inquiro

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The University of Alabama at Birmingham

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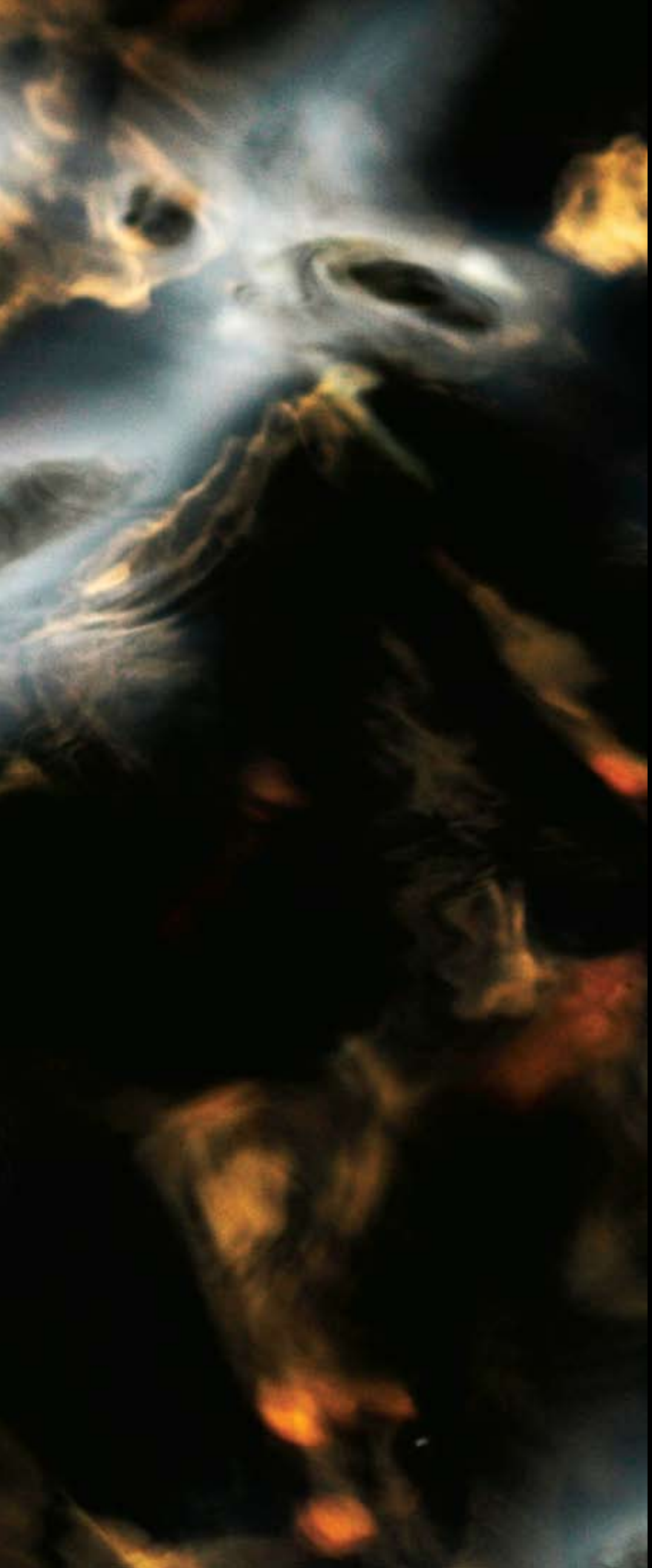

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Founded and staffed by undergraduate students at the University of Alabama at Birmingham, Inquiro is an annual research journal produced as an outlet for the publication of undergraduate scientific research. UAB is an excellent undergraduate research university, and with the addition of a journal such as Inquiro in which to publish their findings, the package is complete. Any undergraduate student at UAB, as well as any student participating in a summer program at the university, is eligible to submit research. The rights to every paper published in Inquiro are retained by the author, leaving each individual free to submit to and publish in a larger national journal or magazine. Students are invited to submit research papers, short reports derived from posters or research narratives throughout the year.

inquiro staff

from the editor

Inquiro: to search; to know.

Curiosity about the natural world has been a defining trait of our species since the beginning of time. Ancient civilizations all over the world developed methods to harness the power of nature and to explain the mysteries of the universe. The human spirit of discovery has survived millennia and flourishes now more than ever before. As we uncover the secrets of the human genome, the laws of modern physics, and the delicate balance of our environment, we embark on unprecedented journeys into the unknown. Furthermore, it is a journey which allows us to escape national borders, age differences, and even language barriers. This journey is for all humanity.

It was this same spirit of inquiry and curiosity about the natural world that spurred me to begin this journey six years ago. Early in my academic career, I strived to realize the real world application of all of the theories, facts, and concepts learned in my science classes. “When will I ever use this in the real world?” was the recurring question posed to my science teachers. As a high school junior, my question received its first clear answer when I began working on my first research project at UAB’s Department of Pathology.

On the very first day when I was instructed to make up a TE buffer, I realized that the stoichiometry I learned in chemistry was essential. When I dissected transgenic mice to determine the areas and extent of metastasis, I knew that I needed a basis in anatomy to differentiate the mouse organs. The entire semester was full of epiphanies like these. I no longer thought of science as just a body of knowledge and facts; I realized that science is a way of thinking that increases our understanding of the world and enables us to save and improve lives.

Now, as a college senior, my initial curiosity has developed into a strong enthusiasm for research. I currently work with Larry DeLucas O.D, Ph.D at UAB’s Center for Biophysical Sciences and Engineering. Having the opportunity to enter a top research lab in my freshman year at UAB and work alongside a world class researcher, astronaut, and mentor is the starting point for many of my achievements. I’ve had the chance to present my research in poster sessions, national conferences, and ultimately be recognized as a 2008 Barry M. Goldwater Scholar. Looking back, none of my accomplishments would have been possible without the environment at UAB that encourages undergraduate research. The lab experiences that I have acquired here have enabled me to lay a foundation upon which I can build my future career as a researcher and health professional. What is most exciting is that I don’t stand alone in my research endeavors.

Many of my peers and undergraduate students in various disciplines throughout UAB's campus are getting to work with top-tier researchers early on. With the newly created Office of Undergraduate Research and other initiatives, the systems in place are being strengthened to increase the level of scholarly activity in which UAB students participate. *Inquiro*, now in its second issue, has exceeded all of the standards set by the inaugural issue. The undergraduate research journal has been well received by the students, faculty, and administration. This year we have had the highest level of involvement and submissions by undergraduates at UAB. The success of *Inquiro* is a testament to the unique atmosphere at UAB that encourages undergraduate students to participate in research.

Inquiro was established because of the need for an outlet through which the high caliber research that UAB undergraduates produce can be featured. Every year, many students participate in departmental honor theses, summer fellowships, and various laboratory experiences. *Inquiro* provides students with the opportunity to display their work before peers and faculty from other disciplines, as well as to the university community as a whole.

The second issue of *Inquiro* marks the continuing of a new tradition at the University of Alabama at Birmingham. This journal gives students the opportunity to partake in the scientific process and prepare their research for publication; each paper is reviewed by one or two faculty members. The concept of the undergraduate journal has previously been embraced by other universities such as Harvard, Columbia, and Yale. With the continued success of *Inquiro*, UAB students now have the opportunity to ascend to the undergraduate publishing ranks with the best and brightest students in the nation. Please join us as we blaze the trail for the future of undergraduate research at the University of Alabama at Birmingham!

