Other (specify nature & breakdown if over $1000)	
SUBTOTAL:	$30,279
J. Total Costs: (Add subtotals, sections A through I) **TOTAL:**	$50,000
K. Amount Requested: **TOTAL:**	$50,000
Project Director's Signature: 	Date: May 17, 2013

INSTITUTIONAL AND OTHER SECTOR SUPPORT
(add additional pages as necessary)

A. INSTITUTIONAL / OTHER SECTOR DOLLARS

Source / Description	Amount

B. FACULTY / STAFF POSITIONS

Description

C. CAPITAL EQUIPMENT

Description

D. FACILITIES & INSTRUMENTATION (Description)

Liposome Delivery of Cancer Killing Nullomers

1. **Boise State University**

2. **Greg Hampikian, Ph.D., Principal Investigator**

3. **This project has not been funded in the past.**

4. **Executive Summary**

Boise State University researchers have pioneered the study of the smallest sequences of nucleotides and amino acids absent from nature. The sequences, called Nullomers, have broad applications from DNA barcodes for forensic samples, to anticancer and antimicrobial drugs. The BSU Nullomer research team of professors from Biology, Physics, Computer Science, Criminal Justice, Chemistry, and Engineering has already published peer-reviewed papers on this Idaho technology. In the past 12 months they have attracted international coverage from professional and popular science journals including *New Scientist*, *Peptides*, and *The Journal of International and Legal Medicine*. BSU has applied for patents on 198 of these compounds, and the PI is forming a company to exploit Nullomers. Dr. Hampikian was recently

inducted as a Fellow in the National Academy of inventors based on these inventions.

This proposal will support a new collaboration between the PI, Dr. Abdelkrim Alileche, BSU Physics professor Dr. Daniel Fologea, and Dr. Jeff Bourman of Westvet to develop Nullomer anticancer drugs for animals. Dr. Fologea has devised a way to deliver the Nullomer drugs in lipid microcapsules that he manufactures in his lab. Initial tests show that they improve the strength of Nullomer drugs 10 to 100 fold.

Peptide (protein) cancer drugs are a new area of therapy with important medical and commercial potential. Nullomer peptides are made from natural amino acids, are extremely small (5 amino acids), and are among the most potent peptide drugs ever developed. The current battle in the Supreme Court over patenting "natural sequences" will not affect Nullomers, since they are based on the smallest sequences that do not exist in nature. This novelty gives us many obvious advantages moving forward to commercialization and clinical use.

5. **"Gap" Project Objective and Total Amount Requested**

For the past year, Dr. Alileche an MD/Ph.D. in the PI's lab has worked at half-pay, without benefits to make the first anticancer Nullomer drugs. He published the study in September of 2012, and has

just received $100,000 from the Pardee Foundation to chemically modify the drugs to improve efficacy. This HERC proposal will allow us to collaborate with Dr. Fologea to test the full series of Nullomer drugs in liposomes against breast, prostate, and skin cancer. Without this critical support we cannot complete this collaboration. Boise State University is solidly behind these technologies, and has helped the PI develop a strategic development and marketing plan with the BSU Innovation Team assigned to Nullomers (PowerPoint, Appendix 6); and our local collaboration with WestVet will speed animal studies.

6. **Description of how resource commitments reflect the priorities of the home institution(s)**

Boise State University has developed this technology through a multi college collaboration that has spawned several new technologies. The partnership has resulted in University patents in Biological and Engineering technologies. Furthermore the University has organized a team of graduate business students dedicated to commercialization of the project. The Office of Technology transfer has applied for patents on 198 of the Nullomer peptides discovered and developed at BSU. The University also supported three months of salary for Dr. Alileche to produce the preliminary results presented here.

7. **Evidence that the project will have a potential impact to the economy of**

Idaho

Our partnership with WestVet was developed though participation in the Idaho Coalition for Innovation (CORE)

http://www.thecoreidaho.com/sites/default/files/vision.pdf

We have decided to start with animal treatment for the following reasons:

- The Treasure Valley has nationally recognized strengths in veterinary medical technology and business development, including MWI Veterinary Supply (total revenues of $563.1 million for the 2nd quarter of 2013) and WestVet (nationally recognized as one of the most progressive veterinary hospitals in the Northwest). These corporations are working together to develop new medical technology.
- There are new opportunities for animal to human medical transition. See *New York Times* http://www.nytimes.com/2012/09/11/science/vets-and-physicians-find-parallels-in-medical-research.html?pagewanted=all&_r=0
- We recognize the challenges facing the development of human drugs in our geographic area with available resources.
- We have established a research collaboration with WestVet in animal cancer drug development.

8. <u>The Market Opportunity</u> – Address the following items:

a. Describe need the project would address:

Peptide drugs are part of the new wave of cancer therapeutics.

Our peptides have been shown to affect cancer metabolism, a key area where few drugs are effective.

b. Describe applications and markets for the technology. Include market size and demand projections.

   Cancer drugs now top $80 billion per year. There is a great need for drugs that affect cancer cell metabolism, which is distinct in many ways from normal cell metabolism (particularly in its anaerobic glucose breakdown). Our Nullomer drugs affect cancer cell metabolism by both lowering ATP, and disabling mitochondria. This metabolic targeting would make Nullomer drugs immune from most of the drug resistance factors that plague other cancer drugs.

c. Describe the product, its potential market audience, the competition, and barriers to market entry.

   We have developed drugs based on the smallest peptides absent from nature, Nullomers. These peptides are made from naturally occurring amino acids, which will facilitate drug approval. By targeting animal cancer first, we hope to expedite the drug's initial market success.

9. **The Technology and Path to Commercialization**

BSU researchers have discovered that Nullomer sequences, called "Too Dangerous to Exist" by *New Scientist* magazine, are in fact cancer killers. This work was published in October of 2012 in the peer-reviewed journal, *Peptides (*see Appendix 4). This finding confirmed the PI's hypothesis that

the smallest protein sequences not found in nature are good candidates to kill cancer. We have also published the first commercial prototype of Nullomer markers in *Forensic and Legal medicine* (2013, in press, see Appendix 5), which we used to tag forensic DNA samples. In April of 2013, this story also garnered recent international attention from *New Scientist* (April 2013), the *Journal of Forensic Science* and many others. BSU has applied for patent protection for 198 of these Nullomer peptides, so the clock for drug development is ticking.

In the proposed work, we will design and synthesize new Nullomer drugs for Dr. Fologea to incorporate in liposomes. Dr. Fologea is widely recognized as an expert in liposomal drug delivery, which allows drugs to be targeted directly to cells. In the past, we simply added the Nullomers to the cell media. However, in our pilot test of liposomes, the liposomes increased the cancer killing effects of the Nullomers from 10 to 100 times, an astounding improvement that makes these drugs very viable candidates for cancer treatment (see Fig. 1).

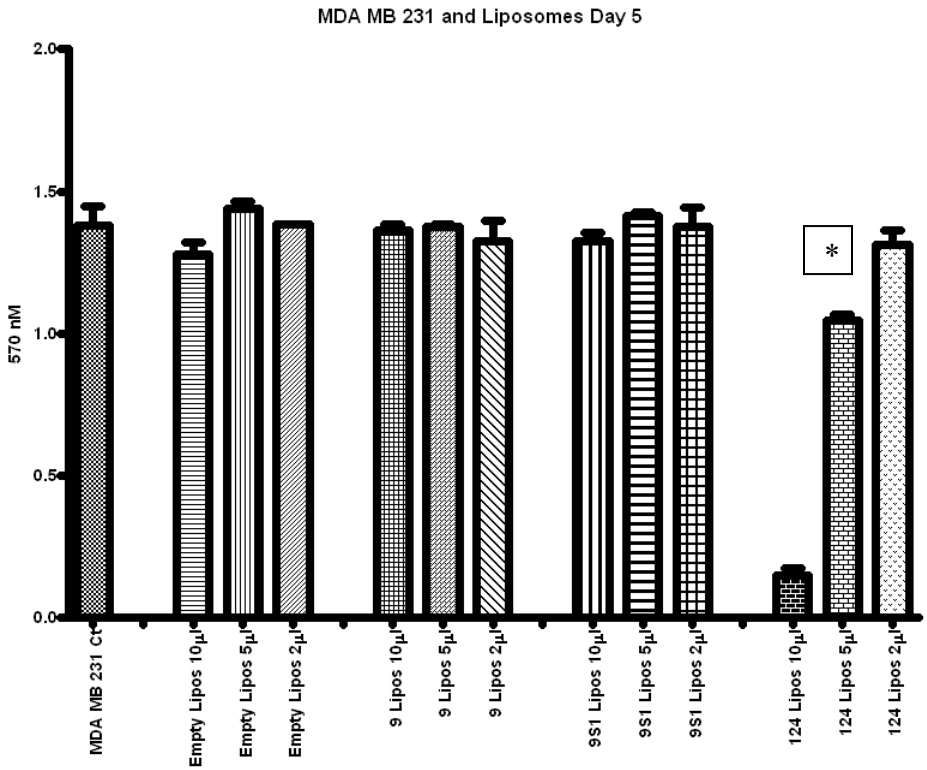


Figure 1. Nullomer Peptide 124 kills breast cancer cells at 3 µM concentration. Breast cancer cells (MDA MB 231) were exposed to Nullomer peptide 124 in liposomes at several different concentrations, and then incubated for 5 days. Survival was then measure by MTT (Y-axis). The untreated control cells appear in the leftmost bar. Nullomer peptide 124 treated cells (three rightmost bars) show completely lethal effects at the 6 µM level (third bar from left); and a significant decrease in viability is seen at the 3 µM level (indicated with *). These results are 10 fold higher than the anticancer effects seen before liposome inclusion.

The PI, in consultation with our Idaho corporate partners, will visit 3 national biotech and drug companies to establish commercial collaborations with

Boise State University and WestVet for drug development and commercialization.

The funds from this proposal will be used for the design and manufacture of the Nullomers for liposome inclusion, and for their testing in cancer cell culture against breast, prostate, leukemia and skin cancer. Once the drugs are made in liposomes that are compatible with animal treatment regimes we will apply to the WestVet experimentation committee for experimental treatments. If the studies on animals are promising, that will open the doors to human drug development.

10. **Commercialization Partners**

We are working with several national companies and have non-disclosure agreements in place with one fortune 500 company and a major biotechnology company. Our primary Idaho corporate partner is Westvet with whom we have been working on animal cancer for the past two years. With our Pardee grant, if this HERC proposal is funded, we will complete the development of Nullomer animal drugs for experimental treatment and large scale testing. Our plan is to secure major corporate partners as we move forward in the proposed activities.

11. **Specific Project Plan and Detailed Use of Funds**

Personnel: Support is sought for one month of summer salary/benefits ($8,001/$2,720) for PI who will be involved in design of the new drugs, analysis of results, and will coordinate development with WestVet and other partners. The other personnel funds ($5,888/$412) are for a student research assistant

who will assist with cancer cell culture tests on the Nullomer-liposome drugs.

Travel: Three out-of-state trips $2,700. We have not detailed the travel as it is contingent on several factors being negotiated with potential corporate partners. We anticipate 3 trips over the life of the grant to meet with potential commercialization partners.

Equipment: Dr. Folgea needs an adjustable sonicator to prepare uniform size liposomes (less than $1,000).

Materials: We anticipate that we will synthesize about 100 peptides, the cost for these is expected to be about $20,000 (list is $40,000 but we are negotiating). Other lab supplies include culture media, serum, MTT assays (viability), serum for culture cells, and cultures of normal cells ($3,779). Dr. Fologea's lab will need materials and chemicals to prepare liposomes which include chemicals to add polyethylene glycol (PEG) to avoid animal cell rejection ($5,500).

12. **Institutional and Other Sector Support:** WestVet will contribute its expertise and will supply tissue samples as needed. BSU has already invested substantially in this project by patenting 198 of the Nullomer peptides, and assigning an innovation team to assist with commercialization (see Appendix 6 for their PowerPoint). BSU also supported Dr. Alileche for 2 months during a funding lag.

Appendices:

1. Facilities and Equipment:
2. Biographical Sketch
3. Support Letter from Westvet
4. Nullomer Cancer paper from *Peptides*
5. Nullomer DNA Forensic DNA Marker (Safeguarding Forensic DNA…) paper from the *Journal of Forensic and Legal Medicine*
6. BSU Innovation Team's Development Strategy PowerPoint

FACILITIES

LABORATORY
Approximately 1000 sq. ft. of newly renovated laboratory space is available to the PI and team this project. Within this space are areas designated for 1) electrophoresis and western blotting, 2) DNA isolation and PCR technology, 3) cell and tissue culture, and 4) general solution preparation and laboratory dishwashing. The PI's laboratory also contains refrigerators, freezers, liquid nitrogen storage, and a chemical fume hood. In addition, shared research facilities are located on all floors of the Science-Nursing Building on the Boise State University campus.

COMPUTER
All research instrumentation for this project includes dedicated computers, which serve as controller and data analysis workstations. In addition, research staff and students have access to desktop computers for data analysis and presentation.

OFFICE
200 sq. ft. of office space is available for the PI. In addition, the departmental administrative and grant assistants track grant budgets, maintain personnel training files, and order equipment.

OTHER
The Simplot/Micron Instructional Technology Center is available for printing and graphics services. A scientific instrumentation shop is available, which includes a complete electronics and machine shop for repair of equipment and instrumentation and for fabrication of items necessary for research.

SCIENTIFIC ENVIRONMENT

The scientific environment at Boise State University is conducive to performing high-quality research. As Boise State strives to reach its goal of becoming a metropolitan research university of distinction, increased emphasis is being placed on providing faculty with the infrastructure and administrative support needed to secure outside research funding and maintain productive research programs. As a result, Boise State has more than doubled its research funding since 2000 and garnered more than $37 million in fiscal year 2009, which makes Boise State's research program the fastest growing in Idaho. Boise State's Division of Research is comprised of several offices that provide support to faculty. These include the Office of Sponsored Programs, which streamlines the grant submission process and manages awards; the Office of Research Compliance, which trains faculty and students on the responsible conduct of research and oversees the Biosafety, IACUC and Human Subjects committees; and the Office of Technology Transfer, which assists faculty with patent submissions and provides opportunities for faculty to network with local and regional businesses. All three of these offices provide regular workshops, seminars and/or classes to assist faculty with

research-related activities. Another asset to the development of productive research programs is Boise State's subscription to the Collaborative Institutional Training Initiative (CITI) program, which provides faculty and students with up-to-date training in the responsible conduct of research. Likewise, Boise State's Environmental Health and Safety Office provides on-line and in-person training in laboratory safety.

The scientific environment in the Department of Biological Sciences is vibrant and diverse. Research interests of the biomedical faculty in the department include cancer progression, developmental toxicity, Alzheimer's disease, vaccine development, forensic biology, pathogenesis of West Nile virus, and nanoparticle characterization. Nevertheless, faculty are united by their interest in working at the cellular and molecular level, which provides a platform for collaborations within the department, as well as with faculty in the Department of Chemistry and Biochemistry, which is housed in the same building. Moreover, faculty in these departments are joined by their participation in the NIH-funded, statewide Idaho INBRE grant, which permeates the daily activities of faculty and students conducting biomedical research at Boise State. For instance, INBRE funding helps support the Biomolecular Research Center, which houses the confocal microscope that is operated by a part-time technician. Likewise, INBRE provides funding for several faculty as "magnet PIs" and currently supports five graduate students in the department on research assistantships. Moreover, there is an INBRE director and full-time administrator in the Department of Biological Sciences, which provides this program with a strong presence on campus. Also, the INBRE program makes it possible for undergraduate students to participate in 10-week summer fellowships each year. These students participate in formal mentoring workshops each week and present their research findings at the statewide INBRE research conference at the end of the summer. Hence, the biomedical research environment at Boise State University is vibrant and strong, which contributes to the high probability of success of the proposed research.

Equipment available for the project

A. The main Hampikian/Alileche shared laboratory consists of approximately 800 square feet. The lab is equipped with: ABI 3130 Genetic Analyzer, ABI 310 Genetic Analyzer (shared with Dr. Kevice Ferris), LI-COR 3100 DNA sequencer with dedicated Optiplex GX280 computer, Qiagen BioRobot EZ-1 DNA processor, BioRad Gel Imager, Coulter Counter, NanoDrop spectrophotometer, Eppendorf Real Plex^4 real time PCR cycler, BioRad cycler, MJ Research Minicycler, 4°C and -20°C refrigerators and freezers, NuAire Class II biosafety cabinet, two NUAIRE CO_2 incubators, Beckman LS6500 liquid scintillation counter, BioRad electroporator, and assorted electrophoresis and centrifugation equipment.

A second lab space (80 square feet) is connected to the main lab and contains microscopy equipment including a Zeis fluorescent microscope with Spot RT3 Camera, and an Olympus stereo microscope.

A third lab space approximately 100 square feet is available for pre-PCR preparation of reactions. A Synergy Biotech MX microplate reader for the MTT assays and other fluorescence assays.

B. Additional shared resources: the Department of Biology has a number of shared instruments including a Sorvall High Speed Centrifuge, Beckman TL100 Ultracentrifuge, Omni GLH Tissue Homogenizer, Savant SC110A/UVS400 Concentrator/Vacuum System, a Gilson HPLC (including size exclusion and reverse phase chromatography), LC (ion exchange and affinity chromatography) 1-D and 2-D gel electrophoresis systems, protein electroelution system, temperature-controlled chromatography cabinet, and Isothermal microcalorimetry (Microcal), Agfa CP 1000 film processor, Gyromax 737 and 737R incubators, Beckman Coulter Epics XL model flow cytometer, Beckman scintillation counter, Ice machine, and autoclave. ABI 7300 and an I-Core Smart Cycler II Real-Time PCR thermocycler are available for real time quantitative PCR. A LI-COR Global IR2 4200 gel-based automated DNA sequencer, with 64-lane capacity, upgradeable to 96 lanes, housed within the 500 sq ft Sequencing Lab. Large scale sequencing is routinely done at the Molecular Core Facility at Idaho State University. In addition the Biology Department has a Confocal microscope facility which our lab frequently uses.

BIOGRAPHICAL SKETCH

NAME	POSITION TITLE
Greg Hampikian, Ph.D.	**Professor, Dept. Biology, and Department of Criminal Justice Boise State University**

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Connecticut, Storrs, CT	B.S.	1982	Biological Sciences
University of Connecticut, Storrs, CT	M.S.	1986	Genetics
University of Connecticut, Storrs, CT	Ph.D.	1990	Genetics

A Personal Statement

For several years I have been interested in the negative space surrounding the tree of life. There are two basic questions that I would like to answer: What sequences has nature left unexplored, and why are some strings of amino acids (and DNA) absent or extremely rare? Philosophically, the question fascinates me because it uses informatics to explore nature's creative processes, and opens up tantalizing questions. Does life explore every niche of information space, or is our planet's biome information-restricted in some way, leaving islands of code unoccupied. Either of these conclusions has enormous consequences for our understanding of evolution on earth.

My research interests cover a broad range of topics, and this reflects both my personal career trajectory, and diverse professional interests. After my second postdoc, in order to support my growing family and my wife's demanding career as a researcher, I took a job at a primarily teaching institution where research was not encouraged. In order to keep active in my field, I took every opportunity to work nights, weekends and summers on research projects at Georgia Tech, the CDC, and Emory University, and I also engaged my students at Clayton State in small scale projects that we could complete in borrowed classroom space. Finally, I was able to use my understanding of genetics to develop an expertise in forensic genetics, and I have specialized in exonerating the falsely convicted using DNA evidence. My work in that field has led to 13 exonerations including American student Amanda Knox, and the apprehension of four men who had escaped justice by letting others take the blame.

Since establishing my research laboratory in 2005 at Boise State University, we have been interested in exploring the unusual qualities of the smallest absent DNA and protein sequences in nature, Nullomers. We have screened the complete set of absent 5-mers (five amino acids long) that we identified in 2008 for bioactivity in a number of biological systems, and have determined the best anticancer and antimicrobial sequences from this group. Since I first proposed the Nullomer approach, there have been a number of articles debating whether it is a sound method of drug discovery. By focusing on the anticancer effects of one nullomer (9R) and its scrambled version (9S1), we recently published the first paper demonstrating that Nullomer peptides can be used to kill cancer. It is our hope that by characterizing the killing effects of these five-amino acid sequences, and modifying them using in-silico approaches, we will shed light on the world of absent sequences, and their potential therapeutic uses. We also have a paper accepted demonstrating the use of DNA Nullomers to tag forensic samples in order to prevent contamination of criminal evidence with reference samples given by victims, suspects and volunteers.

B- Positions and Employment

1983-84	Research Assistant, Dept. Pediatric Dermatology, Yale Univ. School of Medicine
1990-91	US NSF Postdoctoral Fellow, LaTrobe University, Melbourne, Australia
1992	Postdoctoral Assoc., Worcester Foundation for Experimental Biol., Worcester, MA
1993-2004	Assistant, Associate, Full Professor, Dept. Natural Science, Clayton State Univ., Morrow, GA
1994-95	Visiting Scientist, Emory University & The Centers for Disease Control and Prevention (CDC) Atlanta, GA
1997-98	National Science Foundation ROA award. Research Faculty Member, Dept. of Biochemistry, Georgia Institute of Technology, Atlanta, GA
2001-2002	Biology Coordinator, Dept. Natural Science, Clayton State University, Morrow, GA
2003-	Consultant, DNA and Forensic Science; Board member, Georgia Innocence Project
2005-	Associate Professor 2005-2006, Full Professor 2007-, Dept. of Biology and Dept. Criminal Justice, Boise State University, Boise, ID
2005-	Director, Idaho Innocence Project.

