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 **Title: Nullomer Anticancer Peptides**

SPECIFIC PROJECT FOCUS: **This proposal seeks to fulfill investors requests for cancer cell tests on a novel class of anti-cancer peptides, Nullomers, derived from the shortest amino acid strings not found in nature. Two Nullomers tested to date rapidly kill cancer cells through a mechanism that causes mitochondrial destruction, drastic ATP loss, and rapid cell death. Significantly, the killing dose (IC-50) of Nullomers drops in cancer cells over time, but increases in normal cells.**

Funding will provide the resources to satisfy our corporate contacts desire to see more Nullomers tested against a wider array of cancer types, including drug-resistant cancers.

PROJECT START DATE: 7.1.14	PROJECT END DATE: 6.30.15
NAME OF INSTITUTION: Boise State University	DEPARTMENT: Biology

ADDRESS: 1910 University Drive, Boise, ID 83725-1515

E-MAIL ADDRESS: greghampikian@boisestate.edu	PHONE NUMBER: 208-426-4992

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

NAME OF PARTNERING COMPANY: Middlebrook and Bremmer, LLC	COMPANY REPRESENTATIVE NAME: Jeremy Brehmer

	NAME:	SIGNATURE:
Authorized Organizational Representative	Karen Henry, Exec Director Office of Sponsored Programs	6/16/14

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SUMMARY PROPOSAL BUDGET

| Name of Institution: Boise State University |
| Name of Project Director: Greg Hampikian |

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

Name/ Title	Salary/Rate of Pay	Fringe	Dollar Amount Requested

% OF TOTAL BUDGET:			SUBTOTAL:	

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

Item/Description	Dollar Amount Requested
SUBTOTAL:	

G. TRAVEL:

Dates of Travel (from/to)	No. of Persons	Total Days	Transportation	Lodging	Per Diem	Dollar Amount Requested
					SUBTOTAL:	

H. Participant Support Costs:

	Dollar Amount Requested
1. Stipends	
4. Other	
SUBTOTAL:	

I. Other Direct Costs:	Dollar Amount Requested
1. Materials and Supplies	50,000
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:	
J. Total Costs: (Add subtotals, sections A through I) **TOTAL:**	50,000
K. Amount Requested: **TOTAL:**	50000

Project Director's Signature: _(signature)_

Date: 6/16/14

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)

1. **Title: Nullomer Anticancer Peptides, Boise State University**

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

3. This technology has been awarded an Incubation Fund Award in the past.

   a. Previous HERC award: 2013-2014, "Liposome Delivery of Cancer Killing Peptides"

   b. How is this proposal different from the last proposal awarded?

   Our last HERC grant solved the difficult challenge of Nullomer peptide solubility and delivery using our two initial anticancer Nullomers 9R and 9S1. We also established company contacts interested in developing Nullomer Technology. The new proposal is needed to satisfy corporate partners' calls to test several more (15) Nullomers against the full National Cancer Institute's NCI-60 panel of 60 cancers.

4. Executive Summary: 1 – 2 paragraphs describing all the project elements of the proposal.

   This proposal seeks to fulfill investors requests for cancer cell tests on a novel class of anti-cancer peptides, Nullomers, derived from the shortest amino acid strings not found in nature. Two Nullomers tested to date rapidly kill cancer cells through a mechanism that causes mitochondrial destruction, drastic ATP loss, and rapid cell death. Significantly, the killing dose (IC-50) of Nullomers drops in cancer cells over time, but increases in normal cells.

   Funding will provide the resources to satisfy our corporate contacts desire to see more Nullomers tested against a wider array of cancer types, including drug-

resistant cancers.

5. <u>"Gap" Project Objective and Total Amount Requested</u> – Describe the objective of this project.

   We received funding this year from the Pardee Cancer Foundation ($100,000) to assess the effects of 3 of the 198 Nullomer peptides (9R, 9S1R and 124R) on the National Cancer Institute panel of 60 human cancer cell lines (NCI-60) from nine organ systems: kidney, prostate, ovary, hematopoietic, colon, skin, breast, nervous, and lung. We have found that 94.74% of the cancer cell lines are sensitive to 9S1R, and 68.42% are sensitive to 9R. All of the NCI-60 panel cancer cells are resistant to peptide 124R, which is a Nullomer that serves as a control. All of these cells are now growing in our lab, and we have a fantastic opportunity to test 15 new Nullomer peptides (and their modifications) on this diverse panel of cancer cells. Without HERC funding we will have to shut down these lines; and restarting them will be tremendously difficult and expensive. Funding would allow us to continue building corporate support for Nullomer drug development.

6. <u>Description of how resource commitments reflect the priorities of the home institution(s)</u>

   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 PI of this proposal was nominated by BSU and inducted last year as a

foundational member of the National Academy of Inventors.

7. <u>Evidence that the project will have a potential impact to the economy of Idaho</u>

    Idaho lags behind several of its neighboring states in the development of

biotechnology and medial products. This sector of the economy is seen as a growth

area that provides jobs for both highly-skilled technical specialists and production

line workers.

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

    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.

    A recent (May, 2014) report by the IMS Institute for Healthcare Informatics

summarizes the growing oncology market as follows:

    "The pace of annual global spending on oncology medicines – which is

approaching the $100 billion threshold – has moderated over the past five years,

even as a surge in innovative and targeted therapies has brought new

therapeutic options to the growing number of patients being treated for cancer and as survival rates for most tumor types continue to increase, according to a new report released today by the IMS Institute for Healthcare Informatics Growth in global spending on cancer drugs, including those used for supportive care, increased at a compound annual growth rate of 5.4 percent during the past five years, reaching $91 billion in 2013, compared with 14.2 percent from 2003 to 2008. The recent lower growth rate reflects fewer breakthrough therapies for very large patient populations, as well as patent expiries, reductions in the use of supportive care medicines, and stronger payer management. While oncology spending remains concentrated among the U.S. and five largest European countries – which together account for 65 percent of the total market – the rising prevalence of cancer and greater patient access to treatments in pharmerging nations has propelled oncology to the fifth-largest therapy area in those markets."

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

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.

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.

9. <u>The Technology and Path to Commercialization</u>

    a. Describe the technology and the current state of the technology

    We have 198 Nullomer compounds in development. Two of the three

    Nullomers that we described in our initial publication (Appendix 4) have

    anticancer effects against breast cancer, prostate cancer, melanoma, and

    leukemia. Our new initial results show that modified Nullomers are effective

    against several drug resistant cancer cell lines (established from patient

    tumors).

    b. Describe how the technology contributes to the product and market need and

    its intellectual property status.

    Peptide drugs hold promise as new cancer drugs because they are small,

    made of natural amino acids, easy to attach to homing (specific cell type

    targeting) compounds such as antibodies, and have low side effects.

    c. Identify who developed the technology and with what funding.

    The PI and his team at BSU developed the technology as part of

    $3,000,000 of DOD funding to the PI. Additional support was provided

    through the Pardee Cancer Foundation ($100,000 in 2013-2014) and a

    previous HERC grant ($50,000).

    d. Identify the concrete steps to bring technology to market

    Corporate and investor contacts have told us to test more of the Nullomers

against the NCI-60 panel and to include a special focus on the drug-resistant cancers. We are seeking funding to test a wide array of Nullomers (15), with the concrete hope that this will attract a commitment from investors.

10. <u>Commercialization Partners</u> – Identify commercialization partners, if any, or methods to be used in developing commercialization partners. Provide a clear description of the business relationship between institution's personnel and their private partners. Clearly outline responsibilities for project tasks, proposed schedule of meetings and a discussion of potential future activities that would take place upon successful completion of the project. Include description of public and private sector partnerships.

A. Jeremy Brehmer of Middlebrook and Brehmer has visited the lab, and met with the PI over three days in Boise. The investors he represents are interested in developing the Nullomers and a support letter is attached.

B. We have been approached by a Salt Lake City venture capitol company about developing Nullomer drugs.

C. We have established relationships with WestVEt of Idaho for future animal testing (support letters were included in previous HERC), but animal testing is not part of this HERC.

11. <u>Specific Project Plan and Detailed Use of Funds</u> – Describe tasks to be undertaken, the team (personnel and qualifications) milestones and performance metrics. Include a proposed overall budget. Identify items of equipment costing more than $1,000. Round all budget totals to the nearest $100. A description of

the role of personnel or the nature and purpose of other expenditures should be included for each item in the personnel categories; a description of need for and purpose of equipment for all expenditures over $1,000; a description of purpose and destination of proposed travel expenditures; an explanation of role of additional participants and the nature of expenditures involved; and description of expenditures under other direct costs. Grantees should anticipate that awarded funds will be tracked and contingent upon meeting milestones on schedule. Unallowable costs will include foreign travel, patent searches, and facility and administrative (F&A) costs.

Considering the advantages of peptides as cancer drugs, and encouraged by our previous results we will screen 15 more Nullomer peptides from our library. The 15 sequences are: MWCWY, WCWQ, DQWMC, CECWF, WDCWF, NWWCM, WCTWY, HCWHT, MWHCT, MCWDH, MWYMC, WYNPC, WEYHC, WMYCC, WMYWC.

Materials: We anticipate that we will synthesize and have to modify and resynthesize 15 Nullomer peptides. The modifications will include the use of several right-handed amino acids (not usually found in nature) the cost for these is expected to be about $25,000. Other lab supplies include culture media, serum, MTT assays (viability), serum for the NCI-60 culture cells, and cultures of normal cells ($25,000).

Personnel: The PI and Dr. Abdelkrim Allileche will perform all work (Bios in the Appendix).

12. <u>Institutional and Other Sector Support</u>: Summarize the home institution's commitment at a level appropriate to the project. Describe projected support from external partners, including space, funds, facilities, and people for the project. This is not intended to be cost share.

BSU has already invested substantially in this project by patenting 198 of the Nullomer peptides, and assigning an innovation team to assist with commercialization.