—Larry Lawal
Chief Editor

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Nobel's Awarded

The 2008 Nobel Prizes were announced in early October and awarded at a ceremony in Stockholm, the capital of Sweden, in early December. The Noble prize in medicine and physiology was awarded to Harald zur Hausen from Germany for his discovery of human papilloma viruses (HPV) causing cervical cancer, and Francoise Barre-Sinoussiand and Luc Montagnier from France for their discovery of the human immunodeficiency virus (HIV). In Chemistry Osamu Shimomura, Martin Chalfie, Roger Yonchien Tsien from the United States shared the prize for their discovery and development of the green fluorescent protein, GFP. GFP, is composed of 238 amino acids, and it has a quaternary structure. This protein, which was originally extracted from jellyfish *A. Victoria*, will produce a green light, if it is exposed to UV spectrum. This protein has been used in many biological studies, such as transformation of plasmids into bacteria. The Nobel prize in physics was awarded to Yoichiro Nambu from Japan for the discovery of the mechanism of spontaneous broken symmetry in subatomic physics, and Makoto Kobayashi and Toshihide Maskawa, from Japan, for their discovery of the origin of the broken symmetry which predicts the existence of at least three families of quarks in nature.

science news

UAB: Where Scientists Want to Be

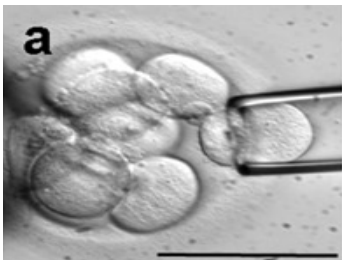
Shweta Patel

According to a survey *The Scientist* magazine conducted, the University of Alabama at Birmingham ranks No. 5 in the U.S. as the "Best Place to Work in Academia." Life science researchers were surveyed on such things as the type of environment they worked in and their relationship with fellow colleagues. Last year, UAB was ranked 47th in the same survey. The drastic change in ranking is attributed to UAB's focus on interdisciplinary collaborations. With such cutting-edge facilities like the new Shelby Interdisciplinary Biomedical Research building and such university-wide research centers like the Comprehensive Cancer Center, researchers have a greater opportunity to work together in solving problems. With an engineer down the hall and a pathologist down the street, making interdisciplinary connections is easier. This positive research environment is especially beneficial to undergraduates interested in graduate school and research opportunities. UAB challenges its students to think outside of the box, to ask the right questions. At such a high ranked university, this is where opportunities are available for the taking; this is where scientists want to be.

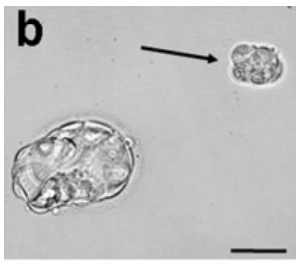
Stem Cells without Embryo Destruction: Ending the Ethical Debate?

Adam W. Scott

Until recently, the derivation of all human embryonic stem cell (hESC) lines involved the destruction of embryos, which has caused considerable ethical concerns. In fact, the process is prohibited in many countries, and in the United States, there is no federal funding for research involving use of hESC lines from destroyed embryos. However, the firm Advanced Cell Technology was able to derive five hESC lines without embryo destruction. In their technique, a single cell (blastomere) was removed from each embryo. The hESC lines generated appeared to have the same characteristics of other hESC lines, including pluripotency, self-renewing capacity, and ability to differentiate into derivatives of all three germ layers. Their method has the potential to end the ethical debate surrounding the use of embryos to derive stem cells, and could double or triple the number of stem cell lines available within a few months.



Blastomere Biopsy



Biopsed blastomere (arrow) and parent embryo developing next to each other

Key Mechanism behind Cancer Metastasis Is Explained

Atbin Doroodchi

A study in the journal *Cell* reports that scientists have reached one of the most important milestones in the fight against cancer, discovering the mechanism of cancer metastasis. *Metastasis*—Greek, meaning next displacement—is the spread of a tumor from one place to other places. Successful treatment will be less effective and more difficult, if the tumor has begun to metastasize. Recently, a group led by Professor Chris Marshall at The Institute of Cancer has discovered the mechanism of the two key processes that allows cancer cells to change the way they spread in the body. The research team discovered that the competition between two proteins 'Rho' and 'Rac' allows tumors to spread throughout the body. These two proteins belong to a family that is responsible for signaling GTPase which regulates the synthesis of RNA during transcription. The research found that activation of Rac encourages tumor to adopt an elongated shape, and Rho encourages cell to adopt a round shape. Different shapes allow tumors to survive in different situations. For instance, round tumors are able to survive better in the bloodstream. Hopefully, this research will be useful in developing successful cancer treatments.

Einstein's Celebrated Formula, $E = mc^2$ is Finally Corroborated

Atbin Doroodchi

Einstein's mass–energy equivalence theory, $E = mc^2$, which was discovered in 1905 in the paper "Does the inertia of a body depends upon its energy-content?", and led to the invention of the first atomic bomb, has been proven right, with the help of a computational effort by French, German, and Hungarian physicists. This theory emphasizes that any mass has an associated energy and vice versa. A group led by Laurent Lellouch of France's Centre for Theoretical Physics, have assigned the calculations for estimating the mass of subatomic particles. Protons and neutrons compromise smaller particles known as quarks. The irregular fact is the mass of quarks is only five percent of the mass of protons and neutrons. This observation violates conservation of mass. The group found that the missing 95 percent comes from the energy from the movements of quarks in the atom. In other words there is an energy associated with the mass of a quark. In general any mass has an associated energy. Even though this equation was used for the invention of the first atomic bomb, it had yet to be proven right. However, now it is proven for the first time after 103 years.

Minerals on Mars Point to More Recent Presence of Water

Atbin Doroodchi

The presence of water in the form of ice on Mars has been known for many years, as ice spots can be easily seen from space. Since the presence of water can be associated with the possibility of life, this phenomena has been an interesting topic in the scientific community. However, scientists still want to know how long ago Mars was wet. In an article in the November issue of the journal *Geology*, scientists working with data from NASA's Mars Reconnaissance Orbiter (MRO) report that they have spotted extensive deposits of opals on the surface of Mars. These opals were found in an area that appears to be two billion years old, younger than previous areas that contained water. Opals are mineraloid gels known as hydrated silicas. Opals are silicon based compound that contain moles of waters in their molecular formula. Basically, water is wedged into the silica. The water content in opal can vary between three to ten percent. The formation of opals needs liquid water. This data proves the presence of water in younger and over a more widespread area.

(*Minerals like opal are shown in cream in this image.)*

Cloning Frozen Animals

Pratik Talati

Imagine being able to clone an extinct species that has been frozen for over 10,000 years. With the current technology, this futuristic idea does not seem too far away. Not too long ago, research scientists were able to clone the infamous sheep Dolly from an adult somatic cell by transferring the nucleus. Currently, researchers in Japan have been able to apply a similar technique to clone mice that have been frozen for as long as 16 years.

Teruhiko Wakayama and colleagues at the Center for Developmental Biology at Japan's RIKEN research institute in Yokohama are the first to manage to clone the mice even though their cells had burst. Cells normally burst during the freezing cycle, sometimes compromising DNA integrity, and many researchers believe that these cells cannot be cloned because no live cells are available. Cryoprotectants can be used to prevent cells from bursting during the freezing process, but only if they are applied before cells are frozen. The process used for cloning the mice is similar to the one used in Dolly: taking the nucleus out of an egg cell and replacing it with the nucleus of an ordinary cell from an animal that is going to be cloned. Under the right conditions—a chemical trigger or an electric shock—

the egg divides as though a sperm has already fertilized it.