Other Experience and Professional Memberships

2009	Chair, Community Relations Committee, Concordia University Law School
2008-	Member, presenter, International Society for Forensic Genetics
2007-	Member, instructor, American Academy of Forensic Sciences
2004	Chair, Georgia Academic Advisory Committee for Biological Sciences
2000-	Member, International Society for Computational Biology

Honors

2013	National Academy of Inventors, Charter Fellow.
2013	Nominated, Boise State University Foundation Scholar in Service and Outreach 2009 Pacific Symposium on Biocomputing, Hawaii 2009, Keynote presentation: *"'DNA Don't Lie':* How Bioinformatics freed some of my best friends, and sent the guilty to prison."
2006	Idaho Science Teachers, annual meeting keynote
2004	Silver Medal in Biography, ForeWord Magazine's Book of the Year Awards for Exit to Freedom by Johnson and Hampikian
2000	First Presidential Faculty Fellow, Clayton State University
1991-92	National Science Foundation U.S.A., Postdoctoral Fellowship Centers of Foreign Excellence, Sex-determination research, La Trobe University, Australia

C- Selected peer-reviewed publications, most relevant to the current application

1. Allileche, A., Goswami, J., Davis, M., Bourland, B., **G. Hampikian**, Nullomer Derived Anti-Cancer Peptides, Peptides, Volume 38, Issue 2, Pages 302–311, (2012).
2. **G. Hampikian** and Tim Andersen; Absent Sequences: Nullomers and Primes, Pacific Symposium on Biocomputing 12:355-366 (2007).
3. C. Bullock, R. Jacob, O. McDougal, **G**. **Hampikian,** T. Andersen, DockoMatic –Automated Ligand Creation and Docking, *BMC Research Notes*, 3**:**289 (2010).
4. Goswami, J., Davis, M.S, Andersen, T., Alileche, A., **Hampikian, G**. Safeguarding Forensic DNA Reference Samples with Nullomer Barcodes, JFS (in press).
5. M. Davis, S. Novak, **G**. **Hampikian,** Mitochondrial DNA analysis of an immigrant Basque population: loss of diversity due to founder effects, American Journal of Physical Anthropology, Vol. 144, Issue 4, p516-525, April (2011).

Greg Hampikian
E-mail: greghampikian@boisestate.edu
208-781-0438

Education

Ph.D. Genetics, The University of Connecticut, 1990
M.S. Genetics, The University of Connecticut, 1986
B.S. Biological Sciences, The University of Connecticut, 1982

Experience

2006-present
Professor of Biology, with a joint appointment in Criminal Justice, Boise State University (BSU), (Associate Professor, August 2004-2006).
Graduate and undergraduate courses: Forensic Biology, DNA Evidence in Cold Cases, Advanced DNA Analysis, Biotechnology, Cell Biology, Genetics.

2006-present
Founder and Director of the Idaho Innocence Project at Boise State University.
Volunteer position. Raised more than $300,000 through grants and donations, organized a Legal Advisory Board with leading lawyers, supervise staff: a full time lawyer, six volunteers, and student interns. Currently working on 10 Idaho cases, worked on 13 exonerations internationally.

2002-present
DNA Expert for the Georgia Innocence Project
Analyze forensic evidence, assist in legal proceedings, testify, work with and train students. Involved in four Georgia exonerations, two of which resulted in the arrests of new suspects more than 20 years after the crime.

1993-2004
Professor, Biology, Clayton State University (CSU)
(Assistant Professor 1993-97, Associate Professor, 1997-2003)
Coordinated the Forensic Science Track for biology major. Courses: Biotechnology, Biotechnology Lab, Genetics, Human Genetics (on-line), Recombinant DNA Laboratory, Bioregulatory Affairs, Microbiology, Microbiology Lab, Anatomy and Physiology (A&P) sequence, A&P Labs, Sex and Reproduction, Introductory Biology (majors and non-majors sequence), Introductory Biology Labs, Biotechnology for teacher education students.

2004
Chair of the Georgia Academic Advisory Committee for Biological Sciences
The Committee included department heads of all Georgia public colleges and universities; coordinated curriculum review, organized corporate partnerships, and responded to the "evolution challenge" in public schools.

2003-2004
Grants Coordinator for the School of Arts and Sciences, CSU
Organized a consortium of six area school systems

2001-2002
Biology Coordinator, Natural Science Department, CSU
Wrote a successful degree proposal for new Bachelor of Science in Biology, which includes tracks in Forensic Science, Biotechnology/Biocomputing, Bioregulatory Affairs/Science Management. Hired five new faculty members.

2000
First Presidential Faculty Fellow, CSU
Coordinate new majors proposals; acted as faculty liaison to campus administration.

1997- 1998
National Science Foundation Research Opportunity Award, Georgia Tech, Biochemistry Dept., Research Faculty Member
Enzymatic nucleotides, and chromatin structural changes caused by anti-cancer drugs, with Loren Williams.

1994-1995
Visiting Scientist, Emory University and **The Centers for Disease Control and Prevention (CDC), Atlanta**
Sex-determination in malarial mosquitoes with John Lucchesi, Biology Department Chair, Emory University; and Frank Collins of the CDC.

1992
Worcester Foundation for Experimental Biology, Postdoctoral Associate with William Crain
Gene expression in mouse embryogenesis, toxicity of antisense therapies on pregnant mice.

1990-1991
U.S. National Science Foundation, **Postdoctoral Fellow with Jennifer Graves, La Trobe University, Australia**
The sequence and expression of mammalian sex-determining genes.

1986-1990
Ph.D. thesis with Linda Strausbaugh, **The University of Connecticut**
Transcriptional regulation of tagged histone genes in relation to the cell cycle in synchronized culture cells. Instructor in the Summer Institute of Molecular Biology, secured all funding for course from corporate sponsors.

1985-1986
Master's research with Paul Goetinck, University of Connecticut.
Cartilage Link protein c-DNA.

1983-1984
Yale University, School of Medicine, New Haven, Conn.
Research assistant, human keratins and drug response, psoriasis research with Joseph McGuire, Head of Pediatric Dermatology.

Selected Publications

Davis, M., Muller, R., Hampikian, G., The PCR enhancer STRboost™ overcomes humic acid inhibition with forensic DNA typing kits (submitted, 2012).

Besecker, J., Cornell, K. A., and Hampikian, G., Dynamic Passivation with BSA Overcomes LTCC Mediated Inhibition of PCR, Sensors and Vol. 176, Pages 118–123 (January 2013).

Allileche, A., Goswami, J., Davis, M., Bourland, B., Nullomer Derived Anti-Cancer Peptides, Peptides, Volume 38, Issue 2, Pages 302–311, (December 2012),

Goswami, J., Davis, M.S, Andersen, T., Alileche, A., Hampikian, G. Safeguarding Forensic DNA Reference Samples with Nullomer Barcodes, JFS (revision submitted, 2012).

Visser, R. and Hampikian G., When DNA Won't Work, Idaho Law Review (in press, Fall, 2012).

Pham-Hoai, E., Crispino, F., Hampikian, G., The first successful use of simple low stringency familial searching in a French criminal investigation, Journal of Froensic Science (Accepted pending revisions, 2012).

Ullakko, K., Wendell, L., Smith, A., Müllner, P. and Hampikian, G., Magnetic shape memory micropump: contact-free, and compatible with PCR and human DNA profiling, Smart Materials and Structures 21 (2012).

Valverde, L., Rosique, M., Köhnemann, S., Cardoso, S., García, A., Odriozola, A., Aznar, JM, Celorrio, D., Schuerenkamp, M., Zubizarreta, J., Davis, M., Hampikian, G., Pfeiffer, H., de Pancorbo, M. Y-STR variation in the Basque diaspora in the Western USA: evolutionary and forensic perspectives, Int J Legal Med, DOI 10.1007/s00414-011-0644-8 (2011).

Zubizarreta, J., Davis, M., Hampikian, G., "The Y-STR genetic diversity of an Idaho Basque population, with comparison to European Basques and US Caucasians", Human Biology, Dec;83(6):685-94 (2011).

Hampikian, G, West, E., Askelrod, O. "The Innocence Network: Analysis of 194 American DNA Exonerations," Annual Review of Genomics and Human Genetics 12, (2011).

Dror, I. and Hampikian, G., Subjectivity and bias in forensic DNA mixture interpretation, Science & Justice, DOI 10.1016/j.scijus.2011.08.004, (2011)

Bourland, W., Vdacny, P, Davis, M., and Hampikian, G., Morphology, Morphometrics and Molecular Characterization of Bryophrya gemmea n. sp. (Ciliophora, Colpodea): Implications for the Phylogeny and Evolutionary Scenario for the Formation of Oral Ciliature in Order Colpodida, Journal of Eukaryotic Microbiology, vol 58, Issue 1, p 22-36, January/February (2011).

Davis, M., Novak, S., Hampikian, G., Mitochondrial DNA analysis of an immigrant Basque population: loss of diversity due to founder effects, American Journal of Physical Anthropology, Vol. 144, Issue 4, p516-525, April (2011).

Karalova, E. M., Sargsyan, Kh.V., Hampikian G.K., Voskanyan , H. E., Abroyan L. O., AvetisyanA. S., Hakobyan, & L. A, Arzumanyan , H.H., Zakaryan H. S., Karalyan, Zaven A., Phenotypic and cytologic studies of lymphoid cells and monocytes in primary culture of porcine bone marrow during infection of African swine fever virus, In Vitro Cell. Dev. Biol.—Animal, (2011) 47:200–204.

Bullock, C., Jacob, R., McDougal, O., Hampikian, G., Andersen, T. DockoMatic - Automated Ligand Creation and Docking, BMC Research Notes 2010, 3:289.

Abu B. Kanu, Greg Hampikian, Simon D. Brandt, Herbert H. Hill Jr., Ribonucleotide and ribonucleoside determination by ambient pressure ion mobility spectrometry, Analytica Chimica Acta 658 (2010) 91–97.

D. E. Krane, et al. (39 authors) "Time for DNA Disclosure", Science, Vol. 326. no. 5960, pp. 1631 – 1632, 18 December, 2009.

Lucian A. Lucia, Lambrini Adamapoulos, Jason Montegna, Greg Hampikian, Dimitris S. Argryopoulos, John Heitmann (2007), "A Simple Method to Tune the Gross Antibacterial Activity of Cellulosic Biomaterials, Carbohydrate Polymers 69"; 805–810.

Greg Hampikian and Tim Andersen (2007), "Absent Sequences: Nullomers and Primes," Pacific Symposium on Biocomputing, 12:355-366.

K. Moeller, J. Besecker, G. Hampikian, A. Moll, D. Plumlee, J. Youngsman and J.M. Hampikian, (2007), "A Prototype Continuous Flow Polymerase Chain Reaction LTCC Device," Materials Science Forum Vols. 539-543 pp. 523-528.

G. Hampikian, (2005), "The Future of Forensic DNA," The Canadian Journal of Police and Security Services, (Spring, 2005).

M. Crayton, C. Ladd, M. Sommer, G. Hampikian, L. Strausbaugh, (2004), "An organizational model of transcription factor binding sites for a histone promoter in D. melanogaster," In Silico Biology 4, 40-45 (October, 2004).

"Exit to Freedom," Johnson and Hampikian (University of Georgia Press, 2003): Calvin C. Johnson, Jr.'s autobiography (written by Hampikian). The true story of a man who served 16 years in Georgia prisons for a rape he did not commit until DNA evidence freed him. Afterward by Barry Scheck.

P. Henderson, D. Jones, G. Hampikian, Y. Kan, and G. Schuster (1999), "Long-distance charge transport in duplex DNA: The polaron-like hopping mechanism," Proceedings of the National Academy of Sciences, USA, Vol. 96, Issue 15, 8353-8358, July 20, 1999.

G. Hampikian, J. Graves, D. Cooper, (1994), "Sex- determination in the marsupial" in Molecular Genetics of Sex Determination, (Ed. S. Wachtel), Academic Press.

M. Gaudette, G. Hampikian, V. Metelev, S. Agrawal and W. Crain, (1993), "Effect on embryos of phosphorothioate modified oligos. into pregnant mice," Antisense Res. & Dev., 3:391-397.

J. Graves, J. Foster, G. Hampikian, F. Brennan, (1993), "Sex- determination in marsupial mammals," in Sex Chromosomes and Sex Determining Genes, (Editors, K. Reed and J. Graves) Gordon and Breach, Melbourne.

J. Foster, F. Brennan, G. Hampikian, P.N. Goodfellow, A. Sinclair, R. Lovell-Badge, L. Selwood, M. Renfree, D. Cooper and J. Graves, (1992), "Evolution of sex determination and the Y chromosome: SRY- related sequences in marsupials," Nature: 359:531-533.

F. Deak, Y. Kiss, K. Sparks, S. Argraves, G. Hampikian and P. Goetinck (1986), "Amino acid sequence of chicken cartilage link protein from c-DNA clones," Proc. National Academy of Science, U.S.A.: 83:3766- 3770.

Patent Awards and Applications

US Patent 8,008,816: Magnetomechanical Transducer, and Apparatus and Methods for Harvesting Energy, Hampikian and Mullner inventors, awarded August 30, 2011.

US 11/24,293 filed December 23, 2004. Reference Markers for Biological Samples. DNA marker to be added to samples as a safeguard. The oligomers are based on sequences not found in GenBank, and can be coded to contain a wide variety of information.

US 13/358,952 filed January 26, 2012 Absent and Rare Peptides and Theraputic Uses Thereof.

US 13/550,386 filed July 16, 2012 Actuation method and Apparatus, Micropump, and PCR Enhancement Method.

Professional Memberships

- American Academy of Forensic Sciences, workshop leader.
- International Society for Forensic Genetics, presenter.
- International Society for Computational Biology.
- American Society of Microbiologists: Editor for education Newsletter (1999-2002), Editor for image archives (1999-2003); Moderator of the Molecular Biology and Biotechnology Education Listserve (1999-2003).
- American Society for Cell Biology, presenter, education committee member, pre-doctoral grants reviewer.

Selected Honors and Awards

Charter Fellow of the National Academy of Inventors (2012)

Keynote Speaker Pacific Symposium on Biocomputing (Hawaii, 2010)

Awarded the 2004 Silver Medal in biography, for "Exit to Freedom," (ForeWord Magazine's Book of the Year Awards).

- Nominated for the 2004 Robert F. Kennedy Book Award.
- Nominated for the 2004 African American Literary Awards.

Recent Professional Education

Tutorial Workshops, 2011 Pacific Symposium on Biocomputing, Hawaii: "Mining the Pharmacogenetics Literature," and, "Identification of Aberrant Pathway and Network Activity from High Throughput Data", Hawaii, January 3-7, 2011

Familial Search Workshop, International Symposium on Human Identification, San Antonio Texas, October 14, 2010

Low Copy Number Analysis Workshop, Ethics and Forensic Science, International Symposium on Human Identification, San Antonio Texas, October 11, 2010

SNP analysis of physical characteristics (ie., eye color) as well as ancestry. HITA/AABB Workshop, International Symposium on Human Identification, San Antonio Texas, October 10, 2010

Ethics and Forensic Science, International Symposium on Human Identification, Las Vegas, October 15, 2009.

Post-conviction DNA Case Management Symposium, US Department of Justice, Office of Justice Programs, National Institute of Justice, invited participant, Tampa, Fla., January 23-24, 2009.

Tutorial Workshops, 2009 Pacific Symposium on Biocomputing, Hawaii: "Open Science: Tools, Approaches and Implications", "Post-Transcriptional Gene Regulation: RNA-Protein Interactions", "RNA Processing" and "mRNA Stability and Localization," 2009.

Applied Biosystems Gene Mapper & ID-X Software Training, Boise State University, May 26-29, 2009.

DNA Mixture Interpretation: Principles and Practice in Component Deconvolution and Statistical Analysis, American Academy of Forensic Sciences workshop, Washington, D.C., Feb. 19, 2008.

Mixture Interpretation Workshop, taught by Gary Schutler, Ph.D., Northwest Association of Forensic Science, Boise, Idaho, 2008.

Forensic Population Genetics Workshop, 19th International Symposium on Human Identification, Hollywood, CA, 2008.

2008 Pacific Symposium on Biocomputing, Hawaii, 2008
Tutorial Workshops: "Multiscale Modeling and Simulation", "Computational Tools for Next-Generation Sequencing."

Applied Statistics Workshop, 18th International Symposium on Human Identification, (covered DNA Mixtures, Statistics, Parentage and Kinship, Pedigree Analysis), Hollywood, CA, 2007.

Pacific Symposium on Biocomputing, Hawaii, 2007: "Computational Proteomics."

DNA Statistics, 17th International Symposium on Human Identification, Workshop, Nashville, TN, 2006.

Advanced Topics in STR DNA Analysis, American Academy of Forensic Sciences, workshop, Seattle, WA, Feb. 20, 2006.

Li-Cor DNA sequencing training for the Li-Cor 4300, Boise State University, 2005.

On-site evaluator training Forensic Science Education Programs Accreditation Commission (FEPAC), American Academy of Forensic Sciences workshop, New Orleans, 2005.

"Symposium: Emerging and Enabling Technologies for Biological and Chemical Detection" and "Federal Bio-Chem Detection R&D Opportunities," 15.5 hours, Information Forecast, Washington, DC, 2005.

Forensic Human Mitochondrial DNA Analysis, American Academy of Forensic Sciences workshop, Dallas, Texas, 2004.

Forensic Science for Medicolegal Professionals Course (co-organizer), Atlanta, 2004.

Mass Fatalities Incident Response Planning Course, (Local coordinator), Atlanta, 2004.

Science in the Courtroom for the 21st Century: Issues in Forensic DNA, Chicago, 2004.

Legal Communication in the 21st Century, 3-hour course, Clayton State University, 2003.

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May 15, 2013

To whom it may concern,

This letter is in support of Dr. Greg Hampikian's research regarding liposome delivery of cancer killing Nullomer peptides. This cutting edge research has the potential for multiple beneficial outcomes including treatment of cancer in both the human and animal populations.

WestVet Animal Emergency and Specialty Center, located in Garden City Idaho, provides advanced medical and surgical treatment to animals with cancer. Our center provides such services to pet owners not only from Idaho but from the surrounding states. Major advances in cancer treatment such as the liposome delivery of Nullomer peptides help lead to cutting edge therapies which can start here and Idaho.

We are very excited about the proposed research and fully support its advancement to the next level.

Sincerely,

Jeff D. Brourman DVM, MS, DACVS

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Nullomer Derived Anticancer Peptides (NulloPs): Differential Lethal Effects on Normal and Cancer Cells *in vitro*

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Nullomer derived anticancer peptides (NulloPs): differential lethal effects on normal and cancer cells *in vitro*

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Abstract

We demonstrate the first use of the nullomer (absent sequences) approach to drug discovery and development. Nullomers are the shortest absent sequences determined in a species, or group of species. By identifying the shortest absent peptide sequences from the NCBI databases, we screened several potential anti-cancer peptides. In order to improve cell penetration and solubility we added short poly arginine tails (5Rs), and initially solubilized the peptides in1M trehalose. The results for one of the absent sequences 9R (RRRRRNWMWC), and its scrambled version 9S1R (RRRRRWCMNW) are reported here. We refer to these peptides derived from nullomers as PolyArgNulloPs. A control PolyArgNulloP, 124R (RRRRRWFMHW), was also included. The lethal effects of 9R and 9S1R are mediated by mitochondrial impairment as demonstrated by increased ROS production, ATP depletion, cell growth inhibition, and ultimately cell death. These effects increase over time for cancer cells with a concomitant drop in IC-50 for breast and prostate cancer cells. This is in sharp contrast to the effects in normal cells, which show a decreased sensitivity to the NulloPs over time.

1. Introduction

This study investigates the biological properties of the smallest absent peptides, nullomer peptides (NulloPs). These peptides were identified after a search of the NCBI databases, which counted all occurrences of peptide strings, and generated a list of the smallest peptide sequences (currently length-5 amino acids) absent from natural databases. While combinatorial and phage display libraries have been used to generate peptide drugs that were not derived from nature, we have taken a completely new approach to look for effective cancer drugs; we look specifically for what does not exist [21] in nature. Acquisti et al. have dismissed this approach [1], claiming that absent sequences are actually a consequence of mutation and will not be useful for medical or labeling purposes; but others have explored the distribution of absent sequences suggesting possible applications [7,31,42]. We have used a new algorithm for this negative *in-silico* selection that enables us to identify small epitopes with possible lethal effects [21]. While others have debated whether this approach might yield lethal sequences [5,42], we have now shown that several of these peptides are in fact lethal at micromolar concentrations. Our approach originally generated hundreds of NulloPs (unpublished data), and we have been characterizing their biological effects, seeking to exploit their killing mechanisms to ultimately produce new anticancer peptides. This report focuses on NulloP 9 (NWMWC), its scrambled version 9S1 (WCMNW); and NulloP 124 (WFMHW). To ensure cell penetration we conjugated the NulloPs with PolyArg (RRRRR).

Cancer therapy is a combination of surgery, chemotherapy, radiotherapy and recently growth factors and cytokines, and has not yet met our dreams of curing this disease [2]. In spite of the application of a wide variety of drugs, anti cancer chemotherapy has always been limited by side effects and chemoresistance [41]. The extreme genetic adaptability of cancer cells in their ultimate quest of immortality and independence gives these cells several survival advantages in harsh conditions; for example they can stop cell division and enter a state of dormancy, or become stem cells capable of regenerating the original tumor [27].

Cancer cells are resistant to apoptosis induction, and a new paradigm in cancer therapy is emerging. Cancer cells survive because their metabolism is altered with significant changes in mitochondrial function, and a lower dependence on pyruvate oxidation, the Warburg effect [6]. The therapeutic approach known as mitochondrial medicine [11,12,16] is justified for several reasons: cancer mitochondria have low oxidative phosphorylation, high $\Delta\psi m$ and reactive oxygen species (ROS) output, and a deregulated apoptotic pathway [12]. The mitochondrial outer membrane permeabilization (MOMP) constitutes a "*point of no return*," in that once it begins, cells are destined to die by apoptosis or necrosis [29]. Chemicals targeting the mitochondria have the advantage to initiate the MOMP independently of upstream signals that are frequently impaired in cancer cells, and thus bypass chemoresistance [12,16].

Already many chemicals and peptides (derived from existing proteins involved in cell death) that target the intrinsic mitochondrial death machinery are being used in clinical trials [12,16]. Other natural peptides such as defensins [10,39,53] (endogenous antimicrobial peptides produced by leukocytes and epithelial cells), have demonstrated anticancer potential. Their mechanism of action involves a stimulation of the immune system [10,56], but it is still not clear what makes cancer cells susceptible to these peptides. The general mechanisms suggested are: lysis of cancer cells [49,23], plasma membrane disruption via micellization or pore formation [50], and induction of apoptosis via mitochondrial membrane disruption [48].

One of the characteristics of the NulloPs described here is their high hydrophobicity due to the presence of TRP and MET. Their poor solubility in water becomes a major obstacle for their use. In order to overcome this, we fused them to a cell penetrating peptide (CPP). This is a well established approach that started with the observation that the human immunodeficiency virus 1 (HIV-1) Tat protein can enter cells efficiently *in vitro* [13]. In 1997, Vives et al. [51] discovered that an 11 amino acids sequence, Tat 48-60 (GRKKRRQRRRPPQ) known as the Tat peptide, can enter cells more efficiently than the full length Tat protein. A PolyArg peptide made from 6 to 9 L-ARG maintains the same cell penetrating capacity as the original Tat peptide [52]. The mechanism(s) of internalization of PolyArg CPP is still a controversial matter. There are two principal routes of internalization: endocytosis (an energy dependent process), and transduction through cellular membrane.