Appendices:

1. Facilities and Equipment:

2. Biographical Sketch

3. Support Letter from Middlebrook and Brehmer, LLC.

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. Latest Results

Facilities and equipment available for the project

A. The main Hampikian/Alileche shared laboratory consists of approximately 800 square feet. The lab is equipped with 3 instruments used for sequencing and related work, each of which has a dedicated computer: ABI 3130 Genetic Analyzer, ABI 310 Genetic Analyzer, LI-COR 3100 DNA sequencer. Other equipment in our lab includes: Cressington 108 Manual Sputter Coater, Olympus BX-53 Research microscope with Canon 6D camera, EpMotion 5070 Robot, 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 Hampikian/Alileche 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 Hampikian/Alileche lab space (100 square feet) is used for pre-PCR preparation of reactions. It also contains a Synergy Biotech MX microplate reader for the MTT assays and other fluorescence assays.

B.   Additional shared departmental 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. 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.

C.   Mass Spectrometry available through the Biomolecular Research Center:

Thermo Scientific Velos Pro LIT Mass Spectrometer is an ion trap mass spectrometer. It offers Trap-HCD (Higher-Energy Collisional Dissociation) combined with CID (Collision-Induced Dissociation), and PQD (Pulsed-Q Dissociation) to enhance coverage and sensitivity of proteomic analysis. An Easy nL nano liquid chromatographic system is coupled to the mass spectrometer through the nanoelectrospray source for protein characterization. In combination with the Thermo

Proteome Discover 1.3 and Sequest and Mascot database search engine, this LC-MS system is our workhorse for routing proteomic analysis.

Bruker Q-TOF Mass Spectrometer is a hybrid tandem mass spectrometer with outstanding performance including fast acquisition rate (up to 30 Hz for small molecules, up to 5Hz dynamic for peptides), high resolution (50,000 Full Sensitivity and Resolution), high resolution EIC (0.5 – 1mDa on typical LC peaks), and excellent sensitivity (1 pg Reserpine >100:1 S/N RMS). This mass spectrometer is coupled with a Dionex Ultimate 3000 HPLC system and an innovative Captive electrospray source. In combination with software tools, including Bruker Compass Data Analysis, Smartformula, ProteinScape, Mascot protein search engine, and Profile Analysis, we use this LC-MS system in small molecule identification, metabolomics analysis and protein characterization.

Bruker HCT Ion Trap Mass Spectrometer is a robust and easy to use mass spectrometer. Coupled with an electrospray sources, it provides good MS and MS/MS spectra quality – in terms of mass resolution, sensitivity and accuracy throughout a wide mass range for applications like: Protein identification, metabolite characterization, and general chemistry LC/MSn. Equipped with an Electron-transfer dissociation (ETD) and CID, HCT provides unique capability to identify post translational modification of protein.

Thermo Polaris Q GC/MSn Benchtop Ion Trap Mass Spectrometer complete with Thermo Trace Gas Chromatograph, external vacuum pump, and XCalibur PC workstation. This combination of power of the MS ion trap technology target specific compounds in complex matrices. The Polaris Q offers unparalleled sensitivity in full scan, plus the power of MS/MS, the PolarisQ provides dependable performance for routine application plus the added horsepower to go further enabling lower detection in complex matrices.

D.   Scanning Electron Microscope used by the Hampikian/Alileche lab is located n the Engineering building: manufacturer: LEO, model: 1430VP, equipped with Energy Dispersive Spectrometry (EDS) System from Oxford Instruments and Nanometer Pattern Generation System (NPGS) manufactured by J.C. Nabity Lithography Systems.

Biographical Sketch

NAME GREG HAMPIKIAN			POSITION TITLE: PROFESSOR BIOLOGY	
EDUCATION/TRAINING				
INSTITUTION AND LOCATION	DEGREE		YEAR(S)	FIELD OF STUDY
University of Connecticut, Storrs, CT	BS		1982	Biological Sciences
University of Connecticut, Storrs, CT	MS		1986	Genetics
University of Connecticut, Storrs, CT	PhD		1990	Genetics
LaTrobe University, Melbourne, Australia	Postdoc.		1990-91	Marsupial Sex Determination
Worcester Foundation for Experimental Biology	Postdoc.		1992	Y Chromosome Mice

Research and professional experience:

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 (with Jennifer Graves)
1992	Postdoctoral Associate, Worcester Foundation for Experimental Biology, Worcester, MA (with William Crane)
1993-2004	Assistant, Associate, Full Prof., Dept. Natural Science, Clayton State Univ., Morrow, GA
1994-95	Visiting Scientist, Emory University & (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

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).

Additional recent publications of importance to the field (in chronological order)
1. F. Deak, Y. Kiss, K. Sparks, S. Argraves, **G. Hampikian** and P. Goetinck, 1986, "Amino acid sequenc chicken cartilage link protein from c-DNA clones," *Proc. Natl. Acad. Sci. USA* 83:3766-3770.
2. 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: *SR* related sequences in marsupials," Nature 359: 531-33.
3. 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.

Patent Awards and Applications
US 13/358,952 filed January 26, 2012 Absent and Rare Peptides and Therapeutic Uses Thereof.

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

Current Support
Title: NulloPs peptides treatment of cancer
Grantor: Elsa U. Pardee Foundation
Duration: June 2013 – June 2014
Amount: $100,000
Time Committed: 1 month

Title: Liposome delivery of cancer killing nullomer peptides
Grantor: Idaho State Board of Education
Duration: July 2013 – June 2014
Amount: $50,000
Time Committed: 1 month

BIOGRAPHICAL SKETCH

NAME **Alileche, Abdelkrim**	POSITION TITLE **Assistant Research Professor**

EDUCATION/TRAINING

INSTITUTION AND LOCATION

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Medicine Faculty of Tunis, Tunisia **Paris XI University, France**	**MD** **PhD**	**1983** **1994**	**General medicine** **Immunology**

Positions and Honors

1994-1998: Metabolism Branch, Cytokine section, NIH/NCI in Bethesda MD, USA with Dr T.A. Waldmann. Post Doctoral position

1998-2001: Bone Marrow Research Laboratories/WEHI in Melbourne Australia with Pr C.G. Begley.Post Doctoral position

2002-2005: Immunology Microbiology Department of the Albert Einstein College of Medicine in Bronx New York, USA with Pr J. Brojatsch. Research Associate position

2006-2008: Biocomplexity Institute of the Indiana University in Bloomington IN, USA with Pr J.A. Glazier. Research Associate position

2008-August 2009 Biology Department of Boise State University, ID with Pr Greg Hampikian. Research Associate position

2009-2011 Research Assistant Professor position

2012- until now: Visiting Scientist Position

Selected peer-reviewed publications (in chronological order)

1. **ALILECHE A**. GOSWAMI J, DAVIS M, BOURLAND W, HAMPIKIAN G. (2012) Nullomer derived anticancer peptides (NulloPs): differential lethal effects on normal and cancer cells in vitro Peptides 38: 302-311
2. AMARIE D, **ALILECHE A**, DRAGNEA B, GLAZIER JA. (2010) Microfluidic devices integrating microcavity surface Plasmon resonance sensors: glucose oxidase binding activity detection Anal. Chemistry 82: 343-352
3. **ALILECHE A**, SQUIRE RC, MUEHLBAUER S, BROJATSCH J. (2006) Mitochondrial impairment is a critical event in anthrax induced cytolysis of murine macrophages. Cell Cycle 5: 100-106.
4. **ALILECHE A,** SERFAS ER, MUEHLBAUER S, PORCELLI SA, BROJATSCH J. (2005) Anthrax lethal toxin mediated killing of human and murine dendritic cells impairs the adaptive immune response. PloS Pathogen 1: e19.

5. BONUCELLI A, BROJATSCH J**, ALILECHE A**, LISANTI MP. (2005)   ATR/DEM8 is highly expressed in epithelial cells lining after Bacillus anthracis three sites of entry: amplification of the pathogenesis of anthrax infection. Am J Physiol Cell Physiol 288: C1402-10.

6. **ALILECHE A** , GOLDMAN CK , WALDMANN TA. (2001)   The differential effects of IL-2 and IL-15 in the induction and expression of IL-2 receptor alpha chain. BBRC 285: 1302-9.

7. **ALILECHE A**. (2003) The IL-2 immunotherapy of cancer : critical analysis of results, problems and expectations   Medical Hypothesis 60, 202-208.

8. **ALILECHE A** , HAHN DS , AZZARONE B. (1994)   IL-2 production by myofibroblasts from post radiation fibrosis in breast cancer patients. I*nt. Immunology* 6: 1585.

9. **ALILECHE A** , PLAISANCE S , AZZARONE B.   (1993) Human melanoma cell line M14 sectretes a functional IL-2 Oncogene 8: 1791.

10. DAMJANOVITCH S , BENE L , MATKO J , **ALILECHE A** , WALDMANN TA. (1997) Preassembly on interleukin 2 receptor subunits on resting kit 225 k6 T cells and their modulation by IL-2 , IL-7 and
IL-15 : a fluorescence energy transfer study PNAS 94: 13134.

11. DOUCET C , MEAZZA R, POTTIN CLEMANCEAU C, SCUDELETTI M, BROUTY BOYE D,FERRINI S, **ALILECHE A,** INDIVERI F.   (1997) Role of interleukin 2 and 15 in the progression of a melanoma cell line MEL P derived from an IL-2 progressor patient. Melanoma SUPPL 7: S7.

12. SEMMEL M, **ALILECHE A**, JASMIN C.   (1992)   Inhibition of human immunodeficiency type 1 multiplication by an avian cellular factor. Arch Virology 123: 309.

13. PLAISANCE S , ALILECHE A, AZZARONE B.   (1994) How interleukin 2 can affect human fibroblast behaviour   Pathol. Res Pract. 190: 942.

14. HAHN D , PLAISANCE S, RUBINSTEIN E, **ALILECHE A**, AZZARONE B. (1995) Non random activation of endogenous interleukin 2, interleukin 2 receptor alpha and interleukin 2 beta genes after transfection of mouse fibroblast with a cDNA for the alpha chain of the human intereleukin 2 receptor. Eur. J Immunology 25: 1905.