According to Wakayama's team, of all of the sources for the cells, they have determined that the brain is the best source. However, no one yet has been able to clone a live mouse from a brain cell, which poses a bit of a mystery for these scientists.

Many animals have been cloned, including sheep, pigs, cattle, mice and dogs. Livestock breeders prefer cloning to produce elite herds of desirable animals, while doctors want to use cloning technology in human medicine. For example, initial studies have shown cloning as a therapeutic option for mice with Parkinson's disease, and if further studies prove fruitful, this technique of somatic-cell nuclear transfer can be used for treatment in Parkinson's and other diseases in humans.

As for now, it still remains to be shown whether nuclei can be collected from frozen bodies without cryoprotectants and if they will be viable for generating offspring after a nuclear transfer. The prospect of resurrecting extinct species, such as woolly mammoths, is quite appealing for many researchers because many of these animals have been found preserved in ice for thousands of years. Perhaps several decades later, we may be able to revive these creatures from their frozen graves. Until then, we must wait patiently for science to lead the way.

New Clues Emerge from Cellular Damages In Huntington's Disease

Pratik Talati

Huntington's disease (HD) is a genetic neurodegenerative disorder that has a late onset, usually in individuals in their 40s. In 1872, George Huntington first recognized the disorder in patients who had physical symptoms such as uncoordinated, jerky movements and stiffness or slow movement. Almost all individuals diagnosed with HD exhibit many of these physical symptoms, and many of them exhibit cognitive decline and psychiatric symptoms including anxiety, depression, and aggression. If many of the physical and psychiatric symptoms are evident in individuals younger than 20, then the condition is known as Juvenile HD.

It is currently known that HD is caused by a mutation in a single gene called the huntingtin gene (HTT). The mutated HTT gene codes for mutated proteins with abnormally long repeats of an amino acid called glutamine (abbv: Q). In many neurons, the defective "polyQ-expanded" protein is misfolded and clumps together, thereby damaging and killing these cells. However, very little is known about the mechanism that causes these cells to be damaged and die.

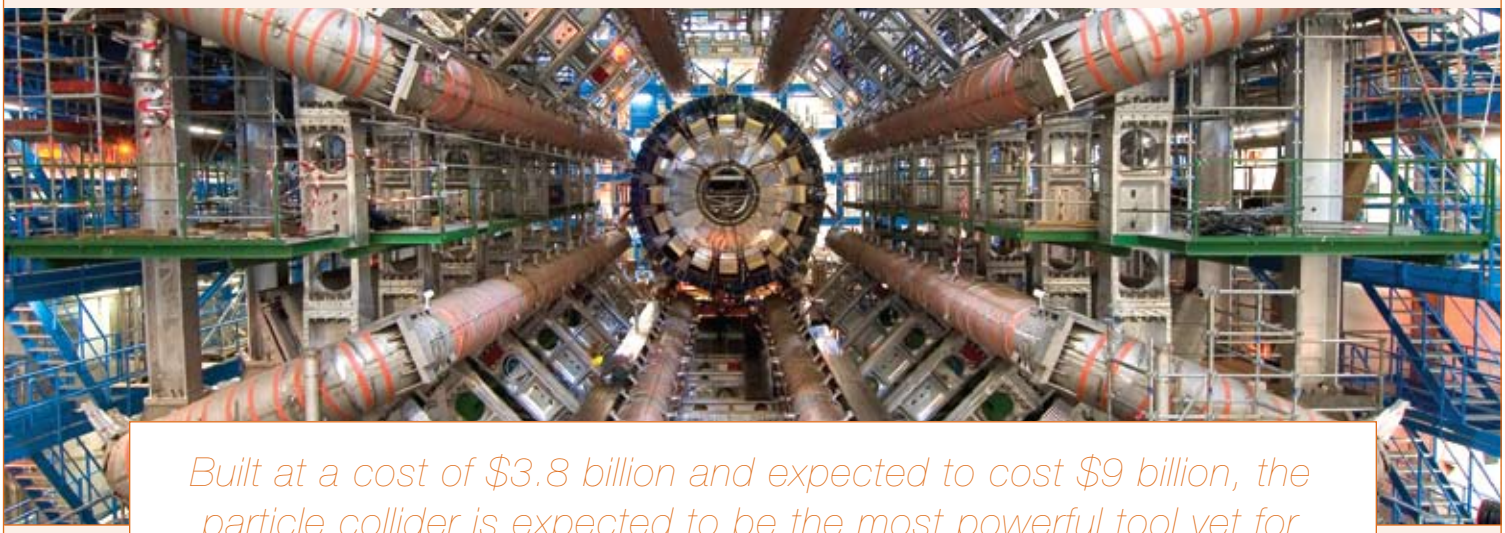
Martin Duennwald, a principal scientist at Boston Biomedical Research Institute in Watertown, Massachusetts, has been using rat cells that model neurons and mouse striatal cells, brain regions involved heavily in HD. When these cells have been generated with polyQ-expanded fragments, they quickly showed problems with proteins that had been marked for degradation in the endoplasmic reticulum (ER), an organelle used in folding proteins. These proteins were not tagged for degradation in areas of the cell outside of the ER. Also, even though there are many mechanisms in the cell that help misfolded proteins refold with the help of a class of proteins called chaperone proteins, polyQ-expanded proteins don't elicit a response for chaperone proteins. Thus, the cell is not able to alter protein folding in mutated proteins, and the low protein quality may be able to cause cell damage and death.

Duennwald then went on to uncover the basis for this breakdown: The polyQ-expanded fragments glom onto the key VCP/Npl4/Ufd1 protein complex that aids in the transport and degradation of the proteins that flunk quality control in the ER. However, when he genetically modified cells to overexpress two crucial proteins in the protein complex, the toxic effect dropped.

These interesting findings may be useful in finding a treatment for the disease. By targeting the cell's protein quality control mechanisms, researchers may be able to provide novel and effective treatment options for individuals with HD or other illnesses with polyQ-expanded proteins.

Repairs on Large Hadron Collider Will Take Longer than Expected

Courtney Sparkman



Built at a cost of $3.8 billion and expected to cost $9 billion, the particle collider is expected to be the most powerful tool yet for physicists hoping to discover the secrets behind the laws of physics.

Repairs on the Large Hadron Collider could take until early summer and cost at least 25 million Swiss francs, which is equal to 20.4 million US dollars.

An electrical malfunction shut down the particle collider in September. According to a spokesman for the European Organization for Nuclear Research (known by the French acronym CERN), James Gillies, fixing the particle collider will probably take longer than expected.

Spokesman Gillies is projecting the massive physics experiment to restart at the end of June or later. "If we can do it sooner, all well and good. But I think we can do it realistically (in) early summer," he said.

The collider, which is in an underground facility close to Geneva, Switzerland, uses a ring of super-cooled magnets to push protons. These magnets push protons to energies and speeds never observed and crash the protons together in order to detect a host of new particles.

Built at a cost of $3.8 billion and expected to cost $9 billion, the particle collider is expected to be the most powerful tool yet for physicists hoping to discover the secrets behind the laws of physics. From the tiny scale of quantum mechanics to the huge scale of galaxies and black holes, the particle accelerator should help scientists better understand these laws.

The collider has had quite a few operational problems even before its brief running in September.

After nine days of operation, a meltdown of a small electrical connection caused the release of a large amount of liquid helium into the 27 kilometer long tunnel, CERN officials reported.

The facility will shut down operations during the winter in order to conserve energy at a time when Europe has a higher power demand.