PolyArg CPP [45] and Tat protein [36] interact with the cell membrane by inducing a Gaussian membrane curvature as a prelude to pore formation through which transduction to the cytosol occurs. In spite of the pore formation, after transduction of PolyArg CPP and its cargo inside the cell, the cell membrane integrity is not compromised and the cell remains negative for propidium iodide (PI) staining [37]. The release of the cargo from the PolyArgCPP-Cargo complex has not yet been demonstrated [34].

We have proposed that by identifying the smallest absent DNA sequences, we can find short peptides eliminated by natural selection. These sequences may have unusual properties that can be exploited for drug development [21]. This report demonstrates the anticancer benefits of the nullomer approach, and shows that NulloPs can affect the growth of several cancer cell lines by mitochondrial impairment.

2. Methods

2.1 *Cell culture*

Cell lines used in this study were obtained from the American Type Culture Collection (ATCC, Manassas, VA). The human and murine cancer lines are: LnCap (CRL-1740) derived from a human prostate carcinoma,

MDA-MB-231 (HTB-26) derived from a human breast adenocarcinoma, B16 (CRL-6322) derived from a C57BL/6J mouse skin melanoma, HUT102 (TIB-162) derived from an ATL (HTLV-1 Adult T cell Leukemia) a gift from Dr. T.A. Waldmann (NIH/NCI), J774A.1 (TIB-67) mouse monocyte/macrophage derived from a BALB/cN reticulum cell sarcoma. Primary normal cell lines were used as well: PCS (PCS-440-010) normal primary prostate epithelial cells, HMEC (ECACC-HMEC 830-05a) normal human mammary cells, WI-38 (CCL-75) human embryonic fibroblasts derived from a 3 months gestation fetus. LnCap, MDA-MB-231, and HUT102 were cultured in RPMI 1640 supplemented with 10% Hyclone Fetal Bovine Serum (FBS) and 1X Penicillin Streptomycin (100X solution, Invitrogen). B16 and WI-38 were cultured in DMEM supplemented with 10% Hyclone FBS and 1X Penicillin Streptomycin. PCS cells were cultured in the Prostate Epithelial Cell Basal Medium (PCS-440-030) supplemented with the Prostate Epithelial Cell Growth Kit (PCS-440-040). HMEC cells were cultured in the Clonetics MEGM BulletKit (CC-3150). Cell cultures were incubated at 37ºC and 5% CO_2. For PCS and HMEC cells we used passages 2-4. The passaging of cells was done at 80% confluence; after a PBS wash, cells were treated with Trypsin-EDTA 0.25% (Invitrogen). Cells were seeded in 96 well plates (Costar) and allowed to adhere to the surface overnight, then the old media was removed and replaced with 90 µl fresh media, with 10 µl containing peptides or other treatment.

2.2 *Cell viability assay*

Cell viability was measured by the colorimetric MTT assay (Sigma, St Louis MO). 3000-5000 cells/well were seeded in 96 well plates and exposed to peptides or other treatments. At each time point, the MTT solution (5 mg/ml in PBS) was added directly to wells and incubated at 37°C for 4 h, then the formazan crystals solubilized with 100 µl dimethyl sulfoxide (DMSO), and incubated for 1h at 37ºC. Absorbance at 570 nm was taken by a plate reader (SynergyMx from Biotek, Winooski VT). Plate readings were exported to Microsoft Excel and GraphPadPrism software. All the wells were analyzed in triplicates. The statistical analysis was done with the GraphPadPRISM*.

2.3 *PolyArg-peptides synthesis*

The PolyArgNulloPs were made by Elim Biopharmacuticals (Hayward, CA) and Pierce Biotechnology (Rockford, IL). HPLC purified peptides (purity >98%) were delivered in 1 to 2 mg/tube (lyophilized) format and stored at -20°C. Peptides were solubilized in 1M trehalose to produce 100 mM stock solutions. Only freshly prepared solutions were used to treat cells. Several concentrations of the peptides were used with a range from 5-10 µM to 100 µM. The trehalose concentration in all reactions was 1 mM (a dose without any effect on cell growth).

2.4 *Preparation for scanning electron microscopy*

Cells were cultured as described above in 35 mm dishes containing cover slips. Cover slips were prefixed in 4% paraformaldehyde in PBS for 30 minutes (mn), washed in distilled water 3 times for 5 mn each and placed in 2.5% glutaraldehyde for 30 mn, followed by washing in distilled water as described. Cover slips were then placed in 0.5% osmium tetroxide for 30 mn and washed again in distilled water. Cover slips were then transferred to 1% tannic acid for 30 minutes, washed in distilled water and transferred a second time to 0.5% osmium tetroxide, and washed again in distilled water. Cover slips were dehydrated in ethanol (30%, 50%, 70%, 80%, 90% and 100% × 3), 5 mn each. The cover slips were transferred to a K850 critical point dryer (Quorum Technologies, Ashford, UK) using 100% ethanol as the transitional fluid. Dried cover slips were sputtered with gold (~20 nm) in a CrC150 sputter coater (Torr International, New Windsor, NY, USA), and examined in a Hitachi S-3400N scanning electron microscope.

2.5 *HCS Mitochondrial Health Assay*

The HCS Mitochondrial Health Kit (Invitrogen, Carlsbad CA) uses two dyes: the MitoHealth stain

(excitation/emission maxima 550/580 nm) accumulates in the mitochondria of live cells proportional to the mitochondrial membrane potential, Hoechst 33342 (excitation/emission 350/461 nm) stains nuclear DNA in live and dead cells. Cells were seeded in 96 well plates and incubated with peptides for 24h. Then 50 µl of MitoHealth stain was added to each well and incubated for 30 mn. After removing the media, Hoechst 33342 and 3.7% paraformaldehyde were added to cells. After washing the cells twice with PBS, colorimetric reading was taken with the plate reader. Relative fluorescence was measured in BioTek Synergy plate reader. This kit was manufactured for imaging, but we standardized it with SynergyMx plate reader for reading 96-well plates.

2.6 *Measurement of total cellular ATP content*

Cells were seeded in 96 well plates (white plates from Nunc to block luminescence bleeding between the wells) and allowed to attach for 24h. Then cells were incubated for 2h, 24h and 48h with peptides (1-50 µM of 9R, 9S1R and 124R), or 100mM sodium azide as an ATP depletion control [26] At each time point a single reagent, Cell Titer Glow™ (Promega), was added to cells.. Complete reagent mixing in 96 wells plates required gentle orbital shaking for 2-10 mn. The plate reading was taken by SynergyMx plate reader. Plate readings were exported to Microsoft Excel and GraphPadPrism software. All the wells were analyzed in triplicates. The statistical analysis was done with the GraphPadPRISM*.

2.7 *Live/Dead assay*

Each peptide's effect on cell vitality was measured by the live/dead assay kit from Invitrogen (Carlsbad, CA). This kit uses Hoechst 33342 as a nucleic acid stain (blue fluorescence) and PI (red fluorescence). Since PI is not permeant to live cells, it is commonly used to detect dead cells in a population. The PI concentration used in this study was 6.25µg/ml. After 2h incubation with PolyArgNulloPs, cells were stained directly in the reaction media with these two stains, and fluorescent images were taken by Zeiss axiovert 40 microscope (Oberkochen, Germany) using a SPOT imaging camera (Sterling Heights, MI).

2.8 *Super Oxide Assay*

After 24h incubation in 96 wells microplates, cells were treated with peptides or control for 2h. Then MitoSOX™ red reagent (Invitrogen) was added for 10 mn, and washed with PBS. The final plate reading was taken in SnyergyMX plate reader.

2.9 *Hemolytic activity*

The hemolytic activity of PolyArgNulloPs was determined on human red blood cells (RBCs) from healthy volunteers. Blood samples were centrifuged and RBCs washed twice with PBS. A 10% RBCs suspension was incubated with 10 µM, 25 µM and 100 µM of peptides (9R, 9S1R and 124R) at 37°C for 24 h with 100 rpm shaking. PBS was used as a negative control (0% lysis: $A_{Control}$), and 1% Triton X-100 as a positive control (100% lysis: A_{Total}). After incubation the samples were centrifuged and supernatants transferred into a 96-well plate to quantify RBCs lysis by a spectrophotometric reading at λ=405 nm [23]. The hemolytic activity [20] in % was calculated using this formula: Hemolytic activity in $\% = \{[A_{Sample} - A_{Control}]/[A_{Total} - A_{Control}]\} \times 100$

2.10 *Statistical analysis*

All results are expressed as mean ± SE (standard error). The statistical analysis was done with the GraphPadPRISM* version 5.03 using one-way analysis of variance (ANOVA) followed by a Tukey's post test. A *p-value* of less than 0.05 was considered statistically significant.

3. Results

3.1 *The in vitro effect of PolyArgNulloPs peptides on cell growth*

Cells were exposed to different doses (10-100 μM) of PolyArgNulloPs (9R, 9S1R and 124R) for 2h, 24h and 48h. For each time period, the exposure of cells to the peptides was continuous with no change of the culture medium. The 2h time point was aimed at detecting toxic effects similar those of the control treatment H_2O_2 (0.01%). The 24h and 48h time points were used to examine peptide effects related to the cell cycle.

The results show clearly that peptide 124R has no effect on the growth of LnCap prostate cancer (Suppl.R1A) or MDA-MB-231 (Suppl.R1B) cells. Peptide 124R is a PolyArgNulloP with the same number of amino acids, and the same poly Arginine content as 9R, 9S1R, and can be considered a control peptide. The peptide 9R at 100 μM has a toxic effect at 2h on LnCap cells, similar to the effect of H_2O_2 (0.01%). At 24h and 48h, 9R has a moderate effect on LnCap at 20-30 μM, and an almost completely lethal effect at 50-100 μM. However, we do not observe a classic dose dependent effect between 5-100 μM with 9R in LnCap cells. With the MDA-MB-231 cells, peptide 9R produces a dose dependent effect (10-100 μM) at 2h, 24h and 48h. The peptide 9S1R shows a dose dependent effect over 10-100 μM at 2h for both the LnCap (Suppl.R1A), and MDA-MB-231 (Suppl.R1B) cells. This toxic effect is also seen at 24h and 48h.

The melanoma B16 cell line was included in this study because skin melanoma is the sixth most prevalent cancer in North America, and its incidence is on the rise [14]. The three peptides 9R, 9S1R and 124R at doses 10-100 μM, and at time points from 2h to 96 h (Suppl.R1C), have no effect on the growth of B16 cells.

Peptide 124R, at doses 10-100 μM, at time points from 2h to 96h, has no effect on the growth of HUT 102 cells. However, peptide 9R and 9S1R have a toxic effect at 100 μM seen from 2h. In addition, a dose response was observed from 10-100 μM at time points from 48h to 72h. In highest range of doses (50-100 μM), a dose dependent effect is only observed at the 96h time point (Suppl.R1D).

The peptides 9R, 9S1R and 124R have no effect on the growth of J774A.1 cells at doses 10-100 μM, from 2h to 72h. At 96h, peptide 124R has no effect; but there is a dose dependent effect with 9R and 9S1R (although the effect is more pronounced with 9R) (Suppl.R1E). Microscopically, the cells swell and contain many vacuoles (data not shown).

We also examined the effects of the peptides 9R, 9S1R and 124R on normal cells. Peptide 124R has no effect on the growth of WI-38 (Suppl.R1F), and PCS (Suppl.R1G) cells up to 48h time point (10-100 μM). However at the highest concentration (100 μM) peptide 124R has an effect on the growth of HMEC cells (Suppl.R1H) at 24h, and to a lesser extent at 48h. Peptide 9R and 9S1R have a moderate dose dependent effect (20-100 μM) on WI-38 cells at 24h, and that effect is restricted to the 50-100 μM treatments at 48h (Suppl.R1F). Peptide 9R and 9S1R have a moderate effect on the growth of PCS cells at 24h and 48h time points (Suppl.R1G). In addition, peptides 9R and 9S1R have a dose dependent effect (10-100 μM) on the growth of HMEC cells at the 24h and 48h time points (Suppl.R1H).

Scanning electron micrographs of LnCap and MDA-MB-231 cells treated with 9R (Fig. 1E, F, respectively) and 9S1 (Fig 1G, H, respectively) for 48 hours (100 μM), show greatly reduced cell density compared to untreated LnCap (Fig. 1A) and MDA-MB-231 (Fig. 1B) cultures. Cells treated with the control peptide 124R (Fig 1C, c, D, d) appear similar to untreated cells (Fig. 1A, a, B, b). At higher magnification the morphology of 9R and 9S1 treated cells show substantial membrane damage including blebbing and tearing (Fig. 1e, f, g, h).

3.2 *The IC-50 evolution of PolyArgNulloPs peptides in vitro*

We calculated the IC-50 of peptides 9R, 9S1R and 124R at every time point where there is an effect on the growth of cells. As shown in Table 1, the evolution of IC-50 for cancer cells is different from that seen in normal cells. For LnCap cells, the 9R IC-50 starts high at the 2h time point (44±2.5 μM), and drops by 48h

(28±1.6 μM). The same pattern of IC-50 evolution for 9R is seen in MDA-MB-231 cells, where the IC-50 is 39 ±1.8 μM at 2h, and falls to 16±0.9 μM at the 48h time point. The same pattern can be seen with peptide 9S1R; for LnCap cells the IC-50 starts at 26±1.3 μM at 2h and falls to less than 8±0.5 μM at the 48h time point. For MDA-MB-231 cells, with 9S1R the IC-50 starts at 18±0.5 μM at the 2h time point, and drops to 10±0.3 μM at the 48h time point. For HUT102 cells, the IC-50 for 9R starts at 93±2.3 μM at the 2h time point, and falls to 25±1.6 μM at 96h time point. This observation shows clearly that the cancer cells are more sensitive to 9R and 9S1R over time. Normal cells show an opposite pattern. For PCS and HMEC cells, the IC-50 for 9R, 9S1R and even 124R rises with time. This means that the sensitivity of normal cells to 9R, 9S1R and 124R diminishes with time, while the sensitivity of the cancer cells increases with time. The obvious potential clinical importance of this finding needs to be validated in animal models.

3.3 *PolyArgNulloPs effects on cellular ATP level*

We studied the effects of peptides 9R, 9S1R and 124R on total cellular ATP content in LnCap and MDA-MB-231 cells. Peptide 124R at doses 1-50 μM, for time points from 2h to 48h, has no effect on the cellular level of ATP in LnCap and MDA-MB-231 cells (Fig. 2). This is consistent with the observation that peptide 124R does not affect cell growth. Peptide 9R has a moderate effect at the 2h time point with 50 μM peptide in both cell lines. This effect persists at the 24h time point. By 48h, there is a 50% and 65% reduction with 25 μM and 50 μM 9R (respectively), this latter effect is similar to that seen with our control 100 mM sodium azide. Peptide 9S1R at 50 μM almost completely shuts down ATP production in both cell lines (LnCap and MDA-MB-231) by the 2h time point, an effect more drastic than the sodium azide control. This is a toxic effect similar to that of anthrax toxin in J774A.1 cells [3,4]. In addition, at 25 μM peptide 9S1R reduces the ATP level by 50% at the 2h and 24h time points, and by 85% at the 48h time point. The effect is even more dramatic in LnCap cells, where 9S1R at 48h produces a 50% reduction of ATP level at 5-10 μM.

3.4 *PolyArgNulloPs effects on mitochondrial function*

Mitochondrial Membrane Potential (MMP) is the driving force behind ATP production by the mitochondrial respiratory chain. As shown in (Fig. 3a) and (Fig. 3b), peptide 124R has no effect on MMP at 24h in either the LnCap or MDA-MB-231 cell lines. Peptide 9R at 50-100 μM reduces by 40% the MMP in both cell lines. Peptide 9S1R has dramatic effects on MMP, producing a 40% reduction at 30-100 μM in LnCap cells. In MDA-MB-231 the reduction of MMP is even more dramatic: 40% reduction at 20-30 μM, and 95% reduction at 50-100 μM.

Superoxide free radicals production is indicative of mitochondrial damage. We investigated the ROS production 2h after exposure to the PolyArgNulloPs, before any cellular mechanisms can scavenge these free radicals. As shown in (Fig. 4a) and (Fig. 4b), for both LnCap and MDA-MB-231, peptide 124R at 10-100 μM has no effect on the production of ROS. However, for both cell lines, peptide 9R at 50-100 μM produces more free radicals than the control H_2O_2 (0.01%). Peptide 9S1 at 10-100 μM induces ROS production in a dose dependent manner in LnCap cells, and produces a dramatic increase of ROS production at 100 μM in MDA-MB-231 cells. It is noteworthy that 9R and 9S1R at 100 μM in both cell lines induces more ROS production than the H_2O_2 (0.01%) treated cells.

3.5 *Effects of PolyArgNulloPs on RBCs*

It is well known that some peptides puncture the cell membrane of RBCs, and this side effect can limit their therapeutic potential. We exposed the PolyArgNulloPs (10μM, 25μM and 100 μM) to human RBCs from healthy donors for 24h at 37ºC. As shown in (Fig. 5), peptides 9R and 124R have no hemolytic effect on human RBCs, and peptide 124R produces only 1% hemolysis at 100 μM. In contrast, peptide 9S1R has significant hemolytic activity: 0.5% at 10 μM, 2.5% at 25μM, and 12% at 100 μM. The hemolytic activity of peptide 9S1R may explain its rapid toxic effects at 2h.

3.6 *Live/Dead Assay*

We performed this analysis at the 2h time point, and used PI as a marker of cell death. Only cells with compromised membrane integrity become PI positive. As shown in (Suppl.R2A) and (Suppl.R2B), both cell lines (LnCap and MDA-MB-231) exposed to H_2O_2 (0.01%) for 2h are nearly 100% PI positive. The untreated cells are all PI negative. Cells treated with 100 μM of peptide 124R for 2h show few PI positive cells (less than 1%). Both cell lines exposed to peptide 9R at 10 μM or 50 μM for 2h show no effect on PI entry. At 100 μM 9R, both cell lines show significant cell death (30-40% become PI positive). 9S1R has stronger effects on both cell lines, as 30-40% become PI positive with 50 μM 9S1R, and almost 100% are PI positive at 100 μM 9S1R. This result is consistent with the effects of 100 μM 9S1R (2h time point) on cellular ATP and cell survival. But the 2h time point is too short for an apoptotic process, and the drop in ATP level also contradicts and apoptotic model of cell death. This response is also not typical of necrosis, since cell swelling is not observed.

4. Discussion

4.1 *Solubilization of PolyArg peptides in trehalose*

The original Nullomer peptides 9 (NWMWC), 9S1 (WCMNW) and 124 (WFMHW) are composed of two types of amino acids: MWF (hydrophobic non polar), and CNH (polar but uncharged). They are insoluble in water, and though they are initially soluble in absolute ethanol, after mixing with culture medium they precipitate. We solubilized the PolyArg conjugated peptides in 1M trehalose (though the NulloPs without PolyArg are insoluble in 1M trehalose). The solubilization process was instantaneous, and PolyArg conjugated peptides remain stable in solution at room temperature. Trehalose has a water replacing capacity and most likely surrounds the hydrophobic peptides with a carbohydrate coat [30]. It has been used extensively in the formulation of therapeutic proteins, because of its qualities as a protein-stabilizer [25]. This is the first time to our knowledge that a CPP conjugated cargo has been solubilized in trehalose. While others [52] report that an R5 tail is not sufficient for cell penetration, the conjugation of an R5 tail to our peptides 9, 9S1 and 124, facilitates solublization in trehalose.

4.2 *PolyArgNulloPs potential for cancer treatment*

The treatment of human cancer is not a single drug therapy. A cocktail of drugs aimed at different targets in cancer cells is used to avoid drug resistance. Potential drugs against cancer are characterized by their IC-50, which is the dose that inhibits the growth of 50% of the cell population exposed to the drug. Protocols have been established in the USA by the NCI/NIH [9,40], and in Japan [55], for testing potential new drugs against a panel of cell lines. The current protocols expose cancer cells to drugs for 48h [57] or 72h [40], followed by cell viability assays. It is noteworthy that the screening systems available like the NCI-60 panel of cancer cells [9,40], the JFCR39 panel of 39 cell lines used in Japan [55] or the recently established CMT1000 (Center for Molecular Therapeutics 1000) panel consisting of 1200 cancer cell lines [46], do not include normal cells as controls. The differential sensitivity of cancer cells and normal cells, to cancer drugs, is a complex area. In some studies, normal cells are not sensitive at all to cancer drugs [38]. In other studies, the response of normal cells to cancer drugs depends on the culture protocol: contact inhibited quiescent 3T3 fibroblasts are not sensitive (to cisplatin and etoposide) [24], while exponentially dividing 3T3 fibroblasts [24] and MCF-10A (normal human mammary epithelial cell line) are sensitive [8].

The results presented here include an analysis of the PolyArgNulloPs differential IC-50 evolution in normal and cancer cells. LnCap and MDA-MB-231 cells were included in our study because they represent, respectively, the most frequent cancer in men (prostate cancer) and women (breast cancer). We looked at the killing potential of PolyArgNulloPs at 3 different time points to assess their cytotoxicity and effect on growth. As shown in Table 1, the IC-50 evolution of 9R and 9S1R in LnCap and MDA-MB-231 demonstrates that these cancer lines have an increasing sensitivity to our peptides over time, while the normal cell lines have a decreasing sensitivity. The LnCap IC-50 of 9R starts at 44 ±2.5 μM at 2h, and falls to 28 ±1.6 μM by 48h. The

LnCap IC-50 for 9S1R starts 26 ±1.3 μM at 2h, and drops to 8 ±0.5 μM at 48h. The same pattern is seen with MDA-MB-231 cells, HUT102 cells and J774A.1 cells. For normal cells like PCS and HMEC, the opposite pattern is seen. The PCS IC-50 of 9R starts at 28 ±1.1 μM at 2h, and reaches 35±1.3 μM at 48h. For HMEC cells, the IC-50 for 9R starts at 20 ±0.8 μM at 2h, and reaches 34 ±1.2 μM at 48h. This differential pattern of the IC-50 evolution for our peptides shows clearly that normal cells, although inhibited by our peptides can recover, while the sensitivity of cancer cells increases with time. Other researchers have found similar patterns of increased sensitivity for taxol and other drugs [18,32,33,35,44]. Unfortunately, these early observations were not investigated further, and these studies failed to include normal cells [18,32,33,35,44]. The parallel evaluation of cancer cells and normal cells in regard to their sensitivity to cancer drugs is important, because the side effects of cancer drugs on normal cells are a major clinical issue. To our knowledge the differential evolution of IC-50 in cancer cells versus normal cells is assessed for the first time here. This comparison of normal versus cancer cell sensitivity over time, may be useful to future screening efforts.