15. SEMMEL M , MACHO A, COULAUD D, **ALILECHE A**, JASMIN C.   Effect of retinoic acid on HL60 cells infected with human immunodeficiency type 1. *Blood* 1994 84 2480.

16. PLAISANCE S , RUBINSTEIN E, **ALILECHE A**, AZZARONE B.   (1993) Human melanoma cells express a functional interleukin 2 receptor   Int J Cancer 55: 164.

17. PLAISANCE S , RUBINSTEIN E, **ALILECHE A**, AZZARONE B. (1993) The interleukin 2 receptor present on human embryonic fibroblast is functional in the absence of p64 interleukin 2 receptor gamma chain   Int. Immunology 5: 843.

Current Support
Title: NulloPs peptides treatment of cancer
Grantor: Elsa U. Pardee Foundation
Duration: June 2013 – June 2014
Amount: $100,000
Time Committed: 10 months

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. Also emeritus Board Member of the Georgia Innocence Project (2002-).

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. Served as 2001-2002 Biology Coordinator, Natural Science Department.

2004
Chair of the Georgia Academic Advisory Committee for Biological Sciences
The Committee included department heads of all Georgia public colleges and universities.

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

Pham-Hoai, E., Crispino, F., Hampikian, G., The first successful use of simple low stringency familial searching in a French criminal investigation, Journal of Forensic Sciences, Volume 59, Issue 3, pages 816–819, May 2014.

Muller, R., Hampikian, G., The PCR enhancer STRboost™ overcomes humic acid inhibition with forensic DNA typing kits (in revision).

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

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Patent Awards and Applications
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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.

Editorial Boards

Journal of Forensic Investigation

Selected Honors and Awards

Idaho Fourth District Bar Association's Liberty Bell Award (2013) for contributions to justice

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."

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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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June 13, 2014

Via Email Only

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      Re: Nullomers

Dear Dr. Hampikian,

I look forward to partnering with you on the Nullomers. I am working on biotechnology projects where the use of human absent sequences (Nullomers) are needed.

I commend you on furthering this important project following your descriptive algorithm for identifying absent sequences, and your demonstration of its use by listing the smallest oligomers not found in the human genome or GenBank sequence primes.

I wholeheartedly support your application for the Higher Education Research Council grant which would allow us to collaborate.

MIDDLEBROOK & BREHMER LLP

Jeremy C. Brehmer

Biology Faculty Publications and Presentations Department of Biological Sciences

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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^{TM} (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 $\lambda=405$ nm [23]. The hemolytic activity [20] in % was calculated using this formula: Hemolytic activity in %={[A_{Sample}-$A_{Control}$]/[A_{Total}-$A_{Control}$]}x100

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).

LNCaP MDA-MB-231

Control

Peptide 124

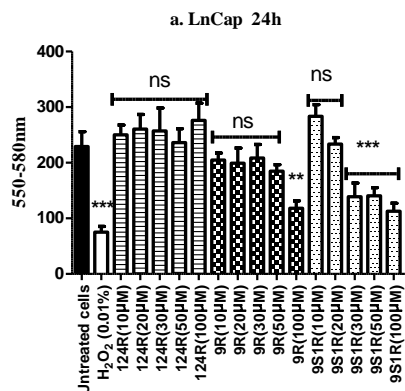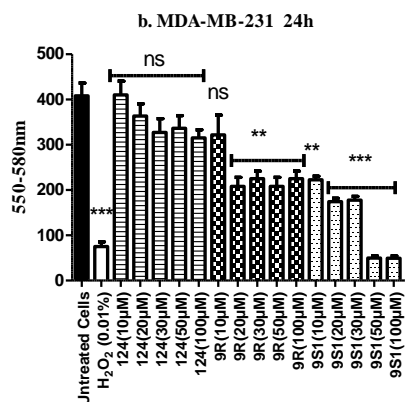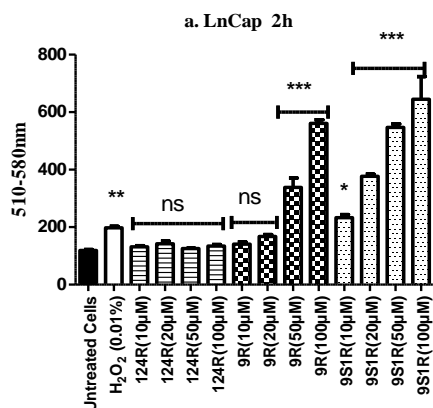Peptide 9R

Peptide 9S1

10
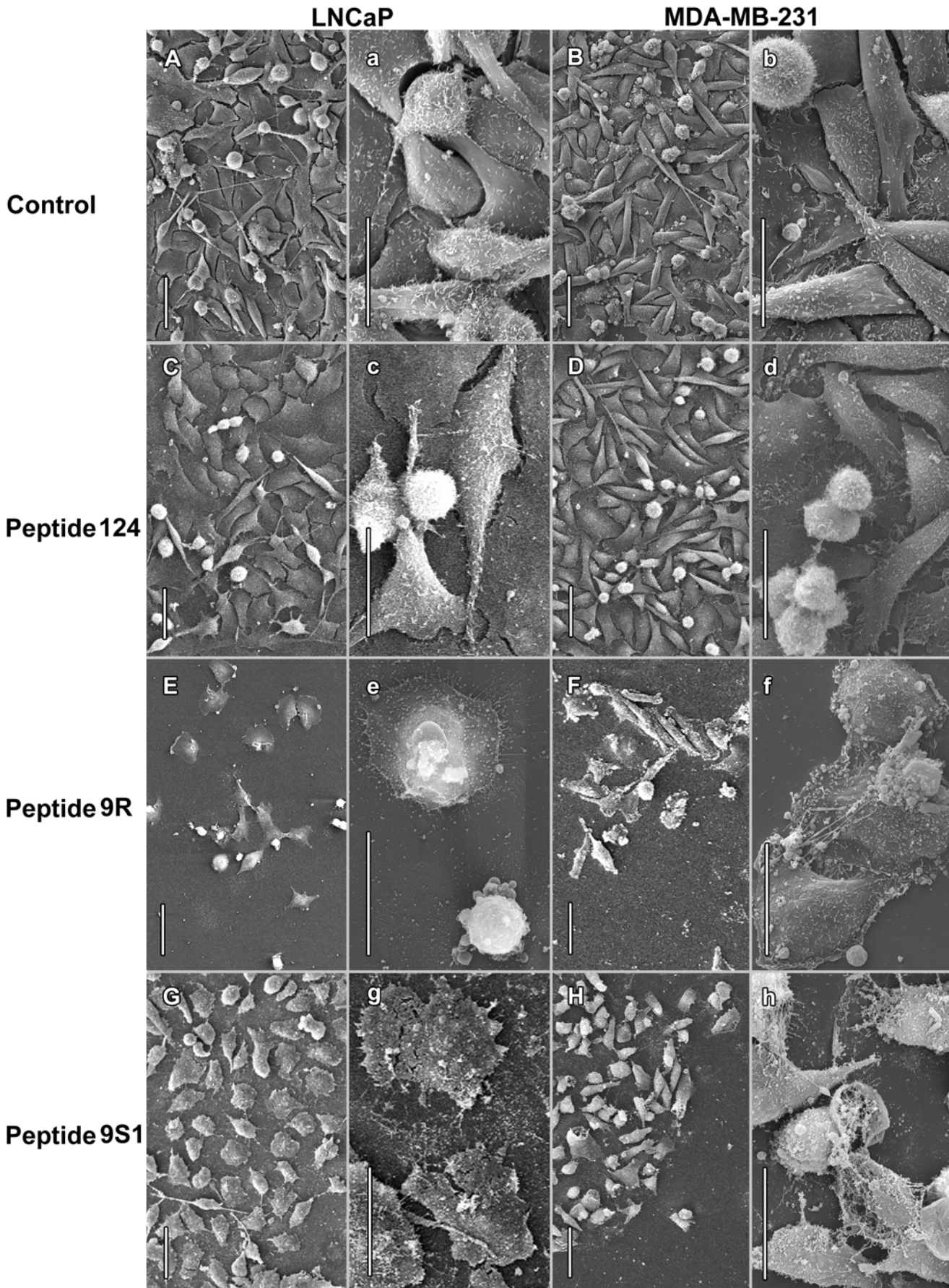
Fig. 2. PolyArgNulloPs effects on cellular ATP level. Cells (3000/well) were seeded in 96 well plates (white plates from Nunc). After 24 h incubation, peptides 9R, 9S1R and 124R were added to the wells. Control conditions included untreated cells, and 100mM sodium azide. At 2h, 24h, and 48h a single reagent Cell Titer Glow^{TM} (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
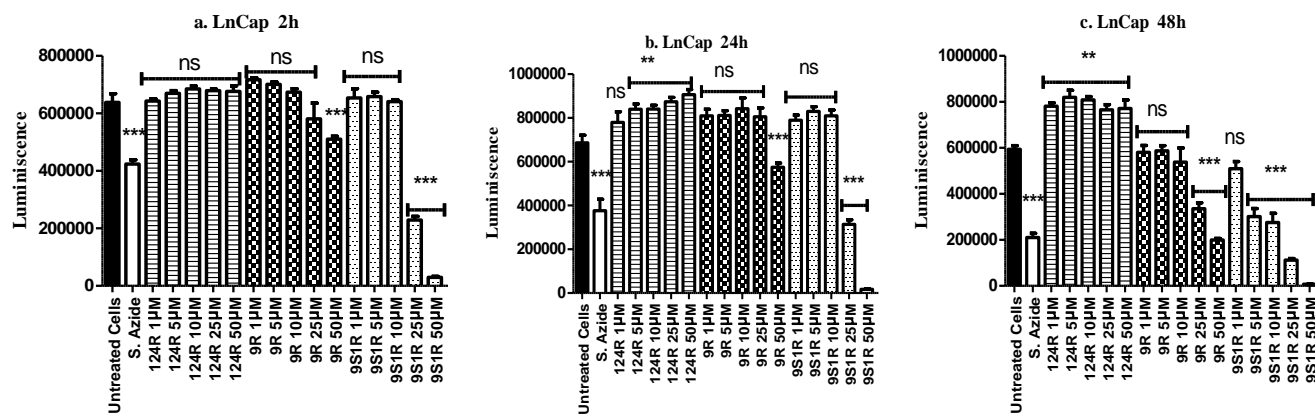
(B) MDA-MB-231