Repair work on this ambitious project is time consuming because it has to be warmed to normal temperatures before any repairs can be made. The magnets are cooled at temperatures near absolute zero to make them superconductive. Such an environment makes particle acceleration easier.

Researchers plan to work on repairs during the winter months and hope to have the magnets cold enough for operation by August 2009. Though the repair work is within the budget, the delays are a blow to the physics community, which has been anxiously waiting for the results from this experiment.

One thing physicists expect to find is evidence of the Higgs boson, a particle believed to impart mass on most other particles.

The Higgs boson is a key component in the Standard Model of particle physics, which has helped explain the interactions of quarks, electrons, photons, and quarks for over thirty years. The particle has never been found, and there is no scientific consensus regarding what the basic characteristics of the particle are.

Out of the Lab and Into the Field

Aaron Neal

It started instantly, jolting me out of a deep sleep. The noise was deafening. The shaking was violent. "What just happened? Is this turbulence? Are we going to crash?" Then, just as quickly as it had begun, it ended. "What is going on here?" My natural bewilderment from just waking up was further exacerbated by a sleepless six-hour layover in Lima. As I finally began coming to my senses, I realized what had happened. The lights flipped on as a stewardess reached for the microphone. "Bienvenidos a Iquitos."

When I first entered the laboratory of Dr. Julian Rayner, Ph.D., I never imagined my research would take me away from UAB, much less out of the laboratory itself. From the moment I walked through the door, my preconceived notions of a research environment were confirmed. Bottles of chemicals occupied every shelf. "Biohazard" and "Radioactive" labels dotted walls and cabinets. Lab coat-clad graduate students hovered from station to station. Dr. Rayner's lab personified the traditional stereotype, or so I thought.

I never imagined my research would take me away from UAB, much less out of the laboratory itself.

As I stood taking in my new surroundings, Dr. Rayner arrived and greeted me. "Welcome to the lab Aaron," he said in a curiously unique accent. "Before we discuss anything, let's go grab some coffee." While I partook in the lab's morning ritual, Dr. Rayner and I exchanged bits of casual conversation. Our discussion slowly migrated from light chatting to the very reason we were meeting. "So you know I work with malaria," he began. I did, of course. I had spent the past week virtually memorizing his webpage, in addition to reading his most current publications and reviewing the disease itself. Dr. Rayner's work targets the blood-stages of the malaria-inducing parasite *Plasmodium falciparum*. It is during this part of its life cycle that the parasite wreaks havoc on the red blood cells of its host, resulting in the clinical symptoms that define malaria. By targeting these stages, the onset of the disease can be prevented and the parasite can be eliminated. In short, Dr. Rayner is seeking the Holy Grail of malaria research: a vaccine. With our cups emptied, the conversation slowly became casual again. "Have you ever been out of the country?" he asked as we left the table. "Stick with the lab and you may find yourself in Peru."

As I stepped off of the plane, the words of Dr. Rayner reverberated through my mind. I never imagined our conversation ten months ago would ever amount to anything. Sure, international field research sounded great, but it was unrealistic to a sophomore who had never left the United States. Besides, even if I did have the opportunity to research abroad, how would I ever be able to afford such a trip? As I pondered the once improbable situation, a stifling wave of humid, jungle air brought me back to reality. Regardless of the circumstances, I was spending the next eighteen days in Iquitos, Peru.

As my taxi literally raced from the airport, I took a quick moment between the coconut trees and thatched huts to ponder my research project. Since August 2007, I have explored the genetics and immunogenicity of a promising malaria vaccine candidate known as PfMSP6. Though experiments in the laboratory have showcased the antigen's potential, lab results do not always translate to the real world. Because of this, Dr. Rayner formed a collaboration with another researcher at UAB, OraLee Branch, Ph.D. Since 2003, Dr. Branch has maintained a longitudinal cohort study in the jungles surrounding Iquitos. In this malaria hypo-endemic environment, villagers are monitored and treated for malaria. For every infection, blood samples are collected, from which *P. falciparum* DNA and anti-PfMSP6 antibodies can be isolated. Using these components, I am able to escape the confines of lab samples and gauge the real-world potential of a PfMSP6-based vaccine. Though most of these samples are available for me to analyze at UAB, there are some that never leave the Iquitos lab. After all, problems do arise when trying to transport malaria samples into the malaria-free United States.

Little was accomplished after I arrived at Dr. Branch's rental house. Even though I was able to meet the project staff and experience the full force of the language barrier, I was unable to start any lab or field work. This initial disappointment only fueled my eagerness to travel into the jungles and begin my research. Over the next two weeks, I had several opportunities to visit the study villages and analyze samples. My first trek into the jungle led to the community of Zungarococha, the largest of the study villages. There, I was able to follow a field doctor as he diagnosed patients and collected blood. Despite the many interesting things I observed, the cooperation of the patients made the biggest impression on me. No questions were asked when signatures were needed. No skeptical glares were given when blood was drawn. Everyone willingly complied with whatever the doctor said. As I began thinking

about this unusual trust, the explanation suddenly hit me. While I discussed the dangers of malaria in my comfortable UAB lab, these people were threatened by the disease every day. The small hope that our work may eventually find a cure was enough to win their complete cooperation.

At the end of a long day of visiting families and collecting blood, it was time for the field team to transport the fresh samples to the Iquitos research labs for analysis. These Peruvian labs had many of the same amenities as Dr. Rayner's lab, save a constant power supply, air conditioning, and clean water. Despite working in such "primitive" conditions, I would do the same at UAB if there were that many carambola, mango, and papaya trees on site. Once at the lab, I separated the samples into erythrocytes, which contained the parasites, and serum. The erythrocytes were further processed to extract parasite DNA, while the serum was stored for later use in antibody-quantifying ELISA assays. After following the sample collection process from patient to freezer, I developed a deep respect for the vials of clear liquid I used daily at UAB. For the first time, I realized that the *P. falciparum* DNA did not simply come from the freezer at the end of the hall; it came from the Amazon rainforest over 3000 miles away.

Eighteen days in the Amazon passed by much faster than I had expected. The samples were collected, my research responsibilities were fulfilled, and I even had sufficient results to prepare a poster for the National Collegiate Honors Conference. As I boarded the plane for Lima, and ultimately Birmingham, I carried with me much more than luggage and souvenirs. I flew out of the jungle with a deep respect for Dr. Branch, Dr. Rayner, and every person enlisted in the war against malaria. Without scientists willing to risk everything on the front lines of disease research, very little would be accomplished in the safety of modern laboratories. The more I thought about it, the more I realized that field research is critical not only to every aspect of what I do at UAB, but to virtually any field of research.

I took my seat on the plane destined for Birmingham. As we ascended, the gentleman next to me began to make conversation. "Where are you coming from?" He asked with slight interest. "The Peruvian Amazon," I replied, capturing his full attention. "Let me tell you a little about my trip…"

Why Research?

Kim Trawick

...the work in the lab has allowed me to be exposed to other biologists working in the same field of research. I am working with a lab in Texas to process some of the tissue samples. Also, I was fortunate to be able to attend an International Echinoderm conference and have corresponded with biologists specializing in sea urchin research as far away as Japan.

My first foray into research came as a high school student, when I was required to complete a research project for my biology class. At first, I regarded the project as a necessary element to succeed in my class. After beginning the project, though, I found the entire research process to be quite interesting. I was able to design a project based on a question that I was genuinely curious about. After spending some time on a lake and noticing a surprising amount of boating oil on the surface of the water, I began to wonder how the organisms were affected by human interaction. In the kitchen of my parents' home, I set up a homemade laboratory, and worked diligently to find an answer to my question. The experiment investigated the effects of boating oil on "elodia densa". I measured the amount of growth of the "elodia densa" at varying levels of oil contamination to see if the pollution caused any negative effects. Not only did this experience allow me to foray into the field of research, but also allowed me to discover what was required to make a scientific presentation both in a written and verbal presentation.