4.3 *The lack of effects of 9R, 9S1R and 124R on melanoma B16 cells*

It is surprising that 9R and 9S1R at 100μM, even at time points up to 96h, have no effect on the growth of melanoma B16 cells. The resistance mechanism(s) of B16 cells to PolyArgNulloPs are not known. It has been reported previously that CPPs other than R5 are more efficient at delivering lethal drugs in melanoma cell lines (Bowes melanoma cells [20], and SKMel-37 melanoma cells [15]). Changing the CPP in future studies of the NulloPs may yield better results.

4.4 *Comparison of the killing mechanisms of 9R and 9S1R*

The analysis of our results support the simple statement that peptides 9R and 9S1R have devastating effects on the mitochondria (as evidenced by their changes in cellular ATP, MMP, and ROS production), and consequently on cell growth. These effects are similar to the effects of amyloid beta peptide on nervous cells in Alzheimer disease, where the amyloid beta peptides are imported into mitochondria [22]. The CPP conjugated peptides do not cross the mitochondrial membrane [43], however the mitochondrial outer membrane has a porine protein which is permeable to compounds up to 5000 Da. Our PolyArgNulloPs are in the 1500 Da range, so it is possible that 9R accumulates in the mitochondria, a hypothesis that is under investigation in our lab. Our results, especially the contrast in effects of 9R and 9S1R on cellular ATP, suggest that 9S1R targets non-mitochondrial cytosolic metabolism, whereas 9R attacks the mitochondria. 9S1R (50 μM) almost completely shuts down the ATP production in LnCap and MDA-MB-231 cells. We know that the respiratory chain in the mitochondria is the main source of ATP production in the cells, but glycolysis is important to cancer cell metabolism. The dramatic effect of 9S1R on ATP production (greater than sodium azide) may be caused by an inhibition of cellular glycolysis which automatically shuts down the respiratory chain in the mitochondria. Therefore, 9S1R is a candidate for the new metabolic strategy of treating cancer through glycolysis inhibition [54] and ATP depletion [28]. Peptide 9R also lowers cellular ATP content by attacking the mitochondria.

4.5 *Successful first use of the nullomer approach for drug development*

As with any in vitro drug screening, we realize that our results will need to be assessed in animal models. The treatment of cancer is based on the log-kill hypothesis established in 1970 by Skipper et al. [47]. Basically, this hypothesis states that at a certain dose, a cancer drug kills only a fraction of a tumor cell population. This leads to treatment cycles, in which every cycle enriches the proportion of resistant cells. So the results obtained *in vitro* for a single dose, may not match the results *in vivo*. Another difference between cell culture and animal studies is that *in vitro* we start with a small number of cells, and see if a cancer drug can inhibit the growth of that small population; but in vivo studies usually begin with established tumors. As the tumor grows in volume, the fraction of cycling cells diminishes; and as the log-kill hypothesis states, the response to cancer drugs declines. For this reason, cancer therapy uses a cocktail of drugs to attack multiple cellular targets. The relatively high doses used in this paper should not disqualify NulloPs as possible drugs for cancer treatment.

Cancer treatment is always a combination of drugs rather than a single drug, and our next phase of research is looking at possible combination drug treatments. The protocols based on the log-kill hypothesis have been improved recently by a new strategy focusing on the amount of drug per unit time, or dose intensity. This approach is based on high dose drug pulses rather than on a continuous regimen [19]. The first screening of potential anticancer drugs usually focuses a single drug treatment, and we have shown that our PolyArgNulloPs kill cancer cells by targeting mitochondrial health and ATP production. We do not envision their use as single anticancer drugs, but rather combined with other drugs. It is important to note that the difference in sensitivity was measured in rapidly growing, rather than quiescent cell cultures. We are currently studying the differential effects of NulloPs on cancer cells, versus dividing and quiescent normal cells.

5. Conclusion

We present here the first drug candidates produced by nullomer research. The bio-informatic approach which identified the NulloPs was based on the idea that rare or absent short sequences may be enriched for toxic or problematic peptides. We have shown that the differential effects of PolyArgNulloPs on cancer cells and normal cells indicate that these new peptides should be investigated and optimized for cancer treatment.

Fig 1. Scanning electron microscopy of untreated and nullomer (124, 9R, 9S1) treated LnCap and MDA-MB-231 cells.

(A-H) lower magnification views. (a-h) higher magnification views. (A,a, B,b) Untreated control LnCap and MDA-MB-231 cells respectively. (C,c, D,d) LnCap and MDA-MB-231 cells, respectively, treated with nullomer 124 (100 μM for 48 hr.). SEM examination of overall cell growth and membrane integrity after treatment with nullomer 124 are unchanged from untreated control cells. (E,e, F,f) LnCap and MDA-MB-231 cells respectively treated with peptide 9R (100 μM for 48 hr.); overall cell growth markedly reduced compared with untreated control cell cultures. Cytopyknosis and membrane blebs appear (e, lower right and F,f). (G,g, H,h) LnCap and MDA-MB-231 cells, respectively, treated with peptide 9S1 (100 μM for 48 hr.); overall cell numbers less noticeably reduced than in cells treated with peptide 9R, but show widespread severe membrane damage consistent with necrosis. Scale bars = 50 μm (A-H), 25 μm (a-h).

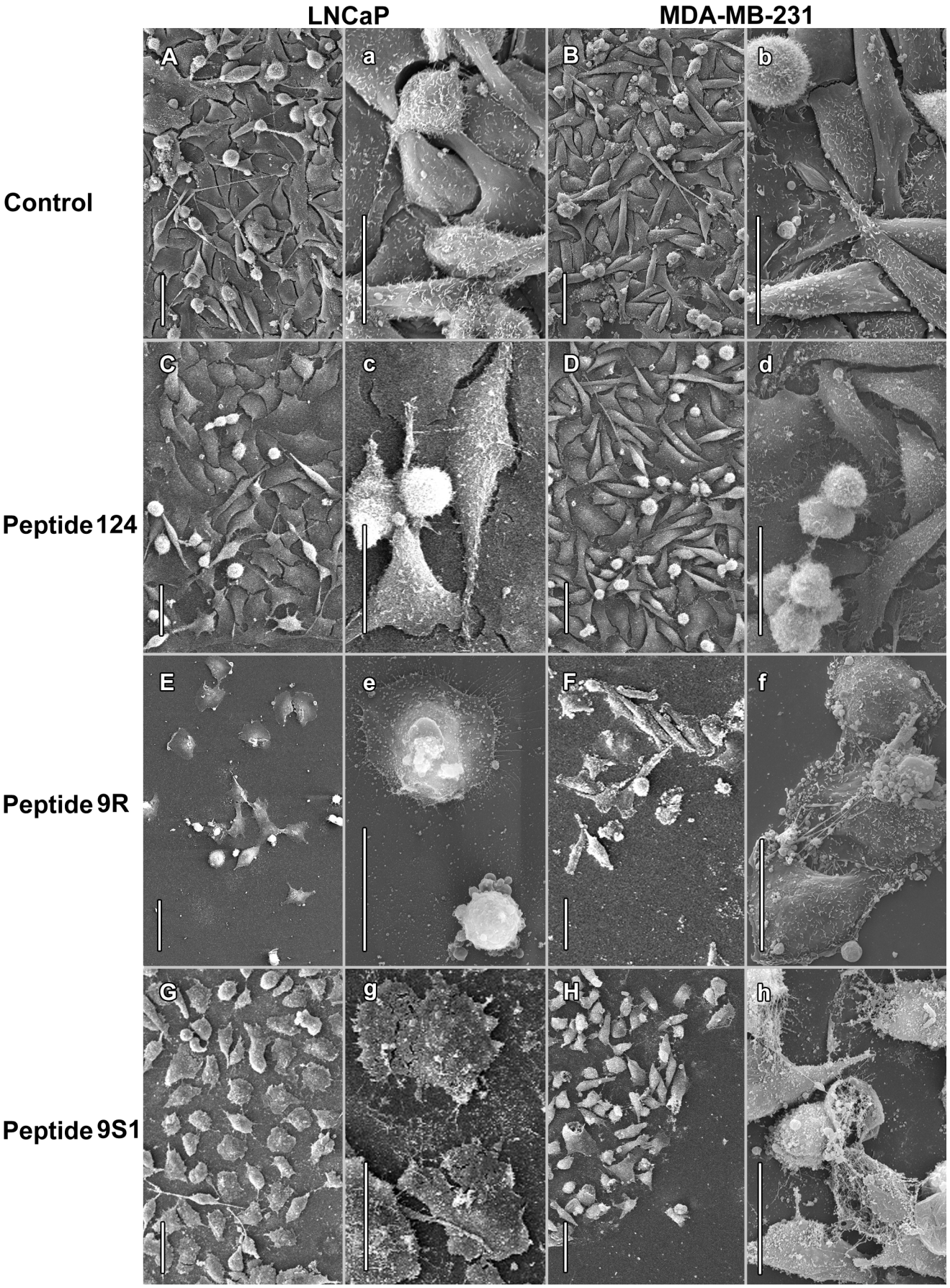
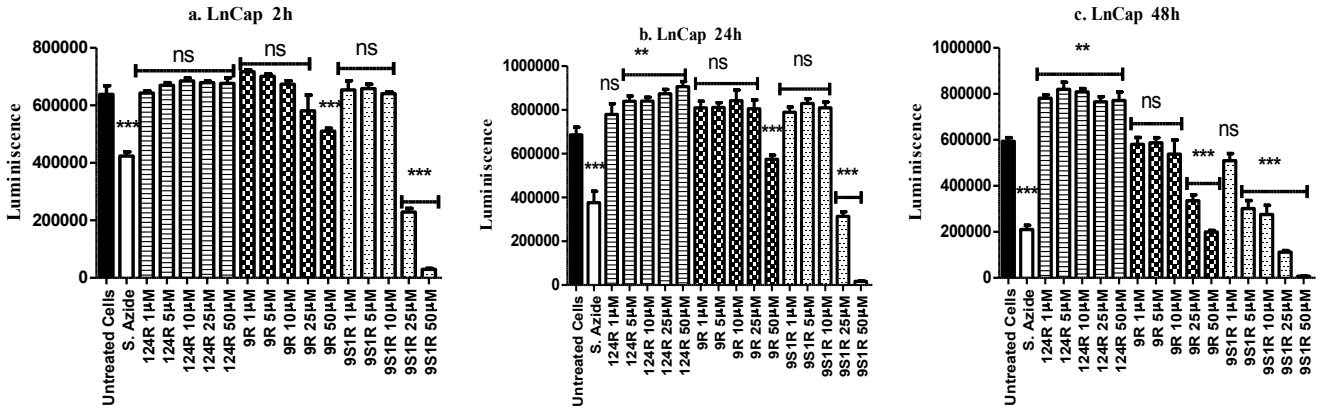


Fig. 2. PolyArgNulloPs effects on cellular ATP level. Cells (3000/well) were seeded in 96 well plates (white plates from Nunc). After 24 h incubation, peptides 9R, 9S1R and 124R were added to the wells. Control conditions included untreated cells, and 100mM sodium azide. At 2h, 24h, and 48h a single reagent Cell Titer Glow™ (Promega,Madison) was added to the wells (v/v ratio with media), and ATP determined with emission reading using the SynergyMx plate reader. The statistical analysis was done with the GraphPadPRISM*. Results are as mean ± SE (standard error) of three different experiments. NS, not significant. ***p<0.05, **p<0.01, ***p<0.001

(A), LnCap, at **a** 2h, **b** 24h, and **c** 48h. (B) MDA-MB-231 at **a** 2h, **b** 24h, and **c** 48h.

(A) LnCap



(B) MDA-MB-231

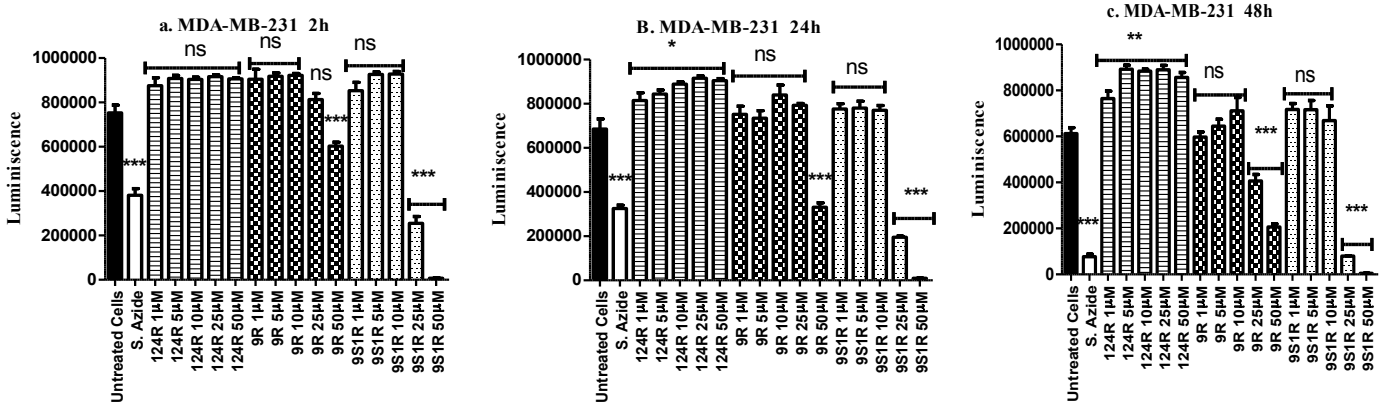
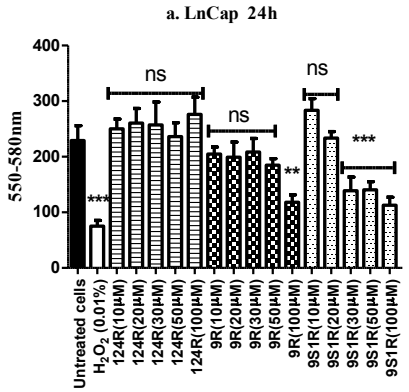


Fig. 3. PolyArgNulloPs effects on MMP. LnCap cells (3000/well) were seeded in 96 well plates (white plates from Nunc). After 24 h incubation peptides 9R, 9S1R and 124R were added to the wells. Control conditions included untreated cells, and H2O2 (0.01%) treated cells. At 24h, 50 µl of mitochondria staining solution (Mito Health) was added per well (following manufacturer's protocol) and incubated for 30 mn. Relative fluorescence was measured in BioTek Synergy plate reader. The statistical analysis was done with the GraphPadPRISM*. Results are as mean ± SE (standard error) of three different experiments.

NS, not significant. *p<0.05, ***p<0.01, ***p<0.001

(a), LnCap, at 24h. (b) MDA-MB-231, at 24h.

(a)



(b)

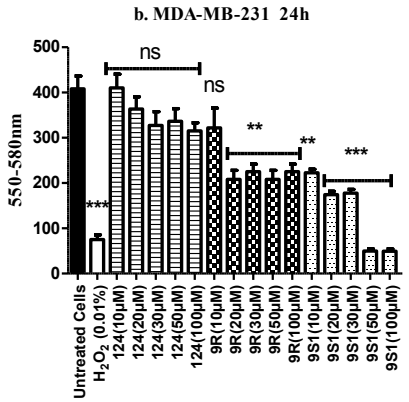
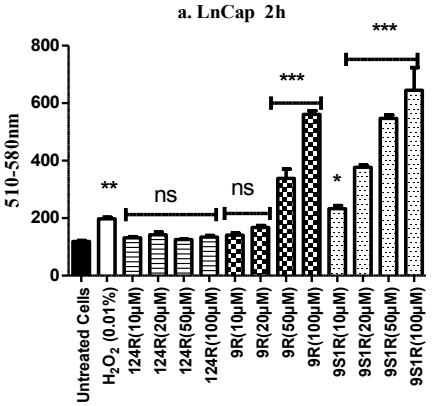


Fig. 4. PolyArgNulloPs effects on ROS production. LnCap cells (3000/well) were seeded in 96 well plates (white plates from Nunc). After 24 h incubation peptides 9R, 9S1R and 124R were added to the wells. Control conditions included untreated, and H_2O_2 (0.01%) treated cells. After 2h incubation, cells were treated with MitoSox red reagent for 10 mn, then washed with PBS, and the plate read in the microplate BioTek Synergy reader. The statistical analysis was done with the GraphPadPRISM*. Results are as mean ± SE (standard error) of three different experiments NS, not significant. *p<0.05, ***p<0.01, ***p<0.001

(a), LnCap, at 2h. (b) MDA-MB-231, at 2h.

(a), LnCap



(b) MDA-MB-231

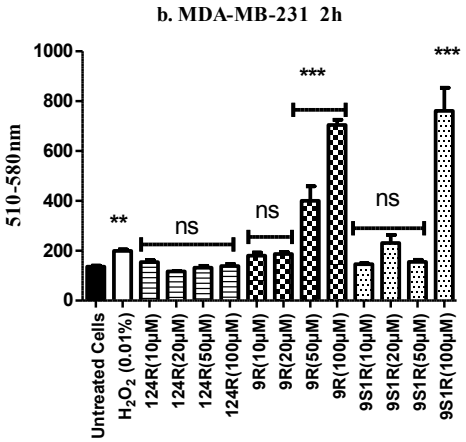


Fig. 5. Hemolytic activity of PolyArgNulloPs. RBCs collected from healthy donors were diluted 1:10 in PBS, and peptides 9R, 9S1R and 124R were added. Control conditions included PBS, and 1%Triton X-100. After 24h incubation at 37ºC with 100 rpm shaking, RBCs were centrifuged and the supernatant collected in a 96 well plates. Absorbance was determined at 405 nm. The hemolytic activity in % was calculated using the formula: Hemolytic activity in %={[A_{Sample}-$A_{Control}$]/[A_{Total}-$A_{Control}$]}x100

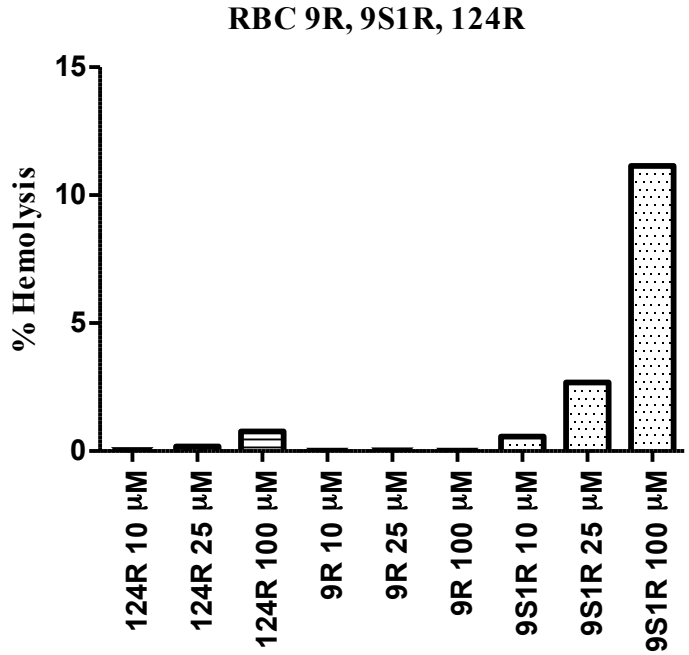


Table 1. IC-50 (μM) Evolution. The IC-50 were calculated from the raw data of MTT readings at 570nm using the GraphPad PRISM* version 5.03. Dose response curves generated by non linear regression with GraphPad PRISM* were used to determine the IC-50s. Basically the x values from the y=f(x) function were transformed into Log10 scale. The y values normalized between 0% and 100% effects and IC-50 calculated. All results are expressed as mean ± SE (standard error). A *p-value* of less than 0.05 was considered statistically significant.

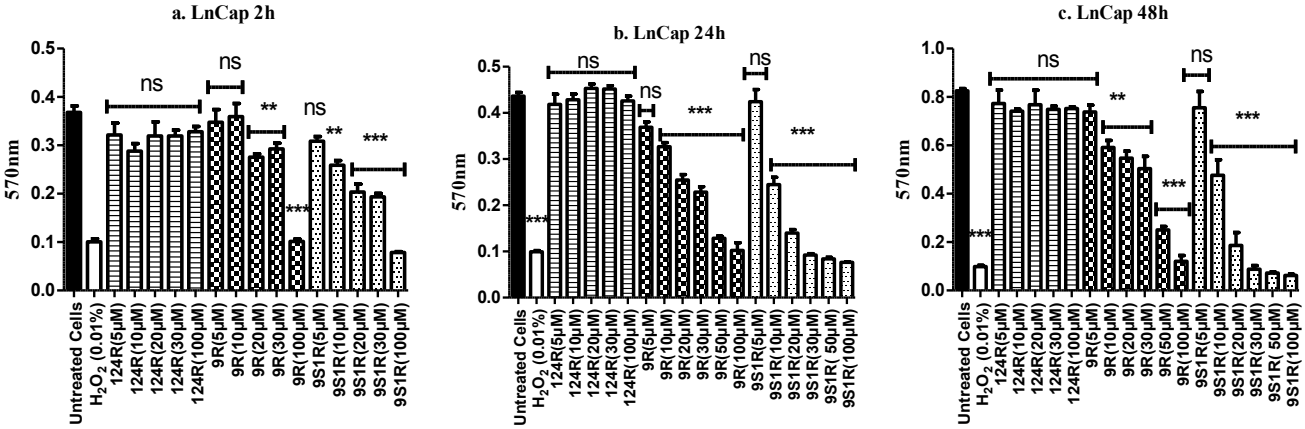
>100* when there is no effects on cell growth for doses range used (10-100 μM).