11
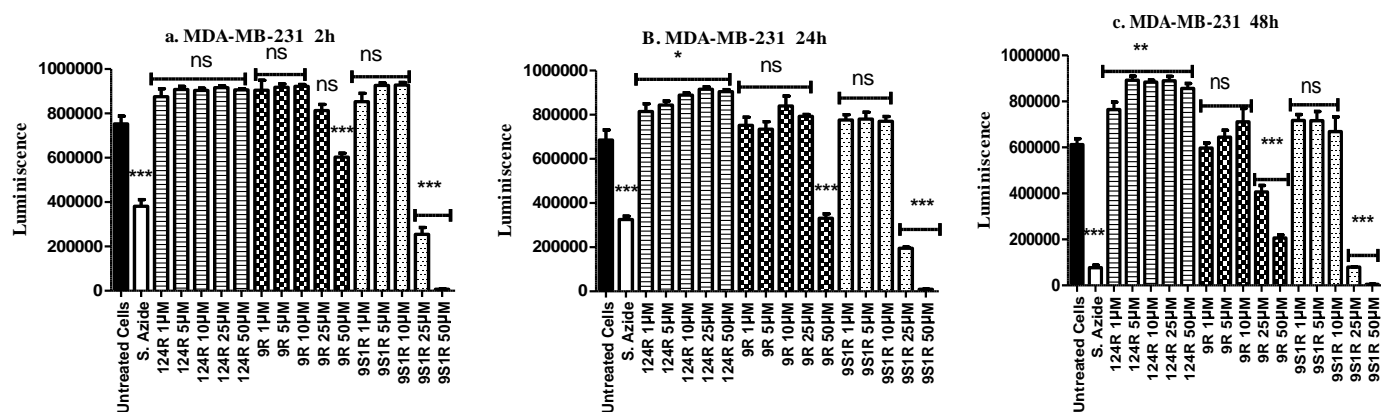
Fig. 3. PolyArgNulloPs effects on MMP. LnCap cells (3000/well) were seeded in 96 well plates (white plates from Nunc). After 24 h incubation peptides 9R, 9S1R and 124R were added to the wells. Control conditions included untreated cells, and H2O2 (0.01%) treated cells. At 24h, 50 µl of mitochondria staining 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)

13

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. *$p<0.05$, ***$p<0.01$, ***$p<0.001$

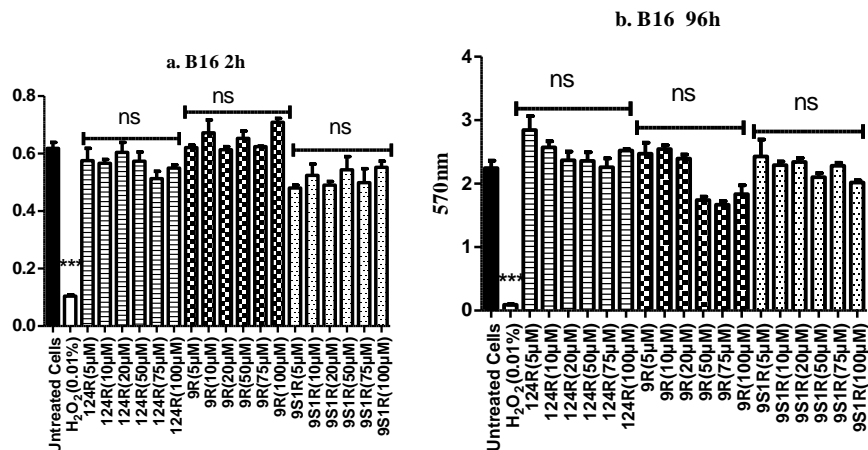(a), LnCap, at 2h. (b) MDA-MB-231, at 2h.

(a), LnCap

a. LnCap 2h

(b) MDA-MB-231

b. MDA-MB-231 2h

14
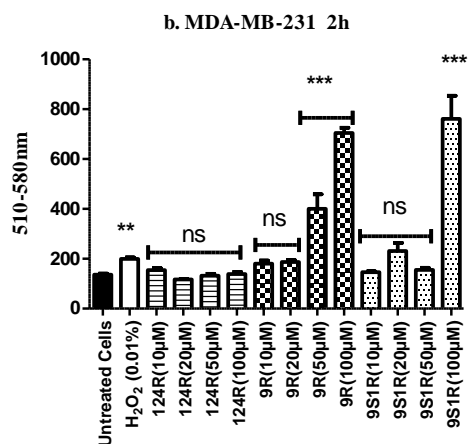
Fig. 5. Hemolytic activity of PolyArgNulloPs. RBCs collected from healthy donors were diluted 1:10 in PBS, and peptides 9R, 9S1R and 124R were added. Control conditions included PBS, and 1%Triton X-100. After 24h incubation at 37ºC with 100 rpm shaking, RBCs were centrifuged and the supernatant collected in a 96 well plates. Absorbance was determined at 405 nm. The hemolytic activity in % was calculated using the formula:
Hemolytic activity in $\% = \{[A_{Sample}-A_{Control}]/[A_{Total}-A_{Control}]\} \times 100$

RBC 9R, 9S1R, 124R
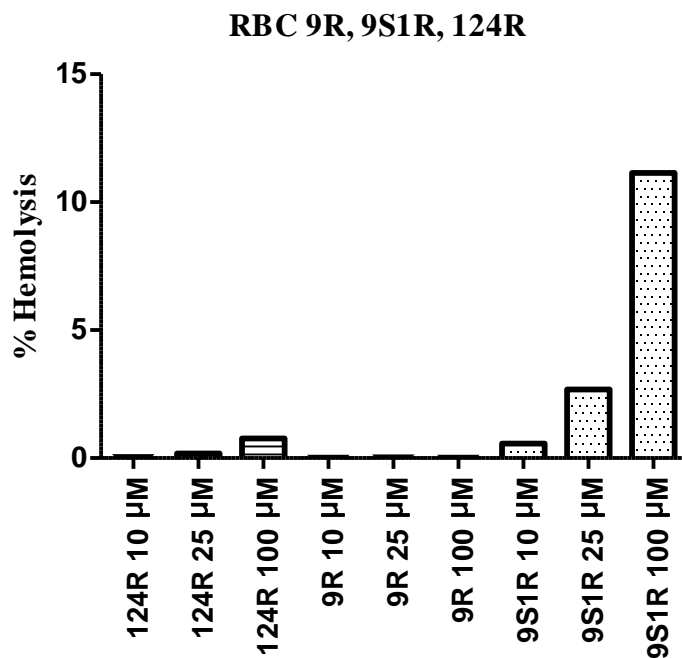
Table 1. IC-50 (µM) Evolution. The IC-50 were calculated from the raw data of MTT readings at 570nm using the GraphPad PRISM* version 5.03. Dose response curves generated by non linear regression with GraphPad PRISM* were used to determine the IC-50s. Basically the x values from the $y=f(x)$ function 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)	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