While this experiment was very simplistic compared to the in depth studies I now conduct, this was the first time that I regarded science as truly exciting. Working to find the answers to my questions became like a puzzle. While I had all of the pieces in front of me, I had to fit the pieces together to be able to understand and see the larger picture. The experiment actually made all the concepts that were just words in a book, come to life. The

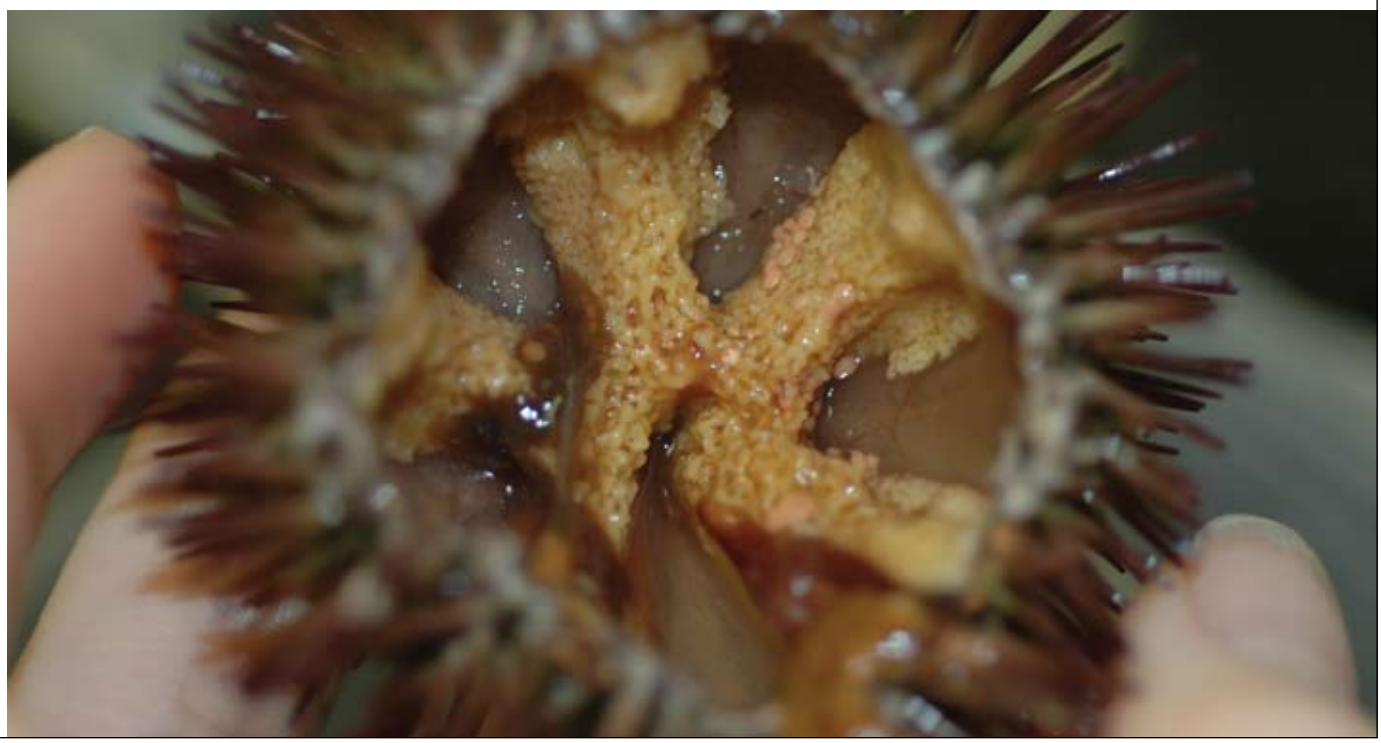
newfound excitement that I experienced when looking at my data and seeing the results of the hard work that I was doing, allowed me to be able to make successful research presentations at local, regional and international levels.

Now, I find the classes that I am taking to be incredibly exciting because I can use my hands-on experience to garner a better understanding of the written material.

In the summer of 2003, I was awarded a research internship with UAB as the result of winning a regional science fair. I started work in Dr. Stephen Watts' biology lab, studying the effects of crude oil on sea urchin embryos. The eight weeks that I spent under his tutelage were invaluable. I finally realized that conducting research was my passion. In addition, I was introduced to research equipment and procedures that I previously did not have access to, such as procedures for spawning and conducting histology on the sea urchin gonads. Work with the sea urchins involved areas such as collecting samples from Florida, reproduction, caring for the embryos, monitoring their growth and the necessary dissections. The collection of sea urchins also sparked an interest in scuba diving, something that I hope to incorporate into my future career. I realized that additional coursework such as cell biology and invertebrate zoology would be necessary to enhance the understanding of the research results to the full extent. I have since continued my work in his lab, though my area of expertise has changed over time. In addition to the actual experiments, the work in the lab has allowed me to be exposed to other biologists working in the same field of research. I am working with a lab in Texas to process some of the tissue samples. Also, I was fortunate to be able to attend an International Echinoderm conference and have corresponded with biologists specializing in sea urchin research as far away as Japan.

Apart from my love of research, I have found a new appreciation for science as a whole. Growing up, I had a difficult time in science classes. I was never able to visualize what the teachers were talking about. Now, I find the classes that I am taking to be incredibly exciting because I can use my hands-on experience to garner a better understanding of the written material. The research experience has also fostered a deeper passion for caring for the environment and the true impact of the coexistence between wildlife and humans. These are areas I hope I can continue and expand upon in the future.

The first dabble into research changed my future. Before research, my goals were to become a lawyer, and now I see that there is nothing more perfect for me than to be a scientific researcher. I highly encourage those students with an inquisitive mind to try research. If nothing else, it will open your mind to appreciating the vast and amazing world of science.



research narrative

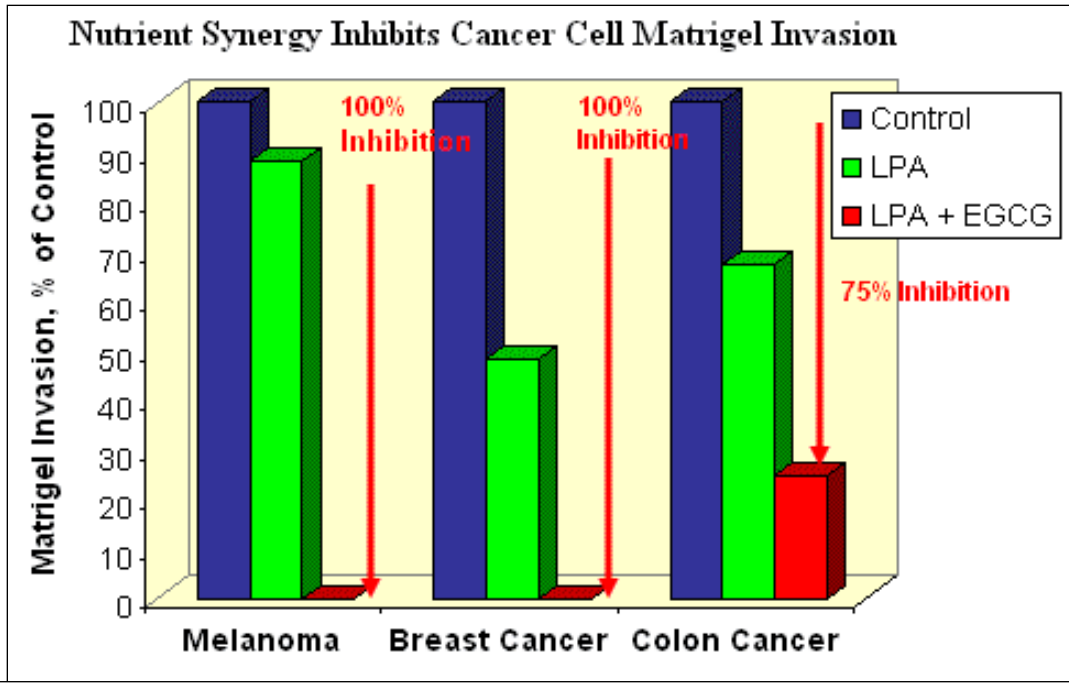
Medicinal Benefits of Green Tea?