Table 1: IC-50 (μM) Evolution

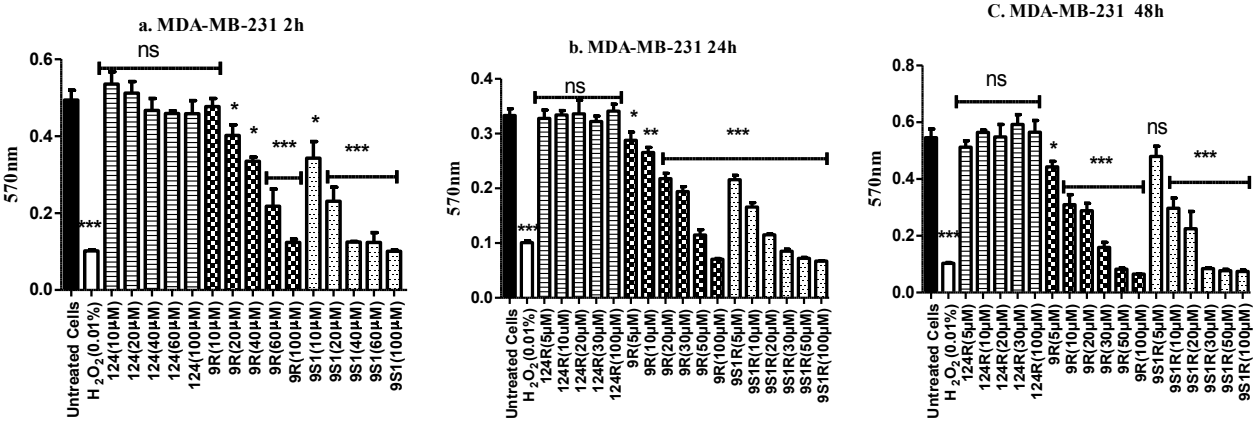
Cell Line	Time (h)	Peptide		
		9R	**9S1R**	**124R**
LnCap	**2h**	**44±2.5**	**26±1.3**	**>100***
	24h	**23±1.2**	**9±0.6**	**>100**
	48h	**28±1.6**	**8±0.5**	**>100**
MDA-MB-231	**2h**	**39±1.8**	**18±0.8**	**>100**
	24h	**29±1.7**	**12±0.4**	**>100**
	48h	**16±0.9**	**10±0.3**	**>100**
PCS	**24h**	**28±1.1**	**26±1.2**	**>100**
	48h	**35±1.3**	**22±0.9**	**88±2.3**
HMEC	**24h**	**20±0.8**	**17±0.8**	**29±1.1**
	48h	**34±1.2**	**19±0.6**	**46±2.2**
HUT102	**2h**	**93±2.3**	**38±1.4**	**>100**
	24h	**39±1.1**	**43±1.4**	**>100**
	48h	**37±2.5**	**43±3.2**	**>100**
	72h	**36±1.8**	**45±2.2**	**>100**
	96h	**25±1.6**	**36±1.4**	**>100**
J774A.1	**24h**	**47±2.5**	**26±1.3**	**66±3.5**
	96h	**12±0.7**	**17±1.2**	**31±1.5**

Suppl.R1. Effect of PolyArgNulloPs on cell growth. 3000-5000 cells/well were seeded in 96-well plates. After 24h incubation, peptides 9R, 9S1R and 124R were added to the wells. Control conditions included untreated, and H2O2 (0.01%) treated cells. Viability of cells was determined with an MTT assay at different time points. (A) LnCap at **a** 2h, **b** 24h, and **c** 48h. (B) MDA-MB-231, at **a** 2h, **b** 24h, and **c** 48h. (C) B16, at **a** 2h, **b** 96h. (D) HUT 102, at **a** 2h, **b** 48h, **c** 72h and **d** 96h. (E) J774A.1, at **a** 2h, **b** 48h, **c** 72h and **d** 96h. (F) WI-38, at **a** 24h, **b** 48h. (G) PCS, at **a** 24h, **b** 48h. (H) HMEC, at **a** 24h, **b** 48h. Results are as mean ± SE (standard error) of three different experiments. NS, not significant. *$*p$<0.05, **$**p$< 0.01,***$***p$<0.001

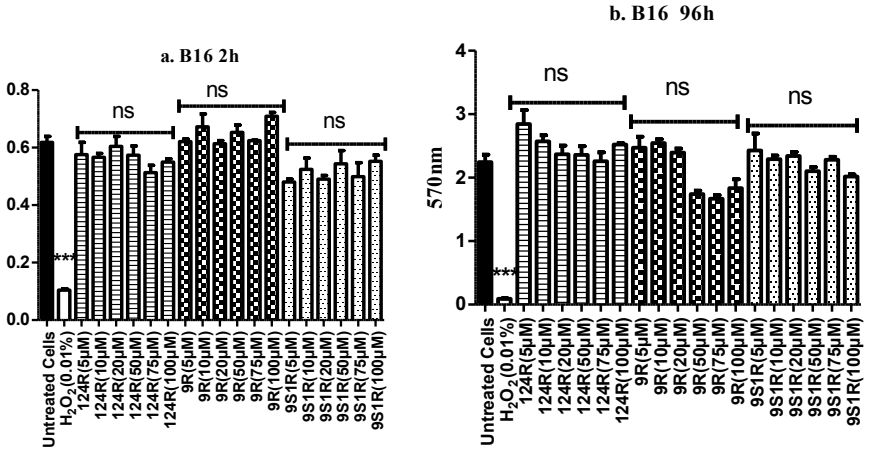
(A) LnCap



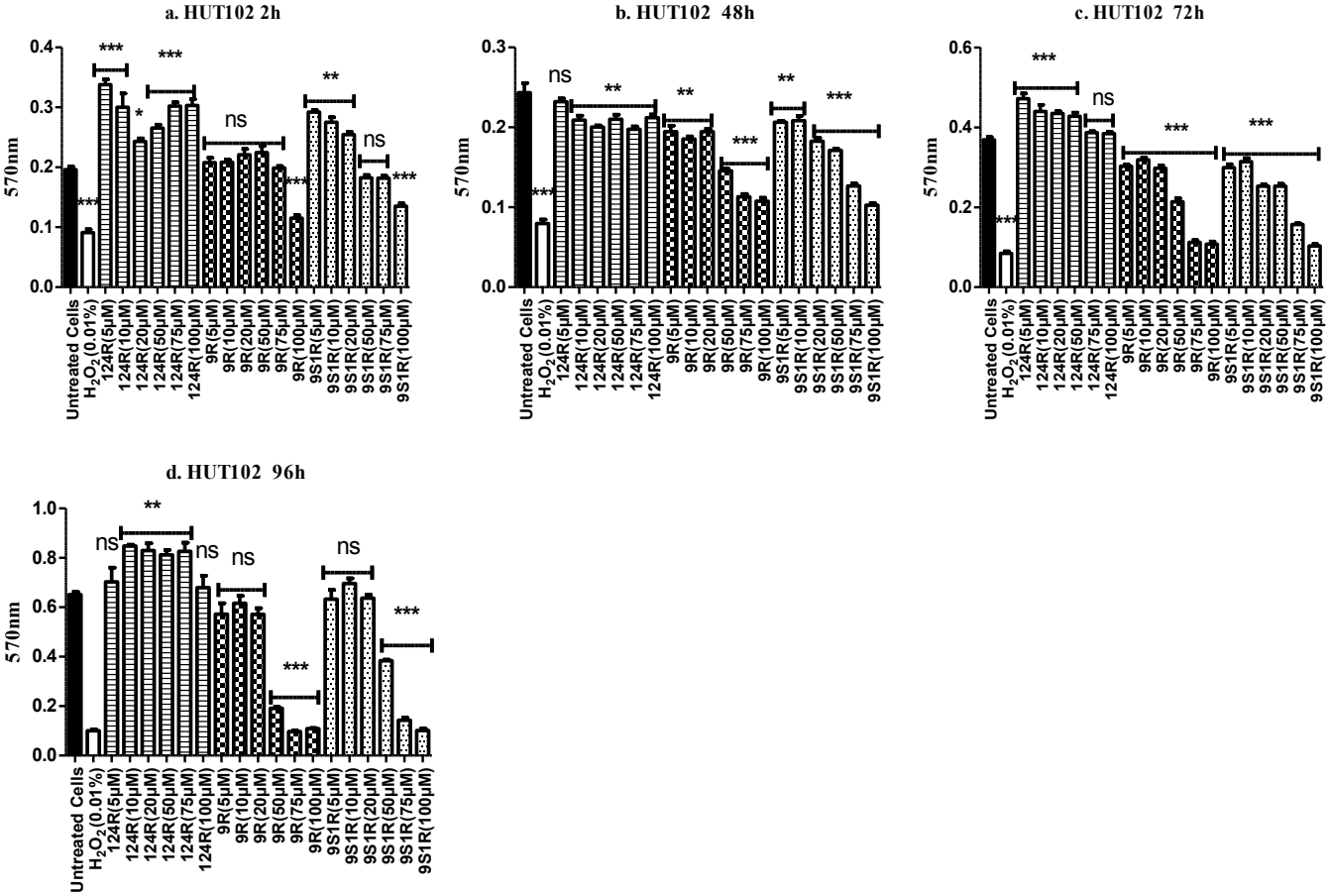
(B) MDA-MB-231



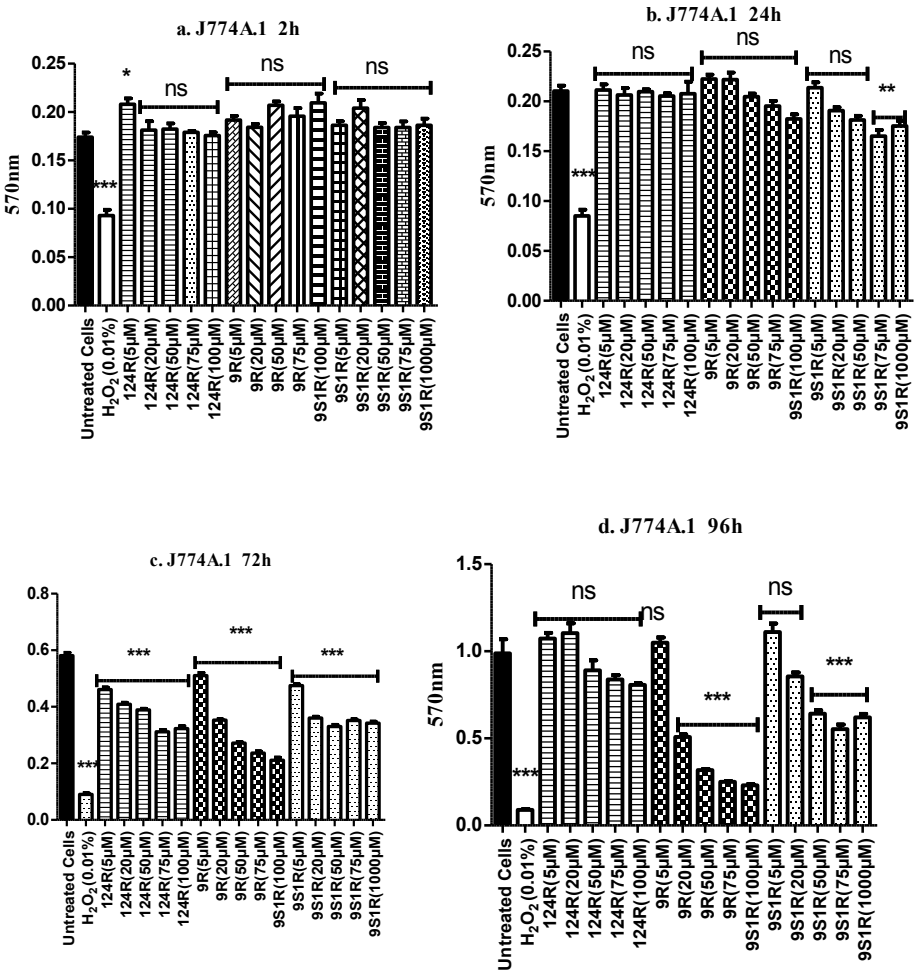
(C) B16



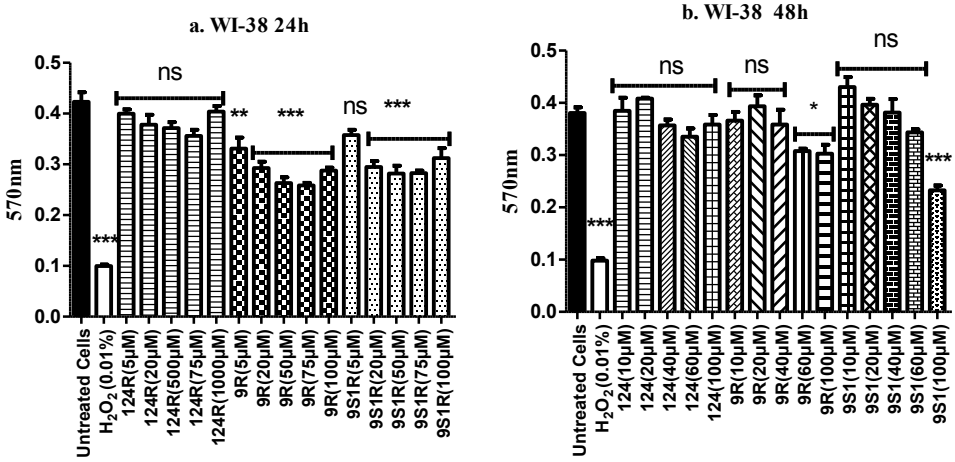
(D) HUT 102



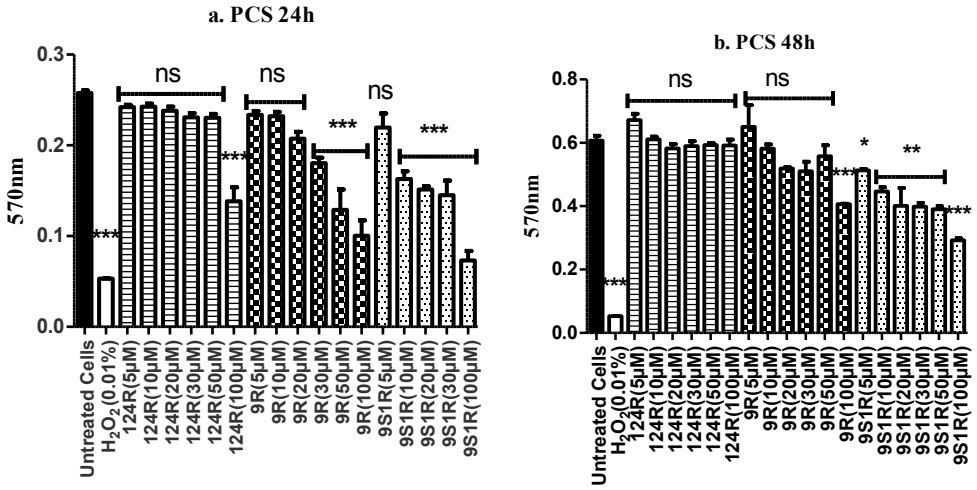
(E) J774A.1



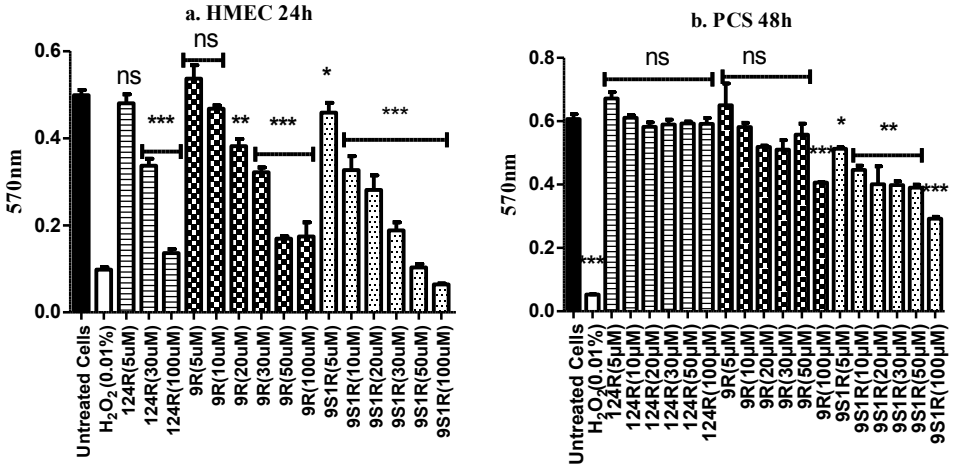
(F) WI-38



(G) PCS

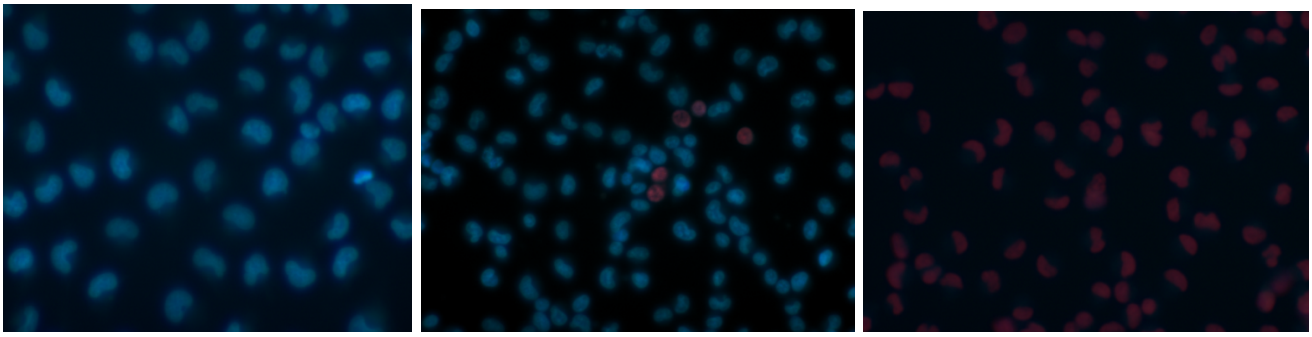


(H) HMEC

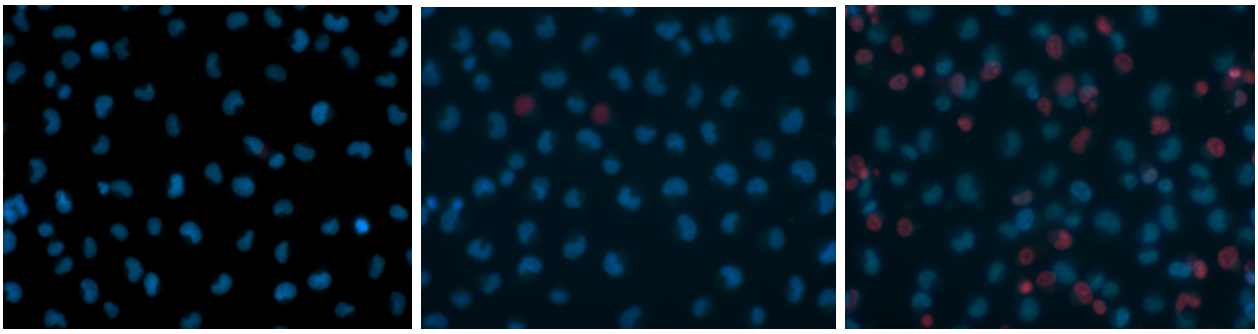


Suppl.R2. PolyArgNulloPs induced cell death (Live/Dead assay). LnCap cells (3000/well) were seeded in 96 well plates (white plates from Nunc). After 24 h incubation peptides 9R, 9S1R and 124R were added to the wells. Control conditions included untreated, and H_2O_2 (0.01%) treated cells. After 2h incubation, Hoechst 33342 and PI dyes were added to the cells, and fluorescent images were taken with a Zeiss axiovert 40 microscope (Oberkochen, Germany) with SPOT imaging camera (Sterling Heights, MI). (A) LnCap. (B) MDA-MB-231