18

(C) B16

a. B16 2h

b. B16 96h

(D) HUT 102

a. HUT102 2h

b. HUT102 48h

c. HUT102 72h

d. HUT102 96h

19

(E) J774A.1

a. J774A.1 2h

b. J774A.1 24h

c. J774A.1 72h

d. J774A.1 96h

(F) WI-38

a. WI-38 24h

b. WI-38 48h

20

(G) PCS

a. PCS 24h

b. PCS 48h

(H) HMEC

a. HMEC 24h

b. PCS 48h

21
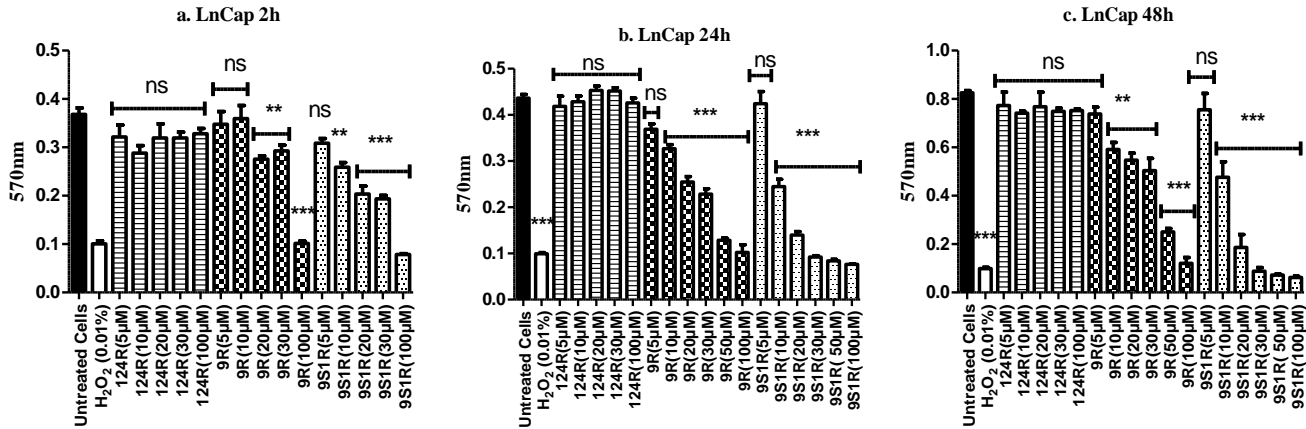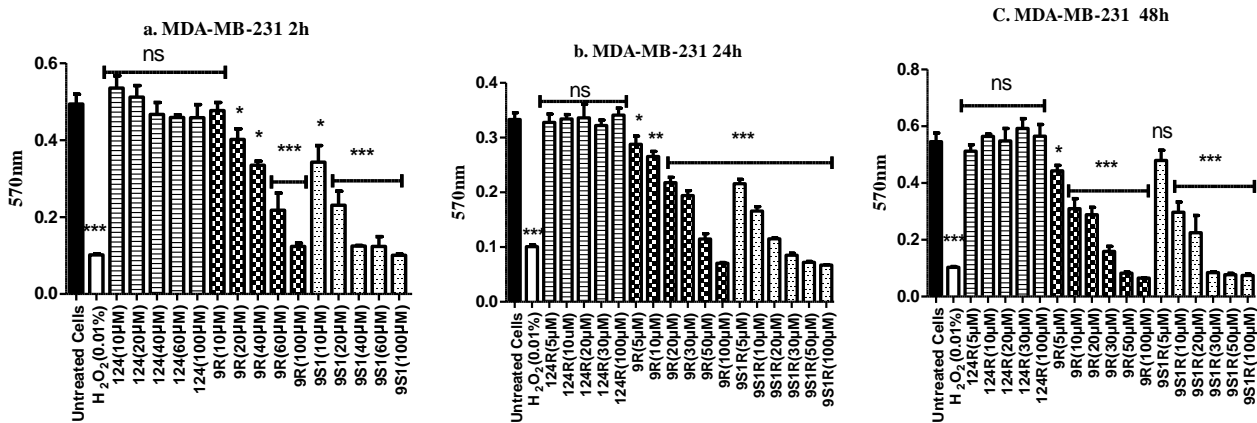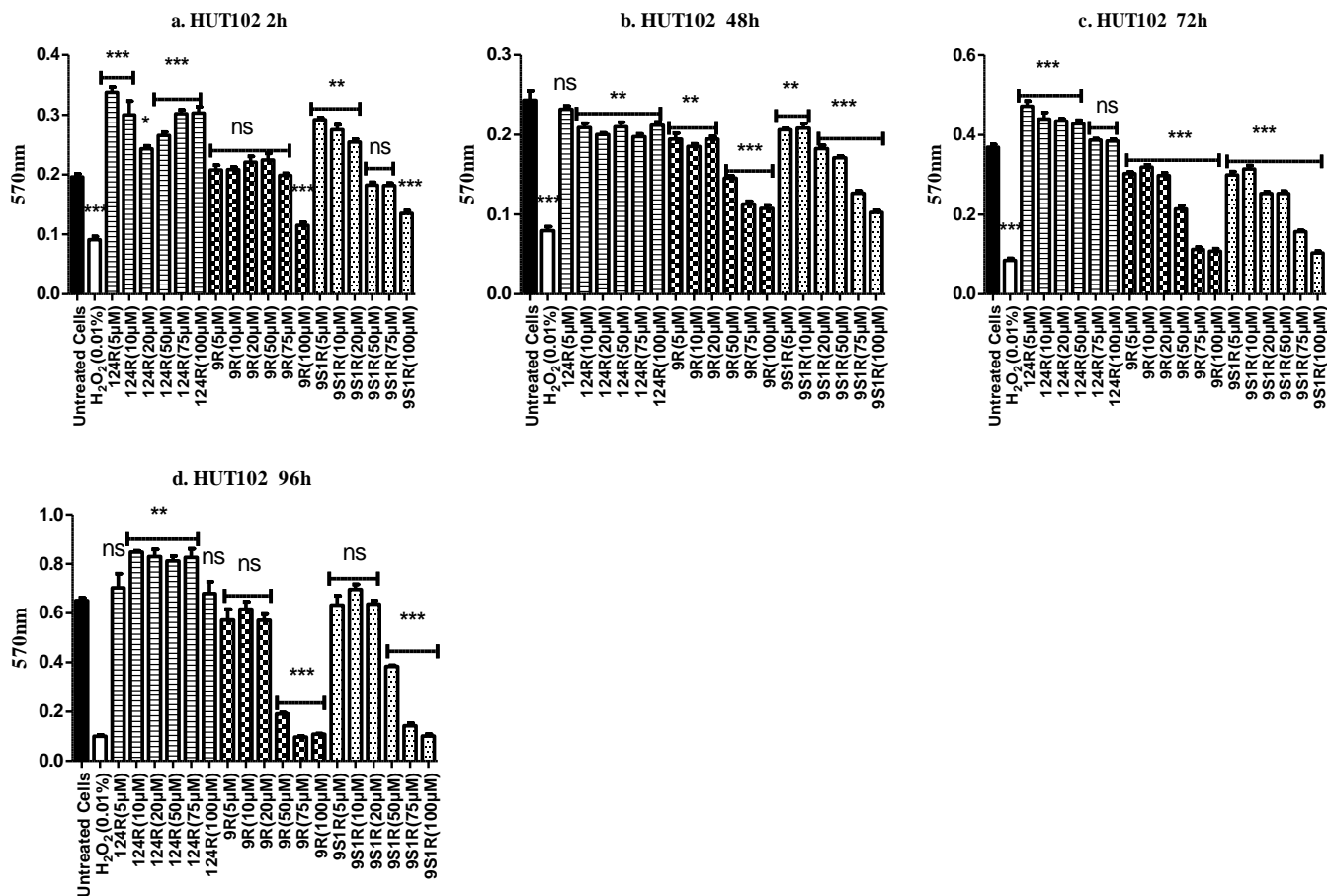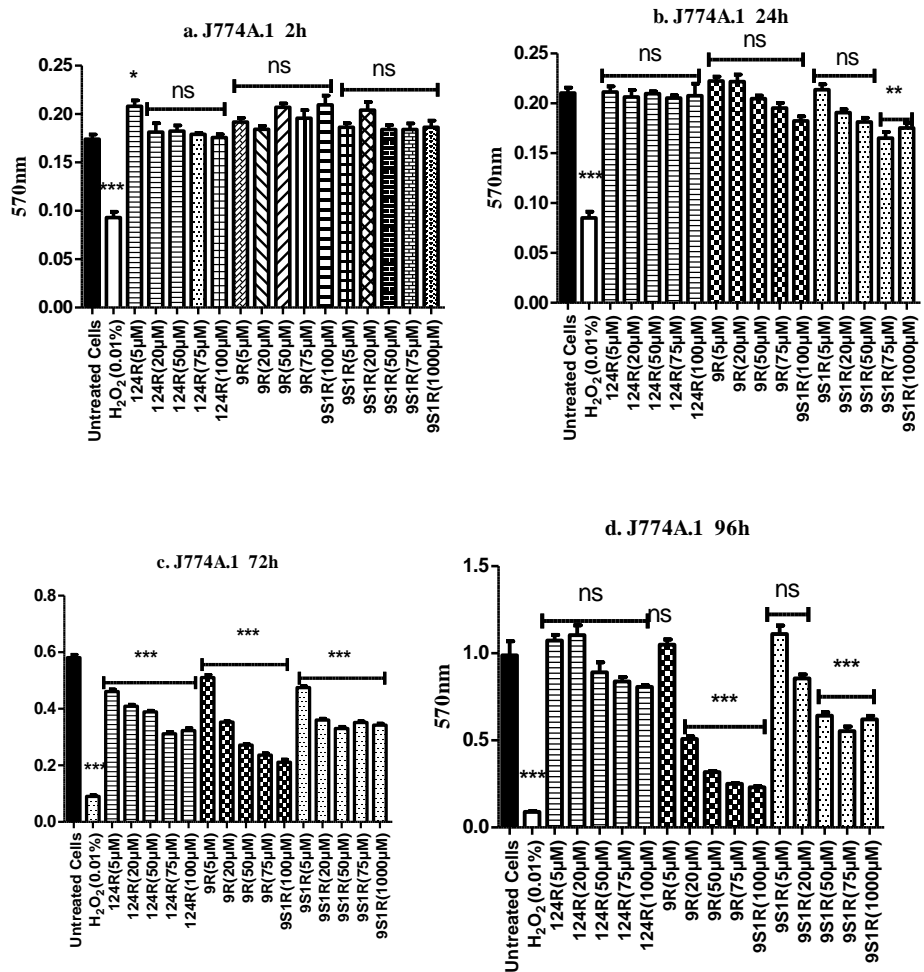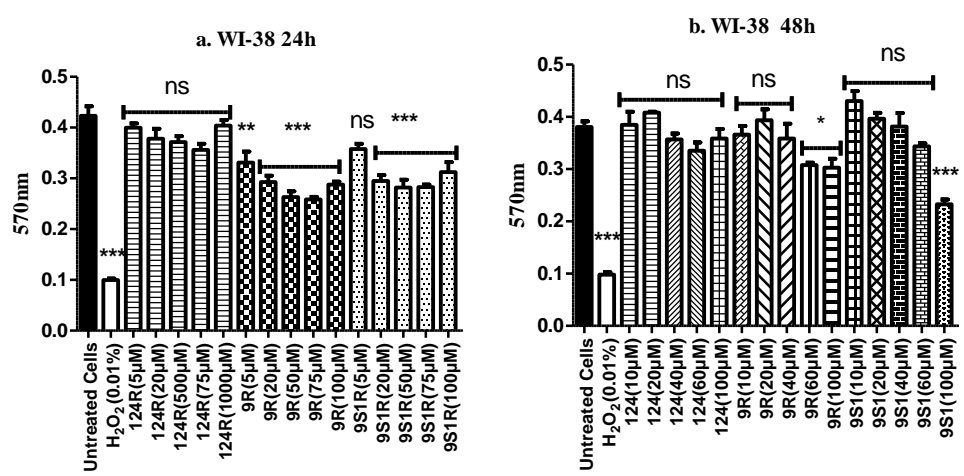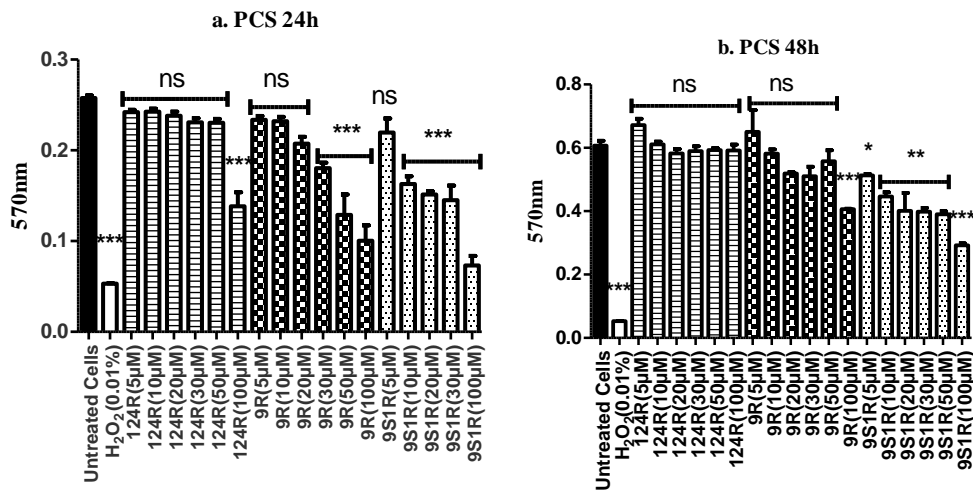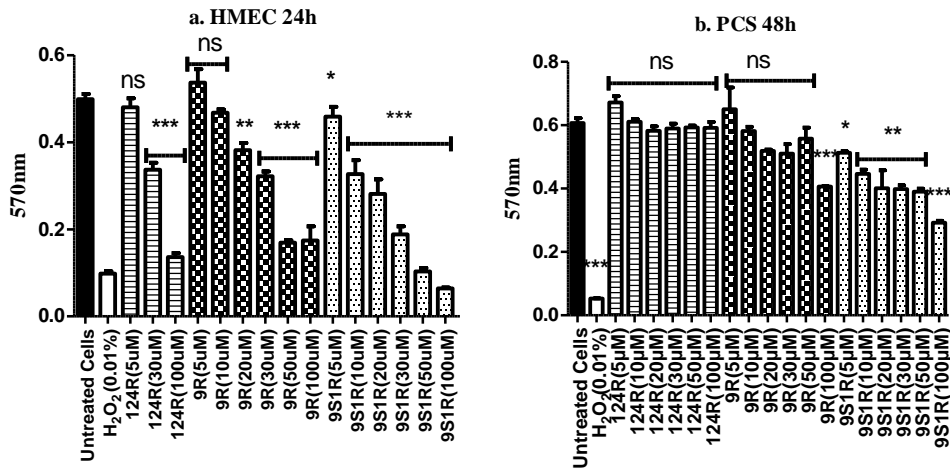
Suppl.R2. PolyArgNulloPs induced cell death (Live/Dead assay). LnCap cells (3000/well) were seeded in 96 well plates (white plates from Nunc). After 24 h incubation peptides 9R, 9S1R and 124R were added to the wells. Control conditions included untreated, and H_2O_2 (0.01%) treated cells. After 2h incubation, 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
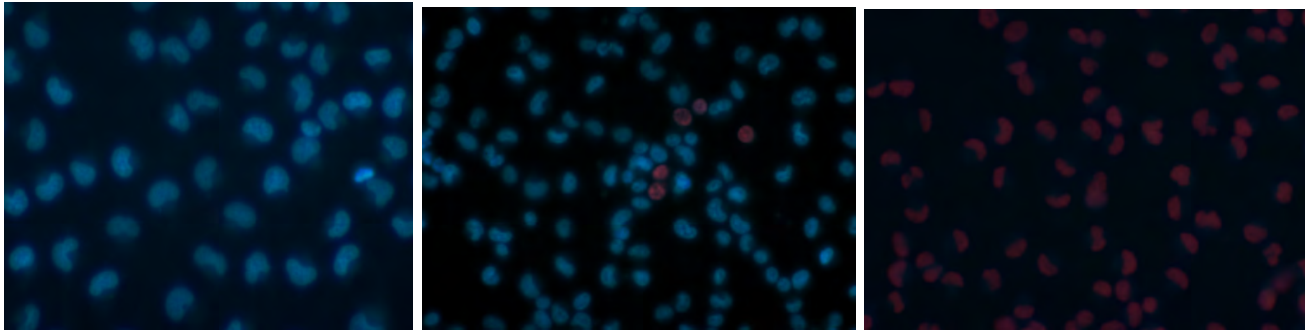
LnCap Untreated   LnCap 124R 100 µM   LnCap H_2O_2 0.01%
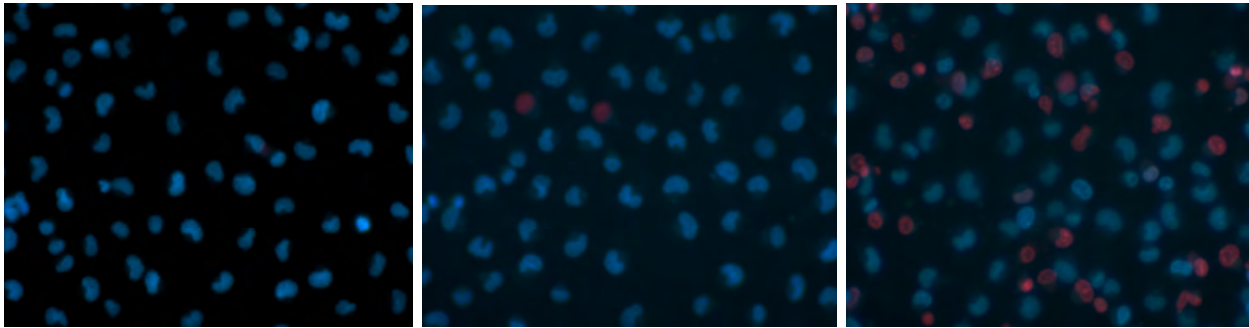
LnCap 9R 10 µM   LnCap 9R 50 µM   LnCap 9R 100 µM
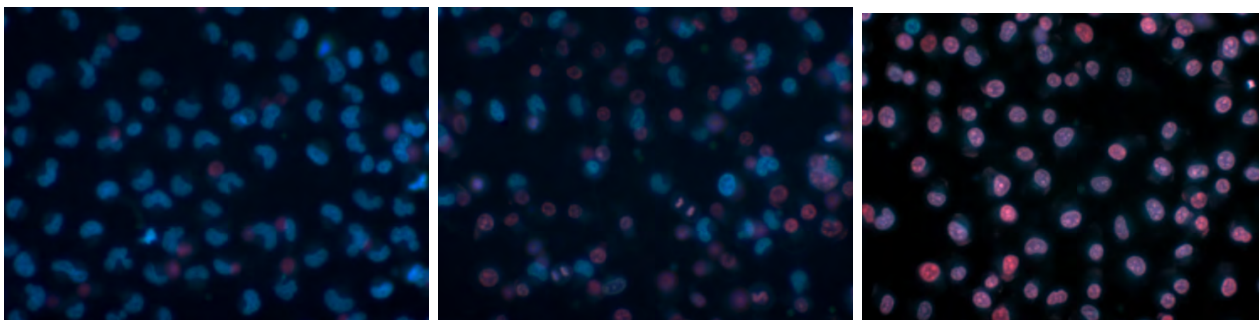
LnCap 9S1R 10 µM   LnCap 9S1R 50 µM   LnCap 9S1R 100 µM
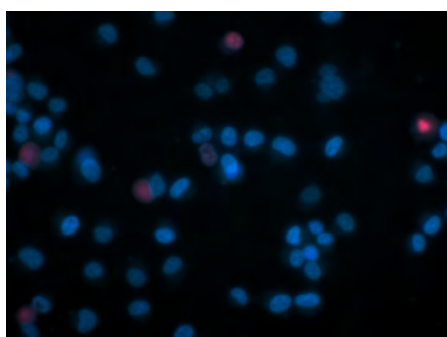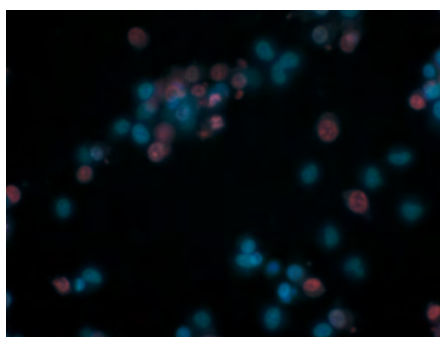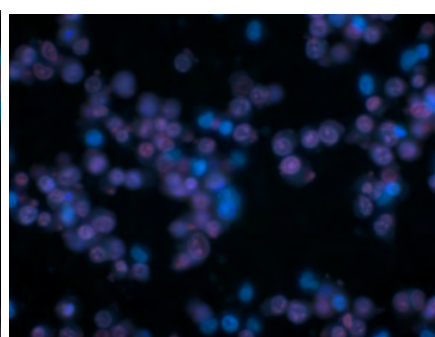
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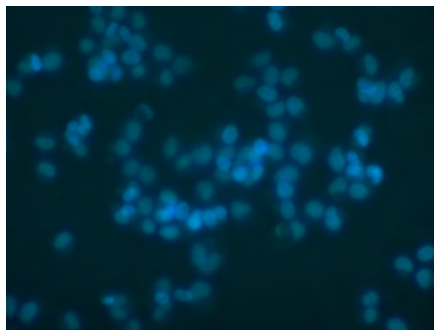
(B) MDA-MB-231