Adam Threet, Natalie Mitchell

Cancer has forever remained a seemingly taboo topic amongst society; however, as scientists advance closer to locating a cure for the disease, the general public continues to develop an extremely positive attitude toward efforts in the laboratory. Undoubtedly, as forward progression results, the majority of society becomes increasingly interested in this subject matter. Through improved awareness, individuals freely converse about aspects of cancer and develop personal responses to related ongoing events as dictated by media coverage. Yet, the question of when absolute effective treatment will be discovered for the disease still remains uncertain. Dr. Trygve Tollefsbol, of the UAB department of biology, believes a beverage may hold the answer to the aforementioned problem.

The integration of students into a laboratory setting where new research is being conducted provides a valuable experience regarding a rather innovative topic.

Research Focus:
His research concerns aging epigenetics, a process in which heritable changes occur that do not involve mutations but rather modifications of DNA. The proliferation of cancer cells may ultimately be attributed to the presence of telomerase, which typically maintains the length of chromosomes. Located in only stem and cancer cells, the presence of this enzyme allows such cells to continually replicate without death. Green tea consists of several components that may help reduce or treat various medical conditions. The drink contains certain antioxidants (catechins/polyphenols) that may selectively inhibit the growth of cancer. In particular, EGCG affects the activity of telomerase, eventually causing these cells to be unproductive due to the inability to replicate. The inhibition of cancer cells due to the presence of LPA and EGCG is indicated in the following graph completed in a study done by Netke, et. al (2003):



Clearly, the same idea depicted in the data relates to the research currently being conducted by Dr. Tollefsbol. Nearly all of the breast cancer cells ceased growth (indicated in red and labeled 100% inhibition) when introduced to LPA and EGCG.

Laboratory Experience:
Visiting Dr. Tollefsbol's laboratory in conjunction with the Science and Technology Honors Program provided a valuable freshmen experience. This unique opportunity, not provided to many students until junior or senior year of undergraduate level, allows students to learn about current faculty research and participate in hands-on experiences. Certainly, the level of work produced by professors initially seems rather intimidating; however, upon entering Dr. Tollefosbol's laboratory, an extremely calm atmosphere emerged, easing any nerves present. After meeting with Dr. Tollefsbol to discuss his current efforts and our questions, we completed various stations developed by his researchers to simulate important biotechnology techniques. The major aspect of inquiry was whether or not EGCG inhibited DNMT1 expression in cells. Overexpression of DNMT1 has been detected in several cancers due to increased methylation. Following protocols resulted in the ability to emerge with knowledge concerning the significance of correctly adhering to lab procedures. The importance of proper equipment use was stressed more so than actual experimentation results. Thus, we only briefly discussed

the expected outcome present through photographed gels using a UV transilluminator. Armed with the information that EGCG does inhibit DNMT1 expression, students were able to create additional research questions:

1. Is there another polyphenol besides EGCG that might inhibit the growth of cancerous tumors? If so, does it slow down or stop the replication of another gene besides DNMT1 related to cancer growth?

2. Is there another gene or sequence of genes that EGCG needs to target to repress the growth and stop the spreading of cancer?
The development of such questions clearly stimulates independent thinking, a valuable characteristic when working in such a setting.

Dr. Diane Tucker, director of the UAB Science and Technology Honors Program, selected the visit with Dr. Tollefsbol due to the exponential-like growth taking place in the area of cancer therapies. Thus, the integration of students into a laboratory setting where new research is being conducted provides a valuable experience regarding a rather innovative topic. In addition, the development of personal responses to such subject matter encourages the creative thought process. This ultimately provides the opportunity to determine a particular field of interest to complete a research proposal.

Societal Implications:
While recent work regarding ceasing the expression of the DNMT1 gene and inhibiting tumor growth has occurred, much more experimentation must be completed to apply this knowledge to other cancer types. Since EGCG does slow down the replication of DNMT1, one must ultimately question the amount of tea consumption necessary to achieve effective results. Perhaps drinking boiled green tea leaves as opposed to the bottled beverage also yields contrasting outcomes. Undoubtedly, additional research needs to be conducted to obtain concrete data. Until this occurs, it is ultimately up to the consumer to decide the means in which to alter diets to receive the benefits of green tea.

Work Cited

Netke SP, et al., (2003) A specific combination of ascorbic acid, lysine, proline and epigallocatechin gallate inhibits proliferation and extracellular matrix invasion of various human cancer cell lines. *Research Communications in Pharmacology and Toxicology: Emerging Drugs*. 2:37-5.

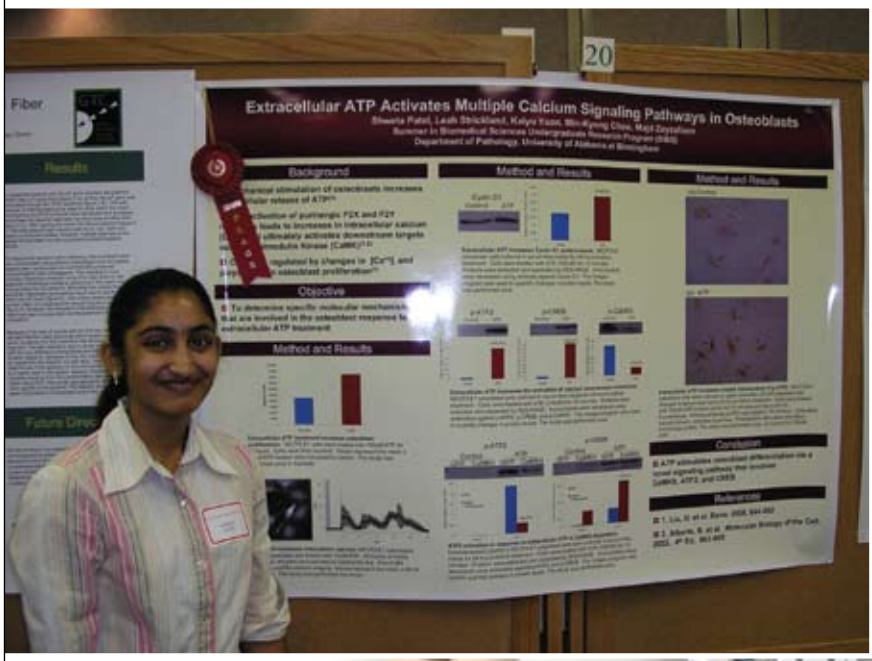
research narrative

Lessons from SIBS

Shweta Patel

Running four 10% SDS-PAGE gels, probing nitrocellulose membranes with a secondary antibody for a Western Blot, and taking care of osteoblast cell lines were all aspects of my work this summer that greatly enhanced my multi-tasking skills. In the summer of 2008, I was accepted in the Summer in Biomedical Sciences (SIBS) Undergraduate Research Program at the University of Alabama at Birmingham. As one of seven, I was involved in cutting-edge research and participated in a series of seminars.