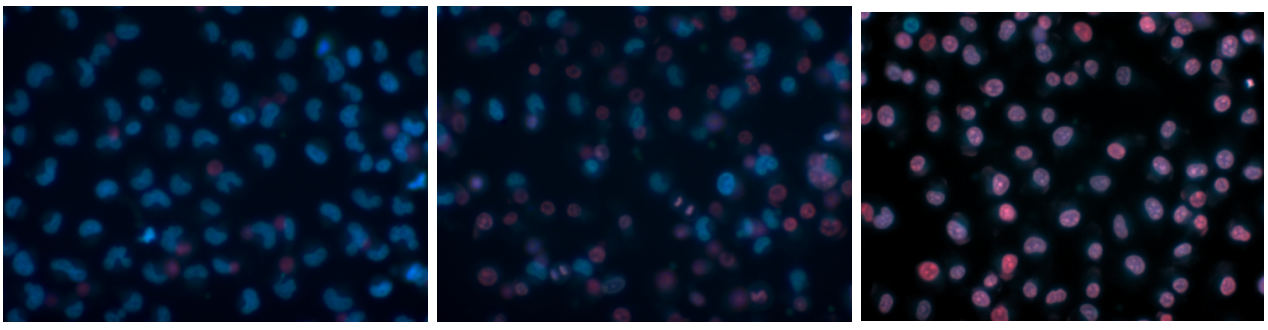
(A) Ln Cap



LnCap Untreated LnCap 124R 100 µM LnCap H_20_2 0.01%

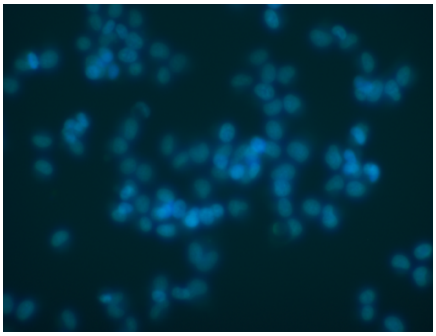


LnCap 9R 10 µM LnCap 9R 50 µM LnCap 9R 100 µM

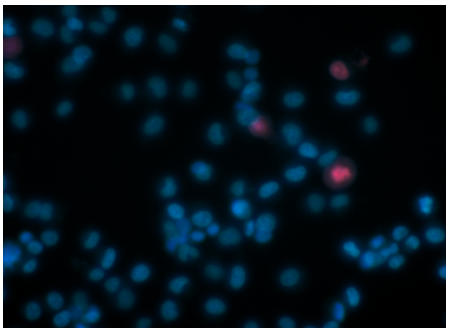


LnCap 9S1R 10 µM LnCap 9S1R 50 µM LnCap 9S1R 100 µM

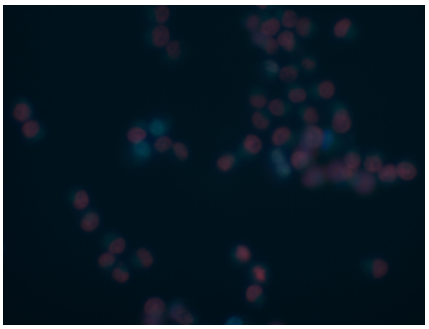
(B) MDA-MB-231



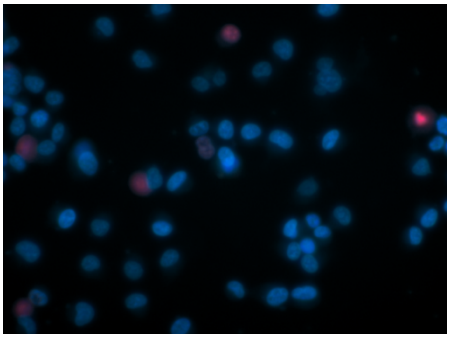
MDA-MB-231 Untreated



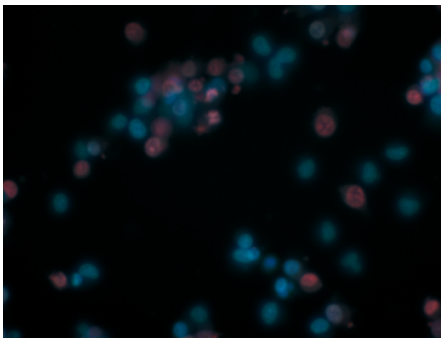
MDA-MB-231 124R 100 μM



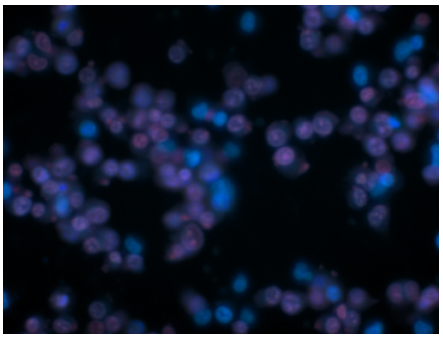
MDA-MB-231 H_20_2 0.01%



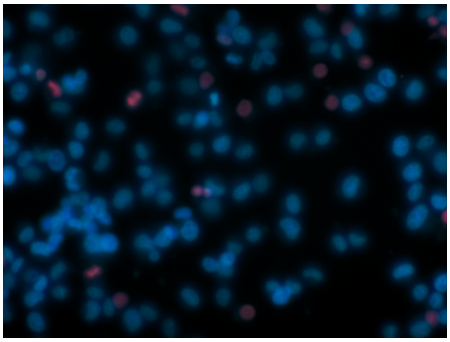
MDA-MB-231 9R 10 μM



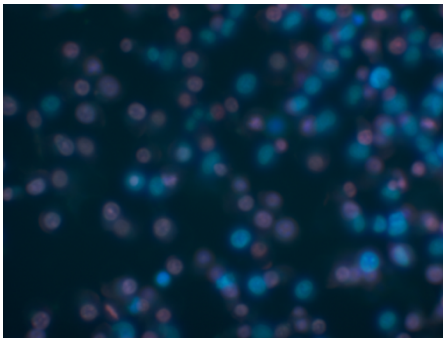
MDA-MB-231 9R 50 μM



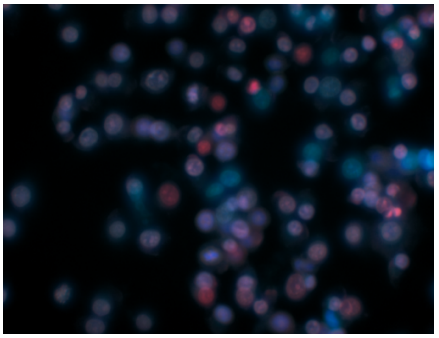
MDA-MB-231 9R 100 μM



MDA-MB-231 9S1R 10 μM



MDA-MB-231 9S1R 50 μM



MDA-MB-231 9S1R 100 μM

References

[1] Acquisti C, Poste G, Curtiss D, Kumar S. Nullomer: really a matter of natural selection? PLoS One 2007; 2: e1022.

[2] Alileche A. Interleukin-2 and cancer: critical analysis of results, problems and expectations Med Hypotheses 2003; 60: 202-208.

[3] Alileche A, Serfass ER, Muehlbauer SM, Porcelli SA, Brojatsch J. Anthrax lethal toxin mediated killing of human and murine dendritic cells impairs the adaptive immune system PLoS Pathogens 2005; 1: e19.

[4] Alileche A, Squires RC, Muehlbauer SM, Lisanti MP, Brojatsch J. Mitochondrial impairment is a critical event in anthrax lethal toxin induced cytolysis of murine macrophages Cell Cycle 2006; 5:100-106.

[5] Bari K, Petrik J, Rumora L. "Biochemistry of apoptotic cell death" Acta Pharm 2003; 53: 151–164.

[6] Bensinger SJ, Christofk HR. Review: New aspects of the Warburg effect in cancer cell biology Sem in Cell & Devlpt Biol 2012; 23:352-361.

[7] Capone G, Novello G, Fusano C, Trost B, Bickis M, Kusalik A et al. The oligodeoxynucleotide sequences corresponding to never expressed peptide motifs are mainly located in the non coding strand BMC Bioinformatics 2010; 11: 383.

[8] Cyr L, Langler R, Lavigne C. Cell cycle arrest and apoptosis responses of human breast epithelial cells to the synthetic organosulfur compound *p*-Methoxyphenyl *p*-Toluenesulfonate Anticancer Res 2008; 28: 2753-2764.

[9] Dhar S, Nygren P, Csoka K, Botling J, Nilsson K. Anti cancer drug characterization using a human cell line panel representing defined types of drug resistance Br J of Cancer 1996; 74: 888-89.

[10] Droin N, Hendra JB, Ducoroy P, Solary E. Human defensins as cancer biomarkers and antitumor molecules J Proteomics 2009; 72: 918-927.

[11] D'Souza GMG, Wagle MA, Saxena V, Shah A. Approaches for targeting mitochondria in cancer therapy Bioch Biophys Acta 2011; 1807: 589-696.

[12] Fantin VR, Leder P. Mitochondriotoxic compounds for cancer therapy Oncogene 2006; 25: 4787-4797.

[13] Fawell S, Seery J, Daikh Y, Moore C, Chen LL, Pepinsky B et al. Tat mediated delivery of heterologous proteins into cells PNAS US 1994; 91; 664-668.

[14] Ferlay J, Bray F, Pisani P, Parkin DM. eds GLOBOCAN 2002: Cancer Incidence, Mortality and Prevalence Worldwide. Lyon France IARC Press 2004.

[15] Fischer M, Waizeneggar T, Kohler K, Brock R. A quantitative validation of fluorophore-labelled cell-permeable peptide conjugate Biochim Biophys Acta 2002; 1564: 365-374.

[16] Fulda S, Kroemer G. Mitochondria as therapeutic targets for the treatment of malignant disease Antioxidants& Redox Signaling 2011; 15: 2937-2949.

[17] Garcia SP, Pinho AJ. Minimal absent words in four human genome assemblies PLoS One 2011; 6: e29344.

[18] Georgiadis MS, Russell EK, Gazdar AF, Johnson BE. Paclitaxel cytotoxicity against human lung cancer cell lines increases with prolonged exposure durations Clin Cancer Res 1997; 3: 49-44.

[19] Hagemeister FB. Treatment of relapsed aggressive lymphomas: regimen with and without high dose therapy and stem cell rescue Cancer Chemother Pharmacol 2002; 49 (suppl.1): S13-S20

[20] Hallbrink M, Floren A, Elmquist A, Pooga M, Bartfai T, Langel U. Cargo delivery kinetics of cell-penetrating peptides Biochim Biophys Acta 2001; 1515: 101-109.

[21] Hampikian G, Andersen T. "Absent Sequences: Nullomers and Primes" Pac Symp Biocomput 2007; 12: 355-366.

[22] Hansson Petersen CA, Alikhani N, Behbani H, Wiehager B, Pavlov PF. Alafuzoff I, et al. The amyloid beta-peptide is imported into mitochondria via the TOM import machinery and localized to mitochondrial cristae PNAS US 2008; 105: 13145-13450.

[23] Hilchie AL, Douctte CD, Pinto DM, Patrzykat A, Douglas S. Hoskin DW. Pleurocidin-family cationic antimicrobial peptides are cytolytic for breast carcinoma and prevent growth of tumor xenografts Breast Cancer Res 2011; 13:R102.

[24] Hyot MT, Palchaudhuri R, Hergenrother PJ. Cribrostatin 6 induces death in cancer cells through a reactive oxygen spieces (ROS)-mediated mechanism Invest New Drugs 2011; 29: 562-573.

[25] Izutsu KI. Stabiliztion of therapeutic proteins by chemical and physical methods Methods In Molecular Biology Vol 308, Humana Press Eds 2005 Chapter 22: p287-292.

[26] Koji I, Hiroyuki T. Protective Effect of Benidipine Against Sodium Azide-Induced Cell Death in Cultured Neonatal Rat Cardiac Myocytes J Pharmacol Sci 2003; 93: 163 –170.

[27] Kondo T, Setoguchi T, Tga T. Persistence of a small subpopulation of cancer stem like cells in the C6 glioma cell line PNAS US 2004; 101: 781-786.

[28] Ko YH, Smith BL, Wang Y, Pomper MG, Rini DA, Torberson MS, et al. Advanced cancers: eradication in all cases using 3-bromopyruvate therapy to deplete ATP Biochem Biophs Res Comm 2004, 324: 269-275.

[29] Kroemer G. Introduction: mitochondrial control of apoptosis Biochimie 2002; 84: 100-104.

[30] Kumar M, Roy J, Roy I. Effects of trehalose on protein structure Protein Science 2008; 18: 24-36.

[31] Lucchese G, Stufano A, Trost B, Kusalik A, Kanduc D. Peptidology: short amino acids modules in cell biology and immunology Amino Acids 2007; 33: 703-707.

[32] Lopes NM, Adams EG, Pitts TW, Bhuyan BK. Cell kill kinetics and cell cycle effects of Taxol on human and hamster ovarian cell lines Cancer Chemother Pharmacol 1993; 32: 235-242.

[33] Liebmann JE, Cook JA, Lipschultz C, Teague D, Fisher J, Venzon D, et al. Cytotoxic studies of paclitaxel (Taxol) in human tumor cell lines Br J Cancer 1993; 68: 1104-1109.

[34] Melikov K, Chernormordik V. Arginine rich cell penetrating peptides: from endosomal uptake to nuclear delivery Cell Mol Life Sci 2005; 62: 2739-2749.

[35] Min-Wen H, Ke-Zuo H, Yun-Peng L, Yuan Y. Effect of staurosporine on cycle human gastric cancer cell World J Gastroenterol 2004; 10: 161-166.

[36] Mishra A, Gordon V, Yang L, Coridan R, Wong G. HIV TAT forms pores in membranes by inducing saddle-splay curvature: potential role of bidentate hydrogen bonding Angew Chem Int Ed Engl 2008; 47: 2986-2989.

[37] Nakase I, Takeuchi T, Tanaka G, Futaki S. Methodological and cellular aspects that govern the internalization mechanisms of arginine rich cell penetrating peptides Advanced Drug Delivery Rev 2008; 60: 598-607.

[38] Nor I'zzah W, Zain VM, Rahmat B, Othman F, Yun Hin Yap T. Antiproliferative properties of clausine-B against cancer cell lines Malaysian J of Medical Sciences 2009; 16: 31-36.

[39] Papo N, Shai Y. Host defense peptides as new weapons in cancer treatment Cell Molec Life Sci 2005; 62: 784-790.

[40] Paull KD, Shoemaker RH, Hodes L, Monks A, Scudiero DA, Rubinstein L, et al. Display and analysis of patterns of differential activity of drugs against human tumor cell lines: Development of mean graph and COMPARE algorithm J Natl Cancer Inst 1989; 81: 1088-1092.

[41] Perez-Tomas R. Multidrug resistance: retrospect and prospects in anti-cancer drug treatment Current Med Chem 2006; 13: 1859-1875.

[42] Reches M, Porat Y, Gazit E. "Amyloid Fibril Formation by Pentapeptide and Tetrapeptide Fragments of Human Calcitonin" J of Biol Chem 2002; 277: 35475–35480.

[43] Ross MF, Filipovska A, Smith RAJ, Gait MJ, Murphy MP. Cell penetrating peptides do not cross mitochondrial membranes even when conjugated to a lipophilication: evidence against direct passage through phospholipid bilayers Biochem J 2004; 383: 45-468.

[44] Rowinsky EK, Donehower RC, Jones RC, Tucker RW. Microtubule changes and cytotoxicity in leukemic cell lines treated with Taxol Cancer Res 1988; 48: 4093-4100.

[45] Schmidt N, Mishra A, Lai GH, Wong GCL. Arginine rich cell penetrating peptides FEBS Letters 2010; 584: 1806-1813.

[46] Sharma SV, Haber DA, Settleman J. Cell line-based platforms to evaluate the therapeutic efficacy of candidate anticancer agents Nature Rev Cancer 2010; 10:241-252.

[47] Skipper HE, Shabel FM Jr, Mellett HH, Brockman RW. Implications of biochemical, cytokinetic, pharmacologic and toxicolgic relationships in the design of optimal therapeutic schedules Cancer Chemother Rep 1970; 54: 431-450.

[48] Somalarczyk R, Cichon T, Kamyzs W, Glowala-Kosinska M, Szydlo A. Lukasz S, et al. Anticancer effects of CAMEL peptide Laboratory Invest 2010; 90: 940-952.

[49] Steinstraesser L, Hauk J, Schubert C, Al-Benna S, Stricker I, Hatt H, et al. Supression of soft tissue sarcoma growth by a host defense like lytic peptide PLoS One 2011; 6: e18321.

[50] Tang C, Shao X, Sun B, Huang W, Qin F, Chen Y et al. Anticancer mechanism of peptide P18 in human leukemia K562 cells Org Biomol Chem 2010; 8: 984-987.

[51] Vives E, Brodin P, Lebleu B. A truncated HIV-1 TAT protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus J Biol Chem 1997; 272: 16010-16017.

[52] Wender PA, Mitchell DJ, Pattabiraman K, Pelkey ET, Steinman L, Rothbard JB. The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: Peptoid molecular transporters PNAS US 2000; 97: 13003-13008.

[53] Wiesner J, Vilcinskas A. Antimicrobial peptides: the ancient arm of the human immune system Virulence 2010; 1: 440-464.

[54] Xu RH, Pelicano H, Zhou Y, Carew JS, Feng L. Bhalla KN, et al. Inhibition of glycolysis in cancer cells: a novel strategy to overcome drug resistance associated with mitochondrial respiratory defect and hypoxia Cancer Res 2005; 65: 613-621.

[55] Yamori T. Panel of human cancer cell lines provides valuable database for drug discovery and bioinformatics Cancer Chemother Pharmacol 2003; 52 (Suppl. 1): S74-S79.

[56] Yang D, Lin ZH, Tewary P, Chen Q, De La Rosa G. Oppenheim JJ. Defensin participation in innate and adaptive immunity Curr Pharm Dis 2007; 13: 3131-3139.

[57] Zhang P, Gao WY, Turner S, Ducatman BS. Gleevec (STI-571) inhibits lung cancer cell growth (A549) and potentiates the cisplatin effect in vitro BMC Molecular Cell 2003; 2: 1-9.

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Abdelkrim Alileche-Devised solubility protocols, designed and performed experiments, analyzed results, and was the lead author in writing the paper.

Jayita Goswami-Designed and performed of experiments, analyzed results, contributed in writing the paper.

William Bourland-SEM preparation, imaging and analysis.

Michael Davis-Contributed to experimental design and analysis of results.

Greg Hampikian-Devised nullomer approach, designed experiments, analyzed results, contributed in writing paper.

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Short report

Safeguarding forensic DNA reference samples with nullomer barcodes

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ABSTRACT

Unintended transfer of biological material containing DNA is a concern to all laboratories conducting PCR analysis. While forensic laboratories have protocols in place to reduce the possibility of contaminating casework samples, there is no way to detect when a reference sample is mislabeled as evidence, or contaminates a forensic sample. Thus there is public concern regarding the safeguarding of DNA submitted to crime labs. We demonstrate a method of introducing an internal amplification control to reference samples, in the form of a nullomer barcode which is based upon sequences absent or rare from publically accessible DNA databases. The detection of this barcode would indicate that the source of analyzed DNA was from a reference sample provided by an individual, and not from an evidence sample. We demonstrate that the nullomers can be added directly to collection devices (FTA paper) to allow tagging during the process of sample collection. We show that such nullomer oligonucleotides can be added to existing forensic typing and quantification kits, without affecting genotyping or quantification results. Finally, we show that even when diluted a million-fold and spilled on a knife, the nullomer tags can be clearly detected. These tags support the National Research Council of the National Academy recommendation that "Quality control procedures should be designed to identify mistakes, fraud, and bias" in forensic science (National Academy of Sciences, 2009).

1. Introduction

Unintended transfer of biological samples is an issue of great concern to all laboratories conducting sensitive analyzes. This is particularly true for crime laboratories, where victims, suspects, and even investigators are asked to supply reference samples of their DNA for comparison to evidence profiles. Contamination, or unintended transfer of DNA, can happen at any time in the process of forensic DNA analysis. Instances of unintended transfer and mislabeling by personnel collecting and processing samples, while uncommon, have been documented,[1–3] and concern about supplying reference samples has been raised by individuals and groups including those representing police officers in Connecticut and Missouri.[4,5] This problem is likely to become more pronounced as forensic DNA techniques become ever more sensitive, and as databases grow with the increasing use of forensic DNA.[6] While forensic DNA analysis is considered the "gold standard" of forensic science practice, there is the expectation that the best possible practices of regulation and oversight be implemented. With regard to the forensic sciences in general, the 2009 report by the National Research Council of the National Academy recommends that "Quality control procedures should be designed to identify mistakes, fraud, and bias" as well as "confirm the continued validity and reliability of standard operating procedures and protocols."[1] With this in mind, while recognizing that laboratory mistakes and mishandling are rare events, we present a means by which to provide an additional "safeguard" for reference samples used in forensic DNA analysis, employing sequences known to be rare or absent in nature[7,8] as internal controls. These sequences (known as nullomers) could be used to both verify sample origin (as a reference sample, or any sample not collected from a crime scene) and as way to potentially track contamination if it occurs.

Studies have shown the propensity of DNA to be deposited by individuals via the mere touching of objects, such as DNA from fingerprints[9] or the secondary transfer of touch-deposited DNA.[9–11] Ladd et al. (1999) found an average of 1–15 ng DNA recovered from touched objects (dependent on the object).[10] Assuming that 1 ng of genomic DNA is the equivalent of genomes from ~170 cells,[12] the swabs in the experiments of Ladd et al. picked up the DNA from ~170 to ~2500 cells. With modern techniques that can produce

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profiles from a few or even single cells,[13] it is expected that the detection of DNA from secondary and tertiary transfer will become more common. In the context of clinical genetic testing, a case of DNA contamination has been reported, and routine forensic STR typing was recommended to ensure that all DNA samples are truly from a single individual.[14] Contamination of samples with amplified products has also been a concern, recognized since the early days of PCR.[15] Laboratories which use enhanced techniques for low template DNA analysis have detected contamination in reagents, plastic ware and laboratory samples at levels that are below detection with less sensitive methods.[16] While most crime labs have protocols in place to prevent PCR products from coming in contact with pre-amplified DNA samples, accidents can happen. Unfortunately, it is not presently possible to detect when a reference sample contaminates an evidentiary sample. A specific incident mentioned in the *Final Report of the Independent Investigator for the Houston PD Crime Lab* documents case in which a re-analysis indicated "that the victim's reference sample was contaminated at some point in the handling of this sample."[3] The independent investigator concluded that the contamination probably occurred after extraction, and happened "most likely at the PCR amplification stage".[3]

In order to safeguard against the accidental transfer or contamination of DNA samples collected from members of the public, we have developed a unique type of internal amplification control (IAC) that can serve as a universal tag and barcode and can be modified to further encode a variety of information, such as laboratory location, testing purpose, or date. These tags are based on the smallest sequences absent from all publicly available DNA databases (nullomers), first described by Hampikian and Andersen,[7] and nullomer technology is also being used to design small peptide drugs.[8] These nullomer IACs (also designated "nullomer barcodes") can be embedded in collection devices such as swabs and FTA paper, thus allowing DNA samples to be tagged at the earliest stage in the DNA analysis process. The nullomer approach has generated recent interest in algorithms for counting and tracking biological sequences.[17,18] This paper describes, to the best of our knowledge, the first practical forensic application of nullomer sequences. Our results show that nullomer sequences can be used as an IAC, and as molecular tags and barcodes, successfully integrated into the multiplex PCR reactions of commercially available forensic profiling kits, and used along with PCR for sequencing. The use of IACs (sometimes designated internal positive controls, IPCs) is already commonplace for food and clinical microbiological testing,[19] forensic quantification kits,[20] forensic human identification kits,[21–23] and are particularly useful for detecting PCR inhibitors.[24] For PCR-based tests of food-borne pathogens, the European Standardization Committee has developed guidelines that require the presence of an IAC.[25] The nullomer approach described here is unique in two important ways: it can be used to distinguish between reference and evidentiary samples, and the tags are designed through an algorithm which identifies small sequences absent from the public databases of all sequenced organisms.[8]

2. Methods

2.1. Nullomer sequences and primer design

The algorithm of Hampikian and Andersen[7] is able to process the entire set of biological sequence data found on NCBI's website in less than 8 h, calculating the frequencies of all sequences up to length 17 (longer lengths can be calculated by our methods as well). Basically, all possible sequences up to a given length are generated, and each sequence is compared to those in the databases; any sequences that are not found in the databases are listed as nullomers. At length 17 there are (as of January 2011) approximately 700 million (695,038,288) absent sequences in the NCBI data bases.