MDA-MB-231 Untreated   MDA-MB-231 124R 100 μM   MDA-MB-231 H_2O_2 0.01%
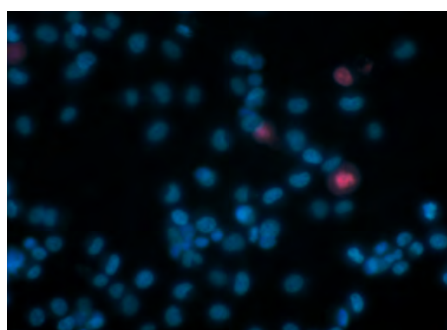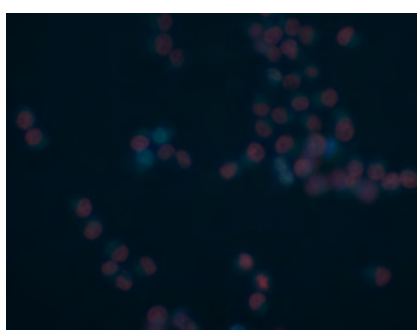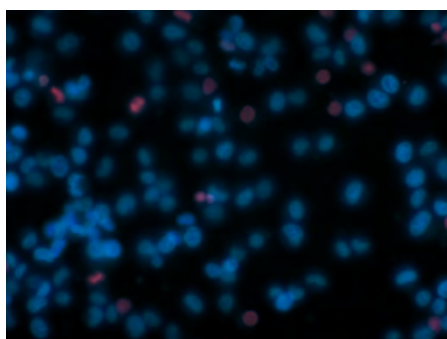
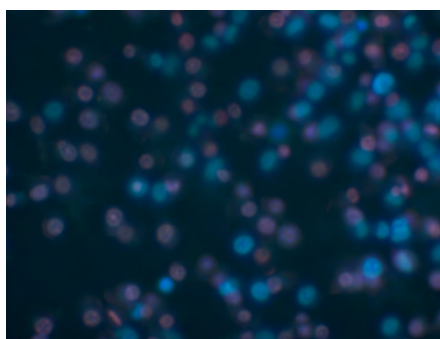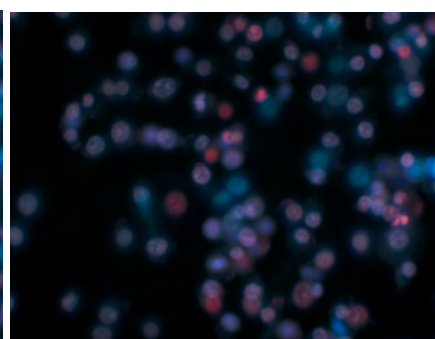MDA-MB-231 9R 10 μM   MDA-MB-231 9R 50 μM   MDA-MB-231   9R 100 μM