Working in Dr. Majd Zayzafoon's lab in the Department of Pathology, I learned about bone-forming osteoblast cells. Specifically, I studied the calcium signaling pathways involved in the osteoblast response to ATP. Through this lab, I refined my pipetting, learned how to make SDS gels, mastered protein assays, and nurtured osteoblast cells. I had to take care of the cells as if they were my children: splitting them, feeding them, and treating them. Besides learning useful laboratory techniques, I came to an important realization…research is 95% failure and 5% success. More importantly, I learned to appreciate that the ounce of success overshadows all the failures, because the miniscule success can lead to the groundbreaking cure.



This summer was full of epiphanies for me. For one, I actually used things I learned in the classroom. For example, calculating concentrations came in handy when making buffers. Dealing with my project, I already had a basis for the signaling pathways because I had an overview of them in my Cell Biology class with Dr. Stephen Watts. Even all the countless organic chemistry lab reports provided help for my final research paper.

Because of SIBS, I gained insight into a possible PhD or MD/PhD career. Through the summer

seminars and participating in a graduate-level class, I learned more about the opportunities available in research. I have always been interested in research. The scientist in me emerged in high school when I had to do science fair projects for four

The best part about doing research for me is that I am participating in biomedical research behind the scenes of medicine. Knowing that the data I collect can someday advance research and ultimately be applied by physicians in their medical practice gives me great satisfaction.

years. Being involved in research labs at UAB since I was in high school, I already had a spark. The SIBS program fueled that spark into a blazing flame. Participating in this program has actually posed a dilemma for me: should I become a doctor or should I become a researcher. Coming into college, I was geared more towards medicine. After SIBS, actually living almost like a graduate student (without the high level of stress), I knew I could not decide between medicine and research. These two fields complement each other; I truly believe you can't have one without the other. So, my decision is made: MD/PhD is the route for me. Knowing that this road is a winding path, I am ready for the bumps, being flexible and open.

My advice to students who have an intellectual curiosity is to try research. Go into a lab and learn. In addition to learning laboratory techniques and protocols you will gain an appreciation of how scientific research is actually conducted and how questions are framed. If you like the experience your array of career choices will certainly be broadened.

The best part about doing research for me is that I am participating in biomedical research behind the scenes of medicine. I am the one contributing to the efforts to find a cure. Knowing that the data I collect can someday advance research and ultimately be applied by physicians in their medical practice gives me great satisfaction.

short report

"Utilizing a Unique Study in the Peruvian Amazon to Assess the Malaria Vaccine Candidate Antigen Plasmodium falciparum Merozoite Surface Protein 6":

Aaron Neal, Stephen Jordan, Ana Oliveira, OraLee Branch, Julian Rayner

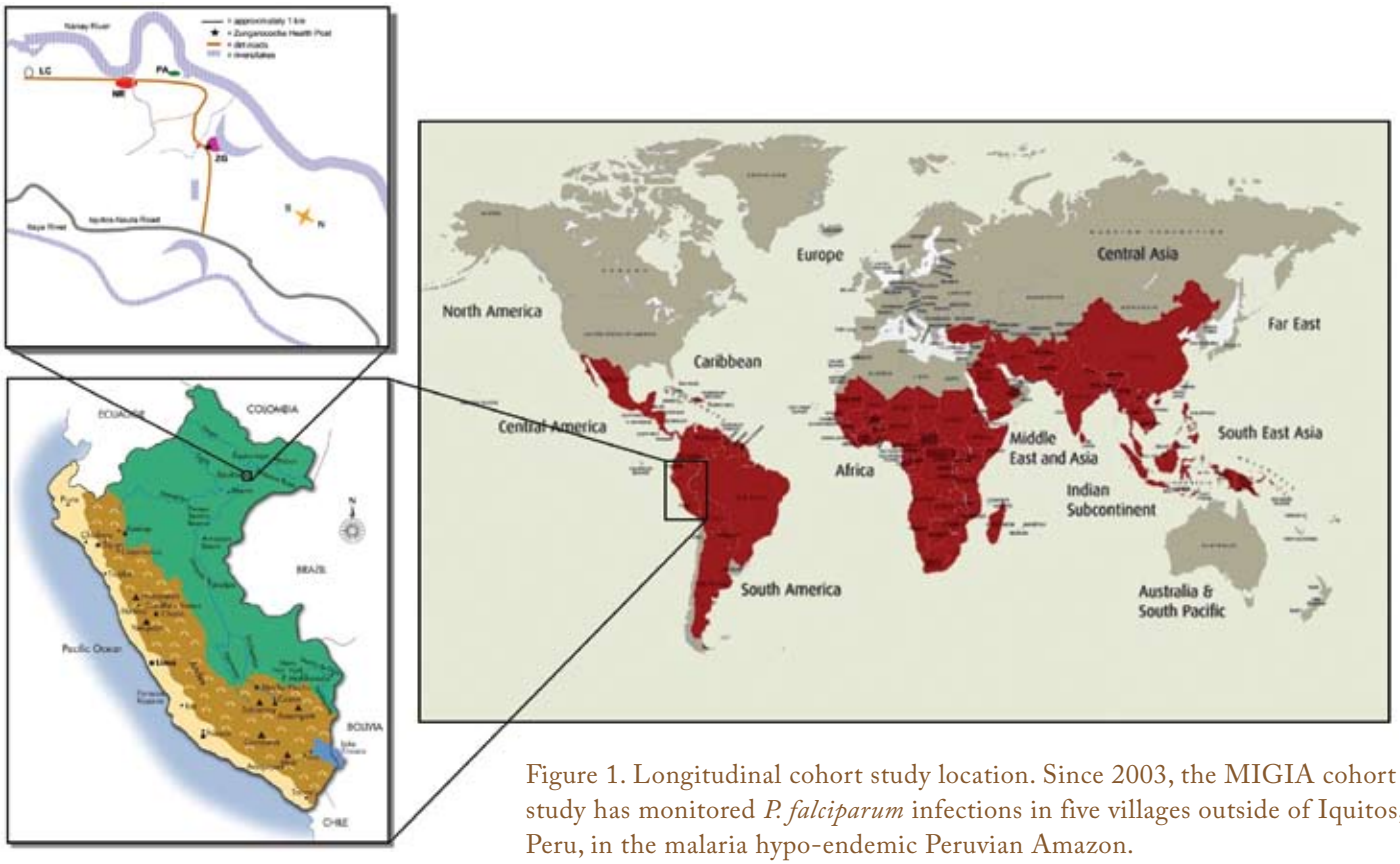


Figure 1. Longitudinal cohort study location. Since 2003, the MIGIA cohort study has monitored *P. falciparum* infections in five villages outside of Iquitos, Peru, in the malaria hypo-endemic Peruvian Amazon.