From a list of absent 15-mers, we concatenated eight sequences to form a 120 bp nullomer "tag". Several permutations of eight 15-mers were analyzed via the Oligoanalyzer tool available at the Integrated DNA Technologies (IDT) website (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/). Sequences were chosen to minimize the formation of secondary structure, and primer-binding regions were chosen so that the annealing temperature would match as closely as possible the 59 °C annealing temperature of the ABI kit protocols (58 °C for Powerplex™ Y kit). Most nullomer sequences have a high GC content; therefore the list of potential 15-mers was reduced to those having a GC content in the 40–60% range. However, since the primer binding region of the sequence is the determining factor in PCR specificity, the internal sequence is amenable to sequence modification, allowing nullomer tags to be used for DNA "cryptography" or barcodes. The sequences of the barcode primers used are shown in the supplementary material. The 120 bp construct was synthesized by IDT (Coralville, IA, USA) as two complimentary single stranded molecules. This complimentary pair was annealed to make a double stranded oligomer. After annealing, remaining single stranded molecules were removed with ExoSAP™ enzyme treatment. Concentration of nullomers (copies/μL) was calculated by measuring ds DNA concentration on a NanoDrop spectrophotometer, and calculating expected copy number from the molecular weight of each nullomer molecule. Primers were designed to yield amplicon sizes of 88, 90, and 114 bp. Several barcode concentrations were tested with different STR kits; we present the main results using 1900 copies per PCR reactions (3800 copies used in the Identifiler™ experiment shown in Fig. 1b), which gave barcode peaks at intensities comparable to the human STR alleles. In order to visualize the amplified nullomer peak on the 3130 Genetic Analyzer, the nullomer primers were ordered from IDT with a 6-FAM modification to the 5′ end of one of the primer pair. In our experiments, we found that a rather high concentration of primers (2.5 μM) was useful for amplification of the nullomer tag such that the nullomer peaks approximated the size of normal amplicon peaks. Although higher than the standard range for PCR primers (0.1–0.5 μM), we saw no evidence for primer-dimer formation or non-specific amplification.

2.2. Co-amplification of forensic loci and nullomer tags

We designed the tag (barcode) DNA amplicons to be of a size outside the range of human STR alleles, so that there is no confusion between the barcode and known STR allele peaks. Human DNA and barcode DNA was amplified according to manufacturer protocols (except for the addition of the nullomer barcode and barcode primers) with the following forensic DNA kits: Quantifiler Duo™ (Fig. 1a), Identifiler™ (Fig. 1b, Fig. S1–S4), Profiler Plus™ (Fig. S5 and S6), Yfiler™ (Fig. S7), and PowerPlex ™ Y (Fig. S8 and S9).[26–30] To test nullomer tag compatibility with mitochondrial DNA (mtDNA) sequencing, we amplified the HV-1 and HV-2 regions of human mtDNA in the presence of nullomers, with and without tag primers. Results from the Quantifiler Duo™ test, as well as results with Identifiler™ (performed in triplicate), were analyzed with unpaired *t*-tests and a one-way ANOVA, using GraphPad Prism version 5.00 for Windows, to test for significant differences, $p \leq 0.05$.

A common technique for the storage and processing of reference samples is to transfer a buccal swab sample to FTA paper and allow it to dry. DNA samples fixed onto FTA paper can be stored at room temperature, and then be extracted from FTA paper punches when needed. We treated an FTA card with a solution of nullomer DNA in nanopure H_2O, allowed it to dry, and then transferred DNA from a buccal swab to the FTA card.

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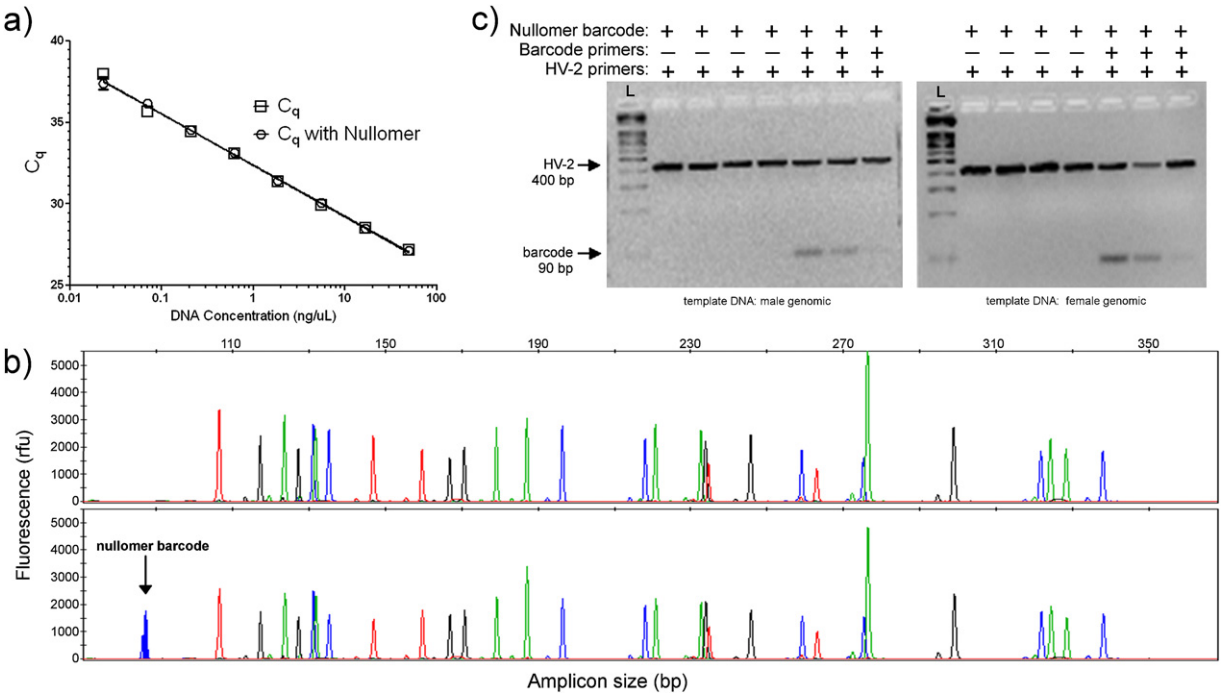

Fig. 1. a. Nullomer tag does not interfere with Quantifiler DUO™ DNA quantification. A standard curve was generated using control DNA supplied with the Quantifiler DUO™ kit from applied biosystems. The DNA standard was diluted, and the real-time PCR performed, according to the manufacturer protocol. Three sets of standards were made without the nullomer tag, and three sets were made with nullomer tag added to the reaction mix. Number of cycles to reach the quantification threshold (C_q) is shown, for DNA with (○) and without (□) 1.9×10^3 copies of nullomer barcode. Results show mean ± SEM. b. STR profile of female genomic DNA in the presence and absence of the nullomer barcode, amplified with the Identifiler™ kit. Top electropherogram, STR amplification without the nullomer barcode. Bottom electropherogram, STR amplification in the presence of nullomer barcode (3.8×10^3 copies). Size of each amplified product is given in base pairs; the locus is indicated by labels above the peaks. The *y*-axis is in RFUs, and is scaled according to maximum peak height. The nullomer peak is the smallest fragment (90 bp amplicon). c. HV2 region of mitochondrial DNA from male (left) and female (right) amplified in the presence and absence of the nullomer barcode. Mitochondrial PCR product was visualized on a 3% agarose, ethidium bromide stained gel. HV2 product amplified properly with nullomer barcode (with and without nullomer primers added to the HV2 PCR reaction).

2.3. Mock contamination experiments with nullomer-tagged reference and post-amplification samples

We setup a mock unintended transfer of Identifiler™-amplified DNA (with nullomer barcode and nullomer primers co-amplified) to a mock evidentiary weapon (knife). The amplified DNA (~0.5 μL) was allowed to dry, and the knife swabbed later with a wet sterile Omni-swab (Whatman). DNA was extracted from the swab and analyzed with the Identifiler™ kit, with nullomer primers added to the PCR reaction.

Another mock contamination experiment was performed, with barcode-tagged reference DNA mixed with a forensic DNA extraction from the surface of a plastic coffee cup lid. A nullomer tag was added to a sample of "reference DNA" extracted from the buccal swab of a volunteer (reference DNA concentration was 36 ng/μL, as measured with a Nanodrop Spectrophotometer). The reference DNA sample was augmented with ~80,000 copies of barcode (~2000 copies of nullomer barcode per μL of reference DNA), and then 1.0 μL of tagged reference sample was added to the forensic swab, prior to DNA extraction. The tagged DNA was extracted along with the forensic sample, to simulate a contamination event involving reference DNA. The nullomer primers used for this experiment generated a 113-bp amplicon.

3. Results

Adding the nullomer tag to a human buccal swab did not affect the quantification of extracted DNA (Fig. 1a). The kit used in our experiment (Quantifiler® Duo) is commonly employed by forensic laboratories to determine the DNA concentration for both the total human and male fraction (Y-chromosome) of a sample. We demonstrate that the nullomer tag does not negatively impact quantification of either total human or male fraction DNA (unpaired *t*-test, $p \leq 0.05$).

When amplified with the STR alleles of the forensic kits we tested, the 90 base pair nullomer barcode appears in the electropherogram as an additional peak outside the first "bin set" (regions where peaks from alleles from the various loci are known to occur). There were no differences in the DNA profile of individuals when barcode DNA was added. The nullomer DNA does not interfere with STR genotyping of individuals (Fig. 1b).

When we extracted and amplified DNA from nullomer-treated FTA paper, we obtained a profile which includes the barcode tag, identifying the DNA as coming from a reference source (Fig. S10), and not from evidence.

Barcode PCR products were co-amplified with mtDNA targets (when barcode primers were added to the reactions), and detected as distinct bands of ~90 bp (Fig. 1c, Fig. S11). Sanger sequencing of the amplified mtDNA was not affected by the presence of the nullomer barcode, whether the barcode was added during initial PCR using HV1 and HV2 primer sets, or if added to the sequencing reactions using HV1 or HV2 amplicons as templates (Fig. S13).

Amplification of barcode DNA along with human DNA in an Identifiler™ reaction did not adversely affect the amplification of the forensic loci, as shown by a comparison of mean peak heights (±SEM) for each allele, with and without the nullomer tag added and amplified (Fig. 2).

The analysis of the DNA collected from the mock forensic contamination event showed a clear signal of nullomer DNA, at two different dilutions. A 10^5-fold dilution of the PCR product showed

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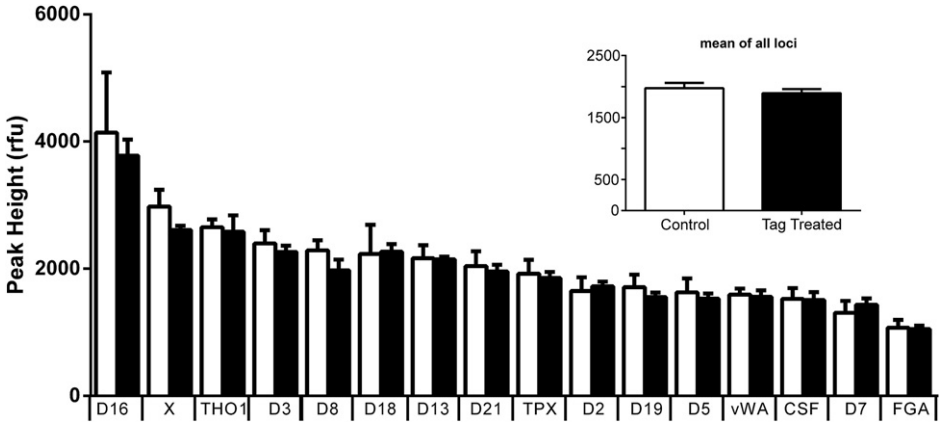

Fig. 2. Average peak heights (per locus) of human DNA amplified with the Identifiler™ kit, with and without nullomer barcode and FAM-labeled primers added. Experiment performed in triplicate. Results shown ± SEM. White bars: control. Black bars: with 3800 copies of nullomer barcode-1 added. Inset: average peak heights of all alleles. No significant differences between control and treatment peak heights, per locus, and over all loci (unpaired *t*-test, $p \leq 0.05$).

amplification of nullomer barcode DNA along with a partial profile of the transferred human amplicons (Fig. 3, top electropherogram). After a 10^6-fold dilution, the nullomer barcode could be detected; even though the human profile was lost at our signal threshold of 100 RFUs (Fig. 3, lower electropherogram, Fig. S13).

Nullomer-tagged reference DNA can be detected when it contaminates a forensic DNA sample (Fig. 4a). Allelic drop-out was observed in this experiment, as is commonly seen in amplifications of low levels of DNA, but there was no correlation with barcode treatment. The amplification of the contaminated DNA mixture was carried out 5 times with and without nullomer barcode primers. A typical example with the barcode primers is shown (Fig. 4a), and two amplifications of the same extract without barcode primers (Fig. 4b, c). Some allelic drop out occurred whether or not the nullomer barcode was amplified (Table 1), however more extensive validation will have to be performed to optimize and validate particular nullomer tags.

We have shown that an artificial DNA barcode can be used in conjunction with forensic genetic analysis kits, without affecting DNA quantification, STR amplification, profile determination, or

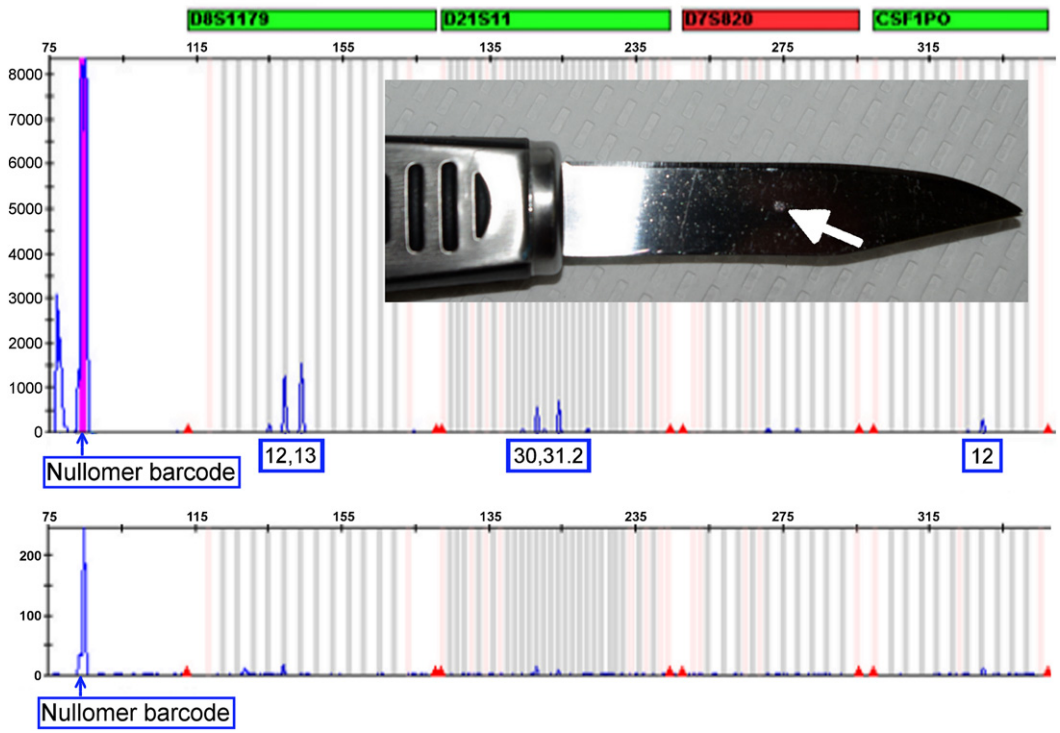

Fig. 3. Contamination of evidence with amplified DNA is detected with nullomer barcode, even when diluted 1,000,000 fold. Top panel and inset: DNA from amplified STR profile (amplified with nullomer barcode) was diluted 100,000 fold in water, and then 1ul of the dilution was applied to a newly purchased knife. This knife was swabbed, and amplified according to the STR kit manufacturer's instructions (with the addition of nullomer primers). The STR profile from the contaminated knife shows both the contaminating profile, and the nullomer barcode peak. Bottom panel electropherogram: original PCR product was diluted 1:1,000,000 in water, and 1 μL of the diluted product was added to a newly purchased knife. The knife was swabbed and processed as above. The nullomer barcode amplicon is prominent, although no alleles from the amplified human profile are detected at this dilution.

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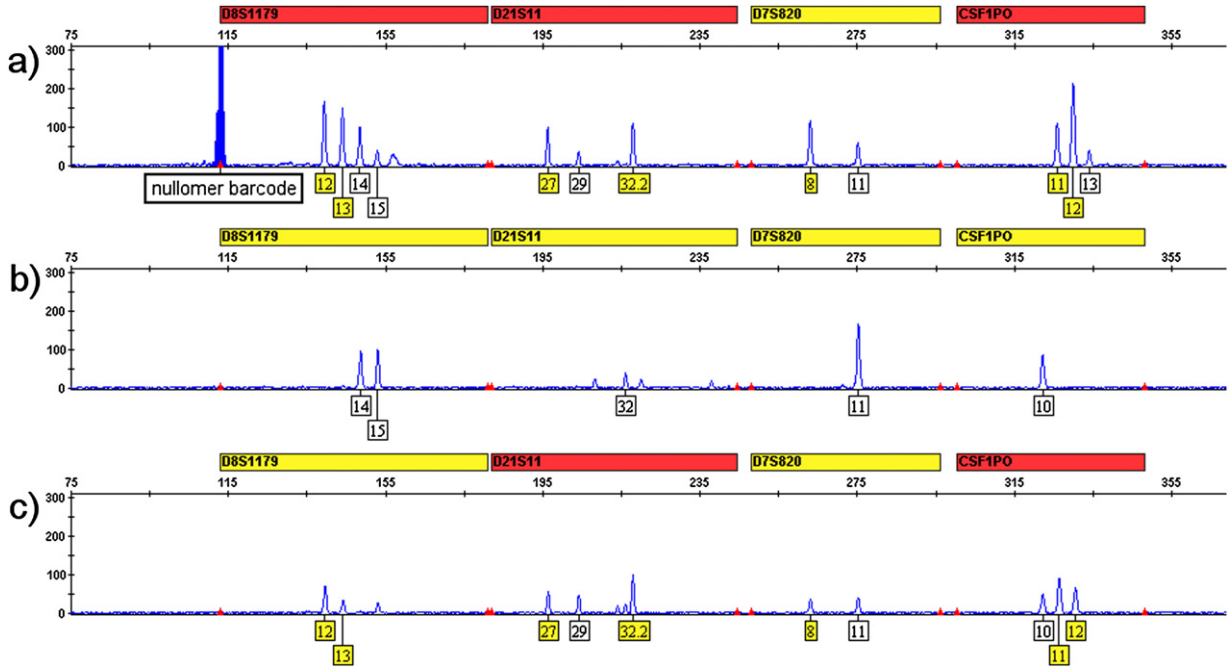

Fig. 4. Contamination of forensic DNA sample with reference DNA is detected with nullomer barcode amplification. a. Identifiler™ profile (blue channel) of DNA from coffee cup (forensic sample) that was contaminated with reference DNA. The presence of nullomer tag peak indicates that a contamination event took place. Allele numbers highlighted in yellow indicate the contaminating reference DNA. b and c. Identifiler™ profile of same mixture, except that nullomer primers were not added to the PCR reaction. Note that stochastic effects from low amount of template DNA result in allele drop-out, with or without nullomer amplification (see text for more details).

mitochondrial sequence, using standard protocols. DNA profiles were obtained by amplification of 0.5—1.0 ng of genomic DNA in the presence of 1900—3800 copies of nullomer barcodes (as indicated). We have stored the barcodes at 4 °C for one year in TE buffer without affecting amplification and detection, and used FTA paper with dried barcodes for six months without any noticeable effect on amplification.

4. Discussion

Extrinsic DNA can enter the laboratory through contaminated reagents, disposables, centrifuges, and water baths.[15,16,31] Reagent controls and routine monitoring can detect many of these examples, however, contamination of evidence with DNA from reference samples taken from suspects, or the switching of reference samples, is more difficult to detect, and may in fact implicate an innocent person in a crime.[2] The nullomer tags described in this paper were developed to assure the public that their reference samples can be marked so as to prevent false incrimination. Further development of the tag technology can be used to code individual samples to further safeguard the public.

The initial tags described here could be added to collection kits so that reference samples are "safeguarded" from the point of collection. Physical and chemical modifications of the tags could be used to stabilize them further[32]; though we demonstrate here that even unmodified synthetic DNA is sufficient.

Synthetic DNA barcoding has been used to make positive amplification controls for applications in a wide variety of fields, such as clinical microbiological testing[33] and food pathogen testing,[19] and has been proposed for a variety of tagging and tracing protocols.[34,35] Internal positive controls are already an important part of forensic genotyping and qPCR quantification kits.[20,23–30] In those kits, the IPC allows the forensic analyst to assess the presence of contaminating PCR inhibitors, such as hematin or humic acids.[25] Unlike our nullomer tagging protocol, current tags are present in the PCR reaction components, and so cannot distinguish reference from evidentiary samples. IPCs currently used in forensic kits have been designed such that the IPC sequence was checked against GenBank to ensure uniqueness—but the methods of sequence design are not always reported.[36] Non-human sequences have been used for this purpose, such as a portion of Sea Pansy (*Renilla reniformis*) luciferase gene,[37] a hydra-specific (*Hydra vulgaris*) actin gene segment,[22,23] or rat DNA.[38] In some commercial kits (e.g. the Quantifiler® Duo kit), the IPC is a trade secret and is simply designated a "synthetic polynucleotide" in the published literature.[20] Our approach of building from these small absent sequences is novel. While these sequences may eventually appear in a natural database, it is highly unlikely that concatamers based on them will—and for such a

Table 1
No significant difference in detectable alleles with or without Nullomer barcode in a low template DNA sample. Stochastic allelic drop out in 5 Identifiler runs with and without nullomer barcode primers. Results are shown for the cup mixture profiles (Fig. 4) using Identifiler, with and without barcode primers. The detection threshold of 35rfu cut-off was used for this low template DNA analysis. No significant difference between treatments was found, using χ^2 test; $p \leq 0.05$, df = 13. D7 and Amel were not used in χ^2 analysis (zero values).

# Alleles dropped out	D8	D21	D7	CSF	D3	THO1	D13	D16	D2	D19	vWA	TPOX	D18	Amel	D5	FGA
Without nullomer primers	4	6	0	5	4	2	3	5	8	4	5	3	6	0	5	4
With nullomer primes	0	4	0	5	2	4	3	3	7	4	1	1	6	0	4	5

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sequence to arise and work with our primers, and produce the proper sized amplicon, would be quite remarkable. An easy verification for checking that the peak does correspond to the correct nullomer tag would be sequencing the PCR products with barcode primers, which would reveal the exact nullomer sequence in a tagged sample. While we don't envision sequencing every reference sample, the nullomer tags could be used to trace the source of contamination. This should be a rare event, but it would be valuable to trace the origin of suspected contamination. The nullomer barcode that we have designed is 120 bases long, and since only ~1900 copies are needed per PCR reaction, the additional cost to sampling kits would be minimal. These barcodes could easily be added to FTA paper, liquid buffer, cotton swabs, or other components of human DNA sampling kits. While DNA has the power to free the wrongfully convicted,[39] it can also be the route of forensic error as illustrated by a recent, highly publicized wrongful conviction and incarceration, due to mislabeling of DNA samples.[2] As DNA sampling and archiving becomes routine, the public needs to be assured that their DNA is being properly collected, stored and interpreted.[1,5,40,41] We propose that nullomer markers can support the National Research Council's recommendations for strengthening and improving forensic science in the United States.[1]

Ethical approval

None.