MDA-MB-231 9S1R 10 μM   MDA-MB-231 9S1R 50 μM   MDA-MB-231   9S1R 100 μM

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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.

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).

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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.
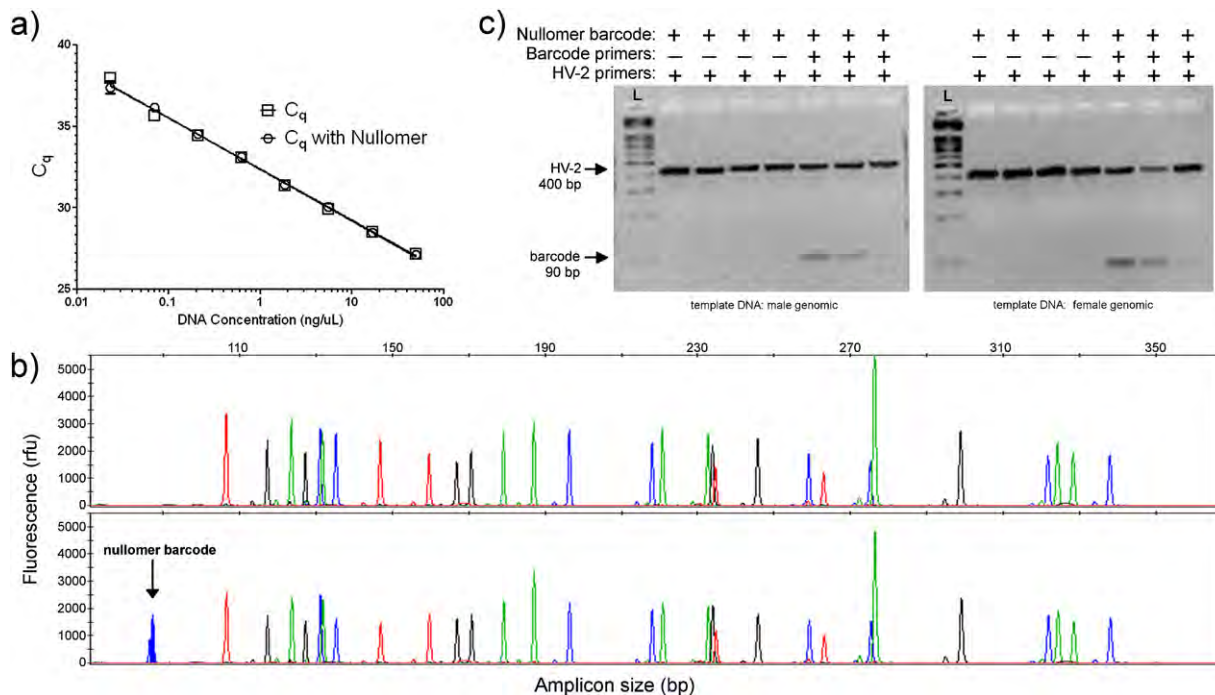
Fig. 1. a. Nullomer tag does not interfere with Quantifiler DUO™ DNA quantification. A standard curve was generated using control DNA supplied with the Quantifiler DUO™ kit from applied biosystems. The DNA standard was diluted, and the real-time PCR performed, according to the manufacturer protocol. 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 ($\bigcirc$) and without ($\square$) 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
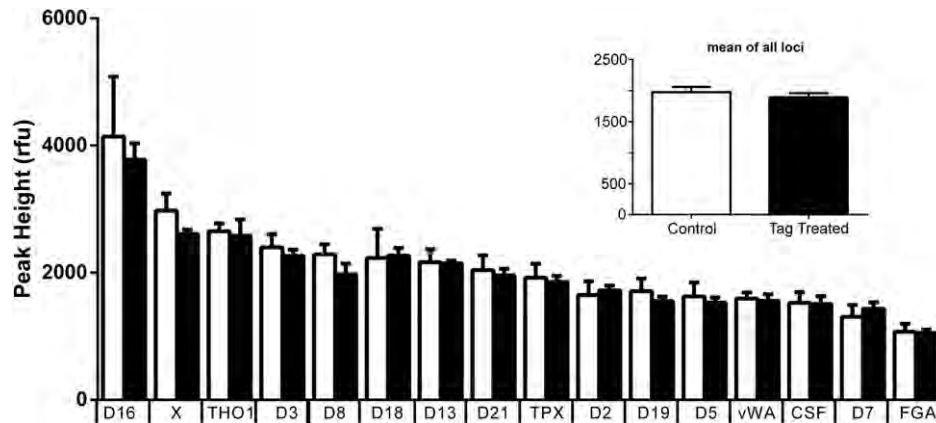
Fig. 2. Average peak heights (per locus) of human DNA amplified with the Identifiler™ kit, with and without nullomer barcode and FAM-labeled primers added. Experiment performed in triplicate. Results shown ± SEM. White bars: control. Black bars: with 3800 copies of nullomer barcode-1 added. Inset: average 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
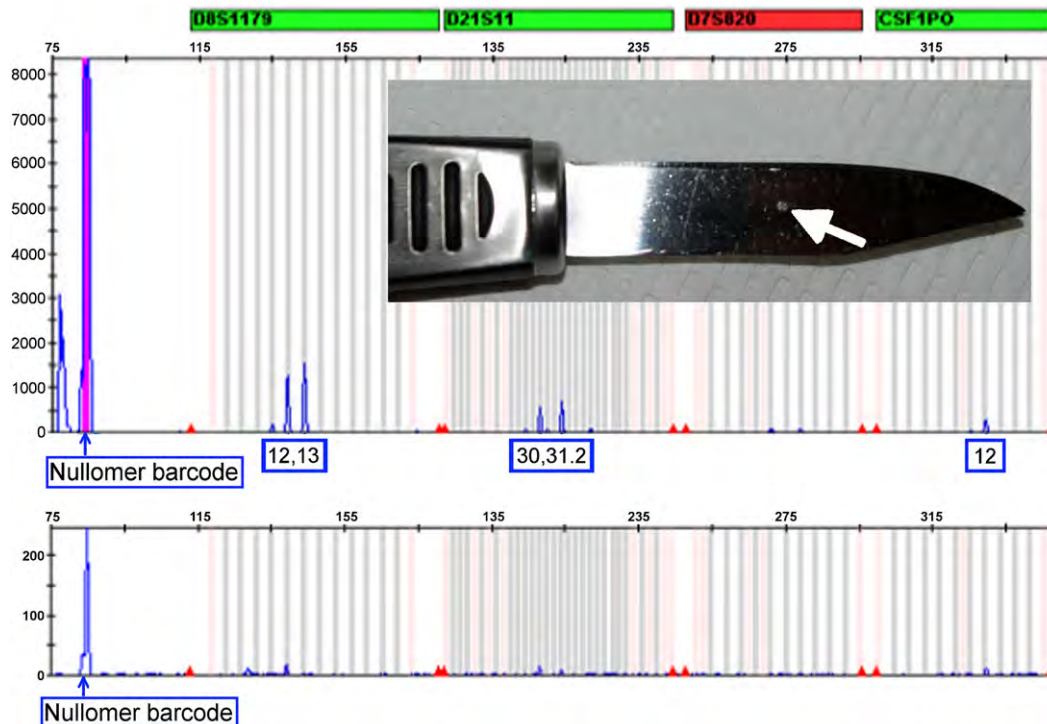

Fig. 3. Contamination of evidence with amplified DNA is detected with nullomer barcode, even when diluted 1,000,000 fold. Top panel and inset: DNA from amplified STR profile (amplified with nullomer barcode) was diluted 100,000 fold in water, and then 1ul of the dilution was applied to a newly purchased 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.
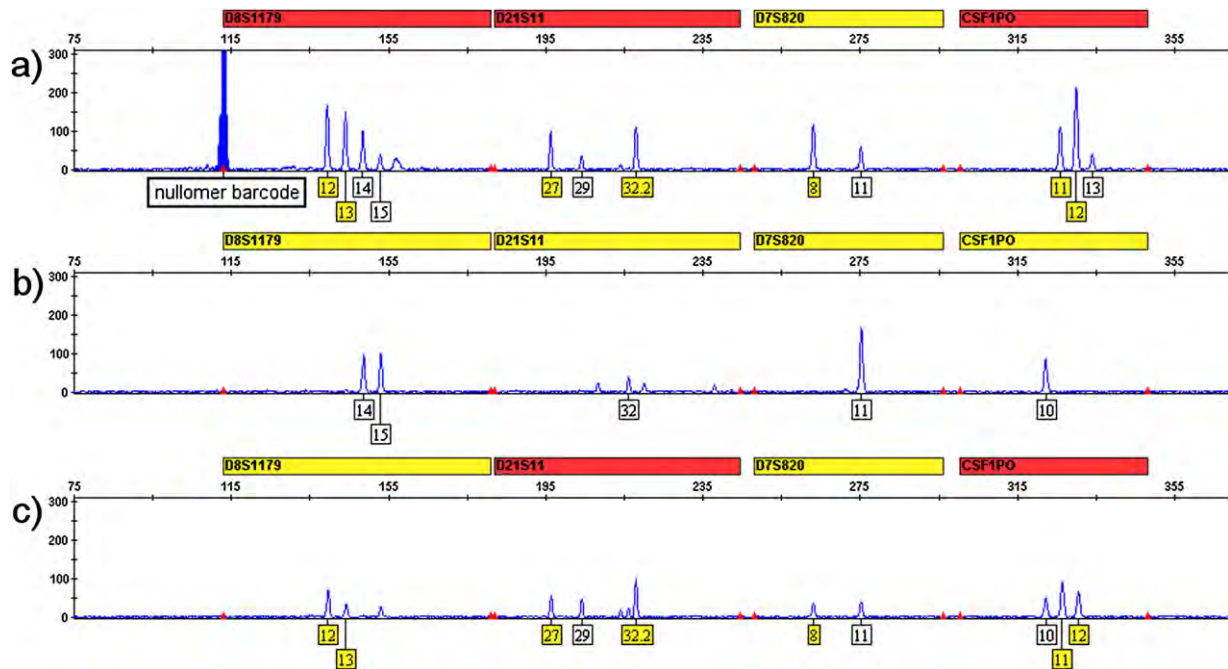
Fig. 4. Contamination of forensic DNA sample with reference DNA is detected with nullomer barcode amplification. a. Identifiler™ profile (blue channel) of DNA from coffee cup (forensic sample) that was contaminated with reference DNA. The presence of nullomer tag peak indicates that a contamination event 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 Identifier runs with and without nullomer barcode primers. Results are shown for the cup mixture profiles (Fig. 4) using Identifier, 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

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]

Conflict of interest

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

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.

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Preliminary results:

Our first Elsa U. Pardee Foundation grant allowed us to test the effects of 3 Nullomer Peptides 9R, 9S1R and 124R on the NCI-60 cancer cell panel. Table 1 shows the results of that testing, and categorizes the response of each of the NCI-60 cell lines to the 3 nullomers. The NCI-60 comprises human cancer cells from nine organ systems: kidney, prostate, ovary, hematopoietic system, colon, skin, breast, nervous system and the lung. We are currently completing our testing of these peptides on a series of normal cell lines as well. Our Pardee Foundation work has shown that the vast majority of NCI-60 cancer types (94.74%) are sensitive to 9S1 at 20 µM. At this concentration peptide 9S1R was effective against 68.42% of the NCI-60 lines. All 60 lines are resistant to peptide 124R, which is a 10 amino acid-length Nullomer that serves as control in our studies. Sensitivity to both 9R and 9S1R is shared by 66.67% of the NCI-60 cancer lines. Cancers that are sensitive to 9S1R but resistant to 9R make up 28.07% of the NCI-60 panel; while only one cell line HCC 2998 (colon cancer) is 9R sensitive and 9S1R resistant. Two cell lines A549 (lung cancer) and HT-29 (colon cancer) are resistant to both 9R and 9S1R. We have categorized sensitivity to 9R and 9S1R in Table 1. Cancers that are sensitive to both are designated I, to only 9S1 are II, to only 9R are III, and to neither are IV.

We were able to increase the efficacy of 9S1R by adding two glycines and extending the poly arginine tail from 5 to 7 amino acids, this peptide is designated 7R-GG-9S1 (Figures 1, 2 and 3). The 7 arginine residues increase cell penetration[1], and the 2 glycines enhance flexibility of the peptide upon interaction with its target[2]. The effects of these improvements can best be seen in colon cancer HT-29 (Figure 1), and drug resistant ovarian cancer SK-OV-3 (Figure 3). Significantly, our preliminary results show that 7R-GG-9S1 is effective against HT-29, which is resistant to both 9R and 9S1R.

We have recently selected another Nullomer, peptide 118 (Sequence WCWYW) to investigate. This peptide has been modified with the GG and 7R modifications and named 7R-GG-118. Both 7R-GG-118 and 7R-GG-9S1 are effective against HT-29 and A549, cancers which are resistant to both 9R and 9S1R.

	9R	9S1R	124R	Category
Kidney SN12C, CAKI-1	+	+	-	I
786-0, UO-31, A498, TK-10, RXF393	-	+	-	II
Leukemia RPMI 8226 HL-60 (TB), MOLT-4, CCRF-CEM, K-562	+	+	-	I
Ovarian NCI/ADR-RES, OVCAR-5, OVCAR-3, OVCAR-4, OVCAR-8	+	+	-	I
SK-OV-3	-	+	-	II
IGR-OV1	-	+	-	II
Melanoma LOX-IMVI, M14, UACC-62, SK-MEL-2	+	+	-	I
MDA-MB-43, SK-MEL-28	-	+	-	II
Breast MDA-MB-231, BT-549, MDA-MB-468	+	+	-	I
T-47D	-	+	-	II
Colon SW-620, HCT-116, COLO 205, HCT-15	+	+	-	I
HCC-2998	+	-	-	III
HT-29	-	-	-	IV
Lung HOP62, EKVX, NCI-H460	+	+	-	I
NCH-H522, NCI-H23, NCI-H226, HOP92, NCI-H322M	-	+	-	II
A549 ATCC,	-	-	-	IV
Prostate DU-145	+	+	-	I
PC-3	-	+	-	II
CNS SNB-19, SF-295, U251, SF-539, SNB-75	+	+	-	I
SF-268	-	+	-	II

Table 1. Sensitivity of the NCI-60 Cancer Cell Lines to Nullomers 9R, 9S1 and 124

HT-29 Colon Cancer
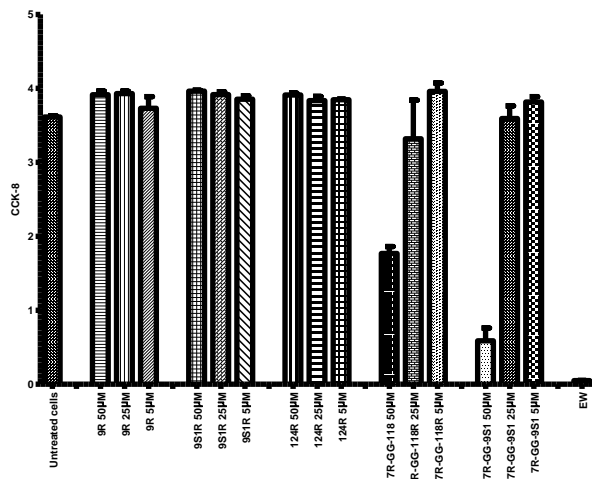**Incubation: 48h, CCK-8 4h**

Figure 1. Colon Cancer (HT-29) responds to new Nullomer 7R-GG-118. CCK-8 assay showing cell viability of HT-29. This cancer is one of only two cancers in the NCI-60 panel that is unaffected by Nullomers 9S1R and 9R. The new Nullomer 7R-GG-118 is effective at 50μM, as is modified 9S1 (7R-GG-9S1).

A549 Lung Cancer
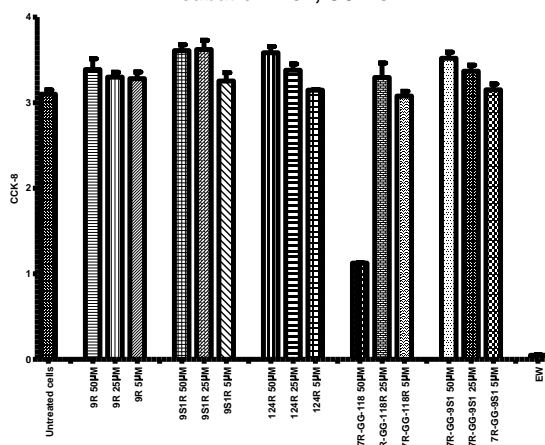**Incubation: 48h, CCK-8 4h**

Figure 2. Lung Cancer (A549) responds to new Nullomer 7R-GG-118. CCK-8 assay showing cell viability of A549. This cancer is one of only two cancers in the NCI-60 that is unaffected by Nullomers 9S1R and 9R. The new Nullomer 7R-GG-118 is effective against A549 at 50 μM.

SK-OV-3 Ovary Cancer
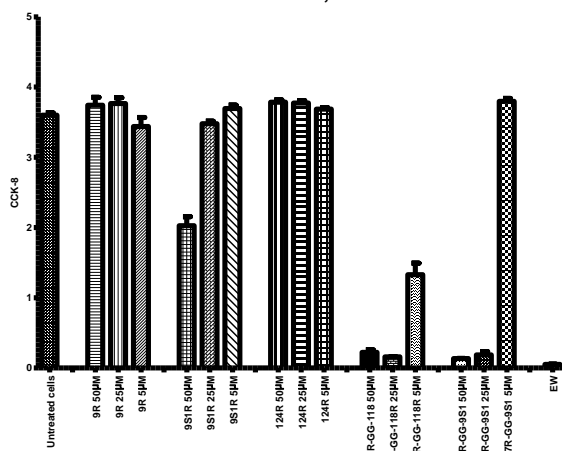**Incubation: 48h, CCK-8 4h**

Figure 3. Drug resistant ovarian Cancer (SK-OV-3) responds to new Nullomer 7R-GG-118. CCK-8 assay showing cell viability of SK-OV-3. This cancer cell line is resistant to a number of cytotoxic drugs including cisplatin and adriamycin. The new Nullomer 7R-GG-118 is however effective against SK-OV-3 at 5 μM; and the modified 9S1 (7R-GG-9S1) is effective at 25μM.