Financial support

None.

Conflict of interest

One of the authors (Greg Hampikian) has also applied for a patent covering the nullomer applications described in this article.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jflm.2013.02.003.

References

1. National Academy of Sciences. *Strengthening forensic science in the United States: a path forward*. Washington, DC: The National Academies Press; 2009. p. 1–254.
2. Valley J. Metro reviewing DNA cases after error led to wrongful conviction. *Las Vegas Sun*. Available from: http://www.lasvegassun.com/news/2011/jul/07/dna-lab-switch-led-wrongful-conviction-man-who-ser/; July 7, 2011 [accessed 3.05.12].
3. Bromwich MR. *Final report of the independent investigator for the Houston police department crime laboratory and property room*. Houston, TX, http://www.hpdlabinvestigation.org/; 2006.
4. Byers C. *St. Louis police riled over collection of their DNA*. STLtoday.com. Available from: http://www.stltoday.com/news/local/crime-and-courts/city-police-riled-over-collection-of-own-dna/article_8adfcf0a-2469-52d9-adad-d20f3805a883.html; November 14, 2011 [accessed 3.05.12].
5. Collins D. *Police cite privacy concerns over their own DNA*. CNSNews.com. Available from: http://cnsnews.com/news/article/police-cite-privacy-concerns-over-their-own-dna; October 19, 2011 [accessed 3.05.12].
6. Smith ME. Lets make the DNA identification database as inclusive as possible. *The Journal of Law, Medicine, and Ethics* 2006;**34**:385–9.
7. Hampikian G, Andersen T. Absent sequences: nullomers and primes. *Pacific Symposium on Biocomputing* 2007;**12**:355–66. Available from: http://psb.stanford.edu/psb-online/proceedings/psb07/hampikian.pdf [accessed 3.05.12].
8. Allileche A, Goswami J, Davis M, Bourland B. Nullomer derived anti-cancer peptides. *Peptides* 2012;**38**(2):302–11.
9. van Oorschot RA, Jones MK. DNA fingerprints from fingerprints. *Nature* 1997;**387**:767.
10. Ladd C, Adamowicz MS, Bourke MT, Scherczinger CA, Lee HC. A systematic analysis of secondary DNA transfer. *J Forensic Sci* 1999;**44**:1270–2.
11. Lowe A, Murray C, Whitaker J, Tully G, Gill P. The propensity of individuals to deposit DNA and secondary transfer of low level DNA from individuals to inert surfaces. *Forensic Science International* 2002;**129**:25–34.
12. Butler J. *Forensic DNA typing: biology, technology, and genetics of STR markers*. 2 ed. Academic Press; 2005.
13. Bruck S, Evers H, Heidorn F, Muller U, Kilper R, Verhoff MA. Single cells for forensic DNA analysis–from evidence material to test tube. *J Forensic Sci* 2011;**56**:176–80.
14. Axler-DiPerte GL, Wurmbach E, Budimlija ZM, Jian B, Fogt F, Prinz M. Forensic STR analysis reveals DNA contamination previously undetected during clinical analysis of chronically inflamed tissues. *Forensic Science International: Genetics Supplement Series* 2009;**2**:14–6.
15. Urban C, Gruber F, Kundi M, Falkner FG, Dorner F, Hammerle T. A systematic and quantitative analysis of PCR template contamination. *Journal of Forensic Sciences* 2000;**45**:1307–11.
16. Scherczinger CA, Ladd C, Bourke MT, Adamowicz MS, Johannes PM, Scherczinger R, et al. A systematic analysis of PCR contamination. *Journal of Forensic Sciences* 1999;**44**:1042–5.
17. Pinho AJ, Ferreira PJ, Garcia SP, Rodrigues JM. On finding minimal absent words. *BMC Bioinformatics* 2009;**10**:137.
18. Wu Z-D, Jiang T, Su W-J. Efficient computation of shortest absent words in a genomic sequence. *Information Processing Letters* 2010;**110**:596–601.
19. Rodriguez-Lazaro D, Pla M, Scortti M, Monzo HJ, Vazquez-Boland JA. A novel real-time PCR for Listeria monocytogenes that monitors analytical performance via an internal amplification control. *Applied Environment Microbiology* 2005;**71**:9008–12.
20. Barbisin M, Fang R, O'Shea CE, Calandro LM, Furtado MR, Shewale JG. Developmental validation of the Quantifiler® Duo DNA quantification kit for simultaneous quantification of total human and human male DNA and detection of PCR inhibitors in biological samples. *Journal of Forensic Sciences* 2009;**54**:305–19.
21. von Wurmb-Schwark N, Schwark T, Harbeck M, Oehmichen M. A simple duplex-PCR to evaluate the DNA quality of anthropological and forensic samples prior short tandem repeat typing. *Legal Medicine* 2004;**6**(2):80–8.
22. von Wurmb-Schwark N, Preusse-Prange A, Heinrich A, Simeoni E, Bosch T, Schwark T. A new multiplex-PCR comprising autosomal and y-specific STRs and mitochondrial DNA to analyse highly degraded material. *Forensic Science International Genetics* 2009;**3**(2):96–103.
23. Zahra N, Hadi S, Smith JA, Iyengar A, Goodwin W. Development of internal amplification controls (IACs) for DNA profiling with the AmpFlSTR® SGM Plus® amplification kit. *Electrophoresis* 2011;**32**(11):1371–8.
24. Hoorfar J, Malorny B, Abdulmawjood A, Cook N, Wagner M, Fach P. Practical considerations in design of internal amplification controls for diagnostic PCR assays. *Journal of Clinical Microbiology* 2004;**42**:1863–8.
25. Hoorfar J, Cook N, Malorny B, Wagner M, De Medici D, Abdulmawjood A, et al. Making internal amplification control mandatory for diagnostic PCR. *Journal of Clinical Microbiology* 2003;**41**:5835.
26. Applied Biosystems by Life Technologies™. *Quantifiler® Duo human DNA quantification kit user's manual* 2005. Pub. No. 4391294.
27. Applied Biosystems by Life Technologies™. *AmpFLSTR® Identifiler® PCR amplification kit user's manual* 2011. Pub. No. 4323291.
28. Applied Biosystems by Life Technologies™. *Profiler Plus® and Profiler Plus® ID PCR amplification kit user guide* 2012. Pub. No. 4476688 Revision B 08.14.2012.
29. Applied Biosystems by Life Technologies™. *AmpFLSTR® Yfiler® PCR amplification kit, user guide* 2012. Pub. No. 4358101 Rev. H.
30. Promega Corporation. *PowerPlex™ Y system technical manual* 2012. Part #TMD018.
31. Daniel R, van Oorschot RAH. An investigation of the presence of DNA on unused laboratory gloves. *Forensic Science International: Genetics Supplement Series* 2011;**3**:e45–6.
32. Meng S, Zhan S, Li J. Nuclease-resistant double-stranded DNA controls or standards for hepatitis B virus nucleic acid amplification assays. *Virology Journal* 2009;**6**:226.
33. Das A, Spackman E, Senne D, Pedersen J, Suarez DL. Development of an internal positive control for rapid diagnosis of avian influenza virus infections by real-time reverse transcription-PCR with lyophilized reagents. *Journal of Clinical Microbiology* 2006;**44**:3065–73.
34. Heider D, Barnekow A. DNA-based watermarks using the DNA-Crypt algorithm. *BMC Bioinformatics* 2007;**8**:176.
35. Sabir IH, Haldorsen S, Torgersen J, Aleström P, Gaut S, Colleuille H, et al. Synthetic DNA tracers: examples of their application in water related studies. In: Dassargues A, editor. *Tracers and modelling in hydrogeology*. Wallingford, UK: International Association of Hydrological Sciences; 2000. p. 159–66.

36. Swango KL, Timken MD, Chong MD, Buoncristiani MR. A quantitative PCR assay for the assessment of DNA degradation in forensic samples. *Forensic Sci Int* 2006;**158**:14–26.
37. Benson GA. Improved quantitation of human DNA using quantitative template amplification technology. M.S. thesis, Oklahoma State University; 2007.
38. Costello MT, Schumm JW. A single assay for human-specific quantification of less than 1 pg DNA and detection of the presence of PCR inhibitors in forensic samples. *International Congress Series* 2006;**1288**:753–5.
39. Hampikian G, West E, Akselrod O. The genetics of innocence: analysis of 194 U.S. DNA exonerations. *Annu Rev Genomics Hum Genet* 2011;**12**:97–120.
40. Dror IE, Hampikian G. Subjectivity and bias in forensic DNA mixture interpretation. *Science & Justice* 2011;**51**:204–8.
41. Gaensslen RE. Should biological evidence or DNA be retained by forensic science laboratories after profiling? No, except under narrow legislatively-stipulated conditions. *The Journal of Law, Medicine, and Ethics* 2006;**34**: 375–9.

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BOISE STATE UNIVERSITY

Nullomer, Absent or Rare Peptides

PI, Greg Hampikian

Updated 12/18/12

Overview

- What is the invention?
- What are the potential uses for the invention?
- Are there any similar items on the market?
- How do you deliver the invention to the market? (Commercialization plan)
  - What stage of development does the product need to reach?
  - What are the funding opportunities available from private corporations?

What Is The Invention

What is the Invention?

- Nullomers- short sequences of amino acids (protein subunits) that are rare or absent in a species as stand alone structures.
  - They have a higher potential to be bioactive due to their elimination from the genome through evolution.
  - Any compound eliciting major effects on an organism is likely to be eliminated through evolution if the action is not necessary in common tasks.
- Major argument against this method
  - Nullomers were eliminated through evolution due to their toxic effects, will they be safe to use in humans as therapeutic agents?

What is the Invention?

- How are proteins identified as therapies currently?
  - Random sequences are synthesized and tested for a response (one such test outlined on next slide).
  - For a 5 amino acid sequence, a library that included all possible sequences would contain over 3.5 million compounds.
    - This is too many to test, indicating a need for an elimination method.
  - The identification of nullomers reduces the 3.5 million to 198 compounds that have an increased probability to be bioactive.

What is the Invention?

- How do you identify a random sequence as a potential therapy?
  - Design an assay that tests effects in a model
    - For cancer, IC-50 (concentration required to elicit inhibitory effect on 50% of subjected cells) is used to determine effect on cancer cells.
    - This is a typical screening method for potential cancer therapies.
  - After a potential candidate is identified, an animal model can be utilized to demonstrate effects and safety.
  - Once demonstrated in an appropriate animal model (closely related to target species) then the therapy has potential to attract a sponsor to bring it to market.

Uses and Alternative Products

What are the potential uses for the Invention?

- The nullomers identified, are potentially bioactive and have an unknown number of cellular effects.
- The bioactivity of all of the compounds is not yet known and will not be known until a test for specific diseases is conducted on them.
- The effects could be useful in the treatment of diseases depending on what the effects are.
- Currently two effects have been tested and could be potential therapies for cancer treatment and anthrax treatment (both have been demonstrated in cell culture).

Are there any similar items on the market?

- There are lots of drugs designed to target cancerous cells and most treatments involve a cocktail made up of multiple drugs. There is a focus on biologic therapies, primarily ones that have the ability to target cancerous cells and reduce side effects associated with chemotherapy.
- The IC-50 values of the nullomer compounds act as a starting point to identify a potential therapeutic agent. much more data is needed to characterize a compound as safe and efficacious.
- This compound if demonstrated to be effective and safe could be used in conjugate with current therapies to better cure cancer.

Cancer therapies (biologic)

- Interferons (IFNs) are types of cytokines that occur naturally in the body. They were the first cytokines produced in the laboratory for use as Biological Response Modifiers (BRMs).
- Interferons can improve the way a cancer patient's immune system acts against cancer cells. In addition, interferons may act directly on cancer cells by slowing their growth or promoting their development into cells with more normal behavior. Researchers believe that some interferons may also stimulate NK cells, T cells, and macrophages, boosting the immune system's anticancer function.
- FDA has approved the use of interferon alpha for the treatment of certain types of cancer (Not for breast or prostate).

Cancer therapies (biologic)

- Interleukins (ILs) are cytokines that occur naturally in the body and can be made in the laboratory.
- IL–2 stimulates the growth and activity of many immune cells, such as lymphocytes, that can destroy cancer cells.
- IL-2 is approved for treatment of metastatic kidney cancer and metastatic melanoma.

Cancer therapies (biologic)

- Monoclonal antibodies are naturally occurring, but are custom made for use in cancer treatment.
- Monoclonal antibodies bind to specific foreign particles in the body and can be grown to bind cancer cells, receptors and cell signaling molecules.
- They are used in three ways:
  - MOAB can directly bind to cancerous cells and stimulate an immune response
  - MOABs can target growth factors, reducing cell proliferation
  - MOABs can be linked to anticancer drugs improving drug delivery.

Cancer therapies (Chemotherapeutic agents)

- Broad category of drugs that are designed to elicit cytotoxic effects on cancerous cells.
- Typically target and kill quickly dividing cells in the body, resulting in massive side effects.
  - Some target the cellular mechanism required for cell division
  - The Nullomer peptide is thought to target ATP production (energy production)
    - The two peptide sequences demonstrated different results, one on mitochondrial ATP production and the other on glycolysis ATP production. When licensing, a well known mechanism of action is a plus. This is a possible area for more study.

Nullomer's Stage of Development

- A bank of Nullomers have been identified (198 in total)
- Initial scanning of peptides for potential cancer therapeutics was conducted using IC-50 as a metric on several common cancer cell lines.
- Initial scanning of peptides for anthrax toxin therapeutics was conducted, identifying nullomer sequences as potential candidates for treatment.
- The identified cancer therapeutic was further examined identifying effect on ATP production, Mitochondrial health, super oxide formation, live/dead assays and hemolytic activity.
- Limited funding available to further the research (1-2 months).

Stage of development required for licensing

- Most companies will want data demonstrating a mechanism of action, safety and efficacy in an animal model that accurately represents the end user. Companies also look for strong intellectual property rights and novel treatment concepts that have the potential to serve a large market.
  - To Do list for cancer therapy Nullomer
    - Demonstrate safety in an adequate animal model
    - Demonstrate efficacy in an adequate animal model (comparison to similar therapies that are approved)
    - Possibly look further into the mechanism of action (how does it slow ATP production)

Funding Opportunities

Funding available to progress the technology to a further stage of development

Funding Opportunities

- To develop this product to a stage where a potential license might be obtained, funding will be required.
  - Government grants will likely be the most readily available funding method for research at this stage of development.
  - Corporate sponsorship of very early stage therapeutics usually involve grants offered by the sponsoring companies. They commonly look for a research program developing a product that has a potential as a revenue stream. Easily arguable for the cancer therapy.
  - Foundations- Only a few offer research funding for outside organizations, most primarily fund philanthropic activities or internal research.
- Potential funding opportunities from private sponsorship are identified in the following slides.

Private Research Funding (Industry)

- **Otsuka**
  - Therapeutic categories include: Neuroscience, cardiovascular, oncology, and medical devices.
  - Funding available http://www.otsuka-us.com/Resources/Pages/SponsorshipsandGrants.aspx
    - *Educational Grants:* Requests for unrestricted grants supporting educational activities are being accepted in the therapeutic areas of cardiology, endocrinology, hepatology, nephrology, treatment of major depressive disorder, schizophrenia and oncology.
    - *Research Grants:* "In a continuing effort to address unmet medical needs, our companies support outside research initiatives to complement internal programs. Areas of interest include cardiology, endocrinology, hepatology, nephrology, major depressive disorder, schizophrenia, and oncology."

Private Research Funding (Industry)

- **Eli Lilly**-
  - Background-Eli Lilly is a global pharmaceutical company based out of Indianapolis IN.
  - Focus- Lilly currently offers human pharmaceutical products in oncology, cardiovascular, diabetes, critical care, neuroscience, men's health, and musculoskeletal. They also offer animal pharmaceuticals.
  - Funding opportunities
    - Foundation-involved in funding philanthropic initiatives such as improving the lives of people who lack recourses to obtain quality health care and strengthening public education in the United States. They do not accept unsolicited grant applications.
    - Lilly Grant Office- http://www.lillygrantoffice.com/pages/index.aspx The Lilly grant office accept proposals for a broad range of funding opportunities. In the past it looks like grants for educational use have been awarded between 5000 and 150000 have been awarded.

Private Research Funding (Industry)

- **Baxter International**
  - Funding available:
    - Baxter International Foundation: primary focus is to increase access to healthcare worldwide. Grants awarded in 2011 fulfilled local needs to increase access to dental care, mental health, and other healthcare services for children, the uninsured, veterans, and the elderly. (Amounts from ~$10,000 to $130,000)
  - Baxter BioScience Grants: http://www.baxterbiosciencegrants.com/process_overview.html
    - *Educational:* grants that support educational programs. $1,000-25,000, but have exceeded $100,000 for requests demonstrating exceptional educational need, educational design, and compliance to existing regulations and guidance.
    - *Clinical:* grants that involve the use of therapeutics or medical devices in human study subjects. Immunology, neurology, hematology, biosurgery, pulmonology, regenerative medicine. $5000-125,000. Requests exceeding this amount must demonstrate exceptional qualities and address critically important medical issues.
    - *Non-clinical:* grants that involves non-human subjects (e.g. laboratory animal studies, bench-work). Immunology, neurology, hematology, biosurgery, pulmonology, regenerative medicine. $5000-125,000. Requests exceeding this amount must demonstrate exceptional qualities and address critically important medical issues

Private Research Funding (Industry)

- **GlaxoSmithKline**
  - Areas of interest- Dermatology, Immuno-Inflammatory Diseases, Infectious diseases, Metabolic pathways, Nurodegeneration and inflammation, Oncology, Ophthamology, Rare Diseases, Respiratory Diseases and Vaccines.
  - Funding opportunities-
    - Academic collaboration-"We are keen to partner with scientists to facilitate the rapid discovery and development of new medicines for patients. Academic collaborations are complementary to our in-house efforts, and vary considerably in their size and nature." http://www.gsk.com/partnerships/academic-collaborations.html

Private Research Funding (Industry)

- **Novo Nordisk**, Denmark
- Funding available
  - Diabetes Innovation Award: Committed to supporting novel research in **protein-based therapies** and technologies that have the potential to improve the effective treatment, prevention and/or cure of diabetes. Targets novel research ideas that despite having potentially high impact would not normally attract funding from such institutions as the NIH or CIHR. Two types: "Early Research Exploration" and "Proof of Principle" awards. Nonclinical research only. Projects can be supported for up to two years under the program.
  - Up to $500,000 for 2 years
  - This award program aims to help scientists substantiate early innovation research efforts and clarify if their hypotheses have the potential to result in the effective cure, prevention and/or improved treatment of diabetes and obesity
- The areas of research supported by these awards are:
  - Non-clinical research related to new biologic therapeutics and targets amenable to biologics therapeutics or novel effects of known biologics in the field of Type 1 Diabetes and Type 2 Diabetes, including microvascular complications, and Obesity
  - Protein-based science technologies including oral and injection delivery concepts

Private Research Funding (Industry)

- Sanofi's funding opportunities (offered through Genzyme)
  - "As part of our commitment to expanding medical knowledge and improving standards of health care worldwide, Genzyme distributes many grants and charitable contributions to hospitals, research institutions, disease organizations, and medical education programs." These grants target efforts in our areas of disease and therapeutic focus:
    - Genetic disease
    - Renal disease
    - Cardiovascular disease
    - Neurologic disease
    - Oncology
    - Orthopedics
    - Transplant and immunology
  - http://www.genzyme.com/Research/Research-Grants.aspx all current offers can be found on the above link.

Foundation Research Funding

- Prostate Cancer Foundation funding opportunities
  - Four funding opportunities available including Recognition Awards, Young Investigator Awards, PCF Creativity Awards and PCF Challenge Awards. http://www.pcf.org/site/c.leJRIROrEpH/b.5796011/k.AF96/Funding_Strategy.htm
  - Open requests for applications can be found at the following link http://www.pcf.org/site/c.leJRIROrEpH/b.5849007/k.F70A/Open_RFAs.htm

Foundation Research Funding

- Prostate Cancer Research and Education Foundation funding opportunities.
  - The foundation gathers donations to support seed funding of projects involving prostate cancer.
  - The prospective project page outlines current requests for funding and past recipients. Found here http://www.pcref.org/prospective_projects.php
  - All applications are for seed funding (less than 30K)

Foundation Research Funding

- Avon Foundation for Women funding opportunities
- "The Avon Foundation for Women continues its search for new preventive strategies to address the growing number of breast cancer cases around the globe. To develop new strategies to prevent breast cancer, we need to understand the causes of breast cancer in people, changes in breast cells that give rise to cancer, markers for disease, and how breast cancer progresses. The 2013 Avon Foundation Research Program seeks proposals in these areas to advance understanding of causes of breast cancer and prevention."
- http://www.avonfoundation.org/grants/breast-cancer/research-grant-guidelines/
  - A letter of intent must be submitted by the deadlines outlined on their webpage to register you as a potential grant recipient.
  - Grant proposals are accepted by invitation only. Invitations for proposals are requested based on submitted letters of intent.

Ranking of Funding Opportunities

- Government grants are the most available recourse for funding early stage projects.
- Industry offered grants, in order of likelihood based on areas of interest and stage of development
  - **Otsuka**
  - **Eli Lilly**
  - **Baxter International**
  - **GlaxoSmithKline**
  - **Pfizer**
  - **Novo Nordisk**
  - **Sanofi**
- Foundation grant funding, in order of likelihood based on funding amounts, areas of interest, other qualifying aspects associated with the foundation.
  - Prostate Cancer Foundation
  - Prostate Cancer Research and Education Foundation
  - Avon Foundation for Women

Plan of Action

- Funding opportunities listed must be evaluated by the PI
- The I Team can offer assistance in gathering market data to buff the commercialization aspect for industry grants, but specific focus must be identified first.
- Katy can assist with developing connections at corporations/foundations if needed.

The I-Team, Nullomer, Absent or Rare Peptides

THANK YOU