Abstract

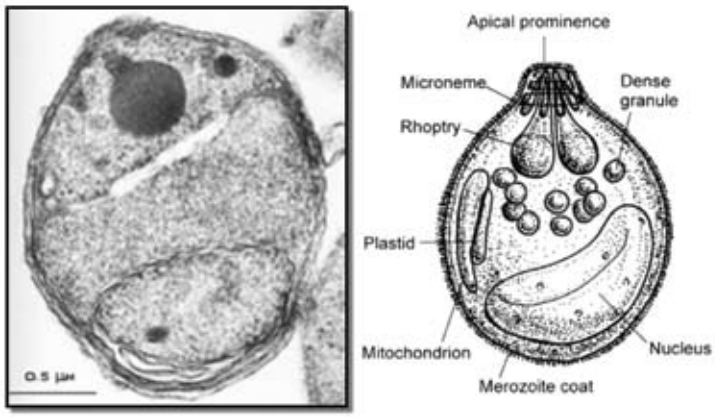
Malaria is responsible for 1-3 million deaths annually, mostly in children under age five. Almost all malaria deaths are due to infection by the protozoan parasite *Plasmodium falciparum*, but no effective vaccine exists to prevent *P. falciparum* malaria. This ongoing study focuses on *P. falciparum* Merozoite Surface Protein 6 (PfMSP6), a dimorphic antigen present on the surface of the *P. falciparum* parasite during erythrocyte invasion. Although PfMSP6 is a promising vaccine candidate, there currently exists no data to establish which PfMSP6 domain(s) should be included in a vaccine. To provide this data, we utilized samples from a unique longitudinal cohort study in the hypo-endemic Peruvian Amazon in which blood and serum samples from *P. falciparum* infected patients have been collected over four years. Using these serum samples, indirect ELISAs were conducted in Iquitos, Peru, to establish which sub-domain(s) of PfMSP6 are targeted by naturally generated antibodies. Serum from 243 patients, separated into time of infection samples and post-infection samples, was tested against the N-terminus of the PfMSP6 allele types HB3 and Dd2. For the time of infection samples, serum from 243 patients was tested, resulting in 79 positive responders, 49 of which responded against both allele types. For the post-infection samples, serum from 56 patients was tested, resulting in 5 positive responders. Though this data is very preliminary, the results indicate that PfMSP6 generates a lower level of positive responders than is seen with other Merozoite Surface Proteins. However, when anti-PfMSP6 antibodies are generated, they appear to cross-protect between allele types, a rarity among *P. falciparum* vaccine candidates. This initial exploration of the immunogenicity of PfMSP6 will provide a foundation for later studies to establish which PfMSP6 sub-domain(s) and/or allele(s) should be included in a malaria vaccine.

Introduction

Mosquito-borne *Plasmodium* parasites cause 300-500 million cases of malaria each year, resulting in a devastating 1-3 million deaths worldwide, mostly in children under the age of five (Breman, 2001). Of the four protozoan parasites that

Figure 2. (left) Conducting indirect ELISAs in Peru. All indirect ELISAs were conducted on-site in Iquitos, Peru, in the labs of the MIGIA cohort study.

Figure 3. (below) SEM image and schematic diagram of *P. falciparum* merozoite. Merozoites are the invasive form of *P. falciparum* during the blood stages. PfMSP6, a component of the surface coat, is exposed to the antibody-mediated immune system.



cause the disease, *Plasmodium falciparum* is responsible for the majority of the infections and almost all of the deaths, making it the most aggressively-combated target. The rapid spread of drug resistance amongst the parasite has further increased the need for an effective *P. falciparum* vaccine.

Of particular interest in vaccine research is the merozoite, the invasive form of the parasite during the blood stages (See Figure 3). Prior to erythrocyte entry, the merozoite is exposed to the antibody-mediated immune system, a critical event for vaccine success. Discovered in early 2001, the vaccine candidate PfMSP6, a 36 kDa secreted antigen associated with the surface of *P. falciparum* merozoites (Trucco *et al.*, 2001), has shown promise but lacks significant data. The antigen consists of a highly-conserved C-terminal domain and a variable N-terminal domain that gives rise to two distinct allele classes, HB3 and Dd2 (Pearce *et al.*, 2004) (See Figure 4). Though individuals protected against *P. falciparum* malaria have a strong antibody response to PfMSP6 (Singh *et al.*, 2005), it is unknown whether individuals develop antibodies against one or both domains, or against one or both allele classes.

The purpose of this project was to collect preliminary data on the anti-PfMSP6 antibody responses of patients in a malaria-endemic environment. To accomplish this, we utilized a unique longitudinal cohort study in the hypo-endemic Peruvian Amazon. (See Figure 1). Since 2003, the Malaria Immunology and Genetics in the Amazon (MIGIA) Project has documented and collected blood samples from natives in communities outside of Iquitos, Peru. The low transmission rate of 0.4215 infections per person per year in these villages has allowed us to examine patient samples both at the time of *P. falciparum* infection and up to two months after the same infection (Branch, *et al.*).

Materials and Methods

Human Serum Samples

P. falciparum-infected patient serum samples were obtained through the MIGIA cohort study. When patient blood is collected, it is centrifuged to separate the serum from the erythrocytes. While the erythrocyte fraction will contain any *P. falciparum* merozoites, the serum fraction will contain any human antibodies. Use of the serum samples was approved by the UAB Institutional Review Board for Human Use (IRB protocol number X080403004).

Recombinant Antigen Generation

We generated two histidine-tagged recombinant PfMSP6 antigens consisting of the N-terminal domain of the HB3 allele and the N-terminal domain of the Dd2 allele. Expression of the C-terminal domain of PfMSP6 was attempted, but failed. Efforts to express this antigen are ongoing. The antigen constructs were made by PCR from Peruvian *P. falciparum* genomic DNA that was genotyped and sequenced to ensure that the recombinant antigens were consistent with the genotypes of the study population. The PCR amplicons were cloned into pET 15-b (EMD Biosciences) and sequenced. The recombinant antigens were then expressed in Rosetta strain *E. coli*. From the soluble fraction of a bacterial lysate, the recombinant antigens were purified using

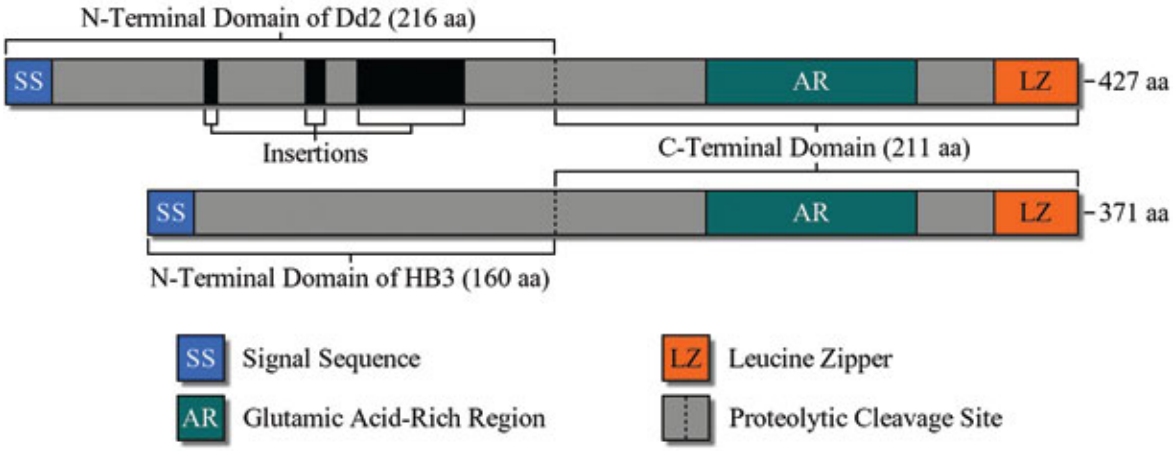


Figure 4. Schematic diagram of PfMSP6 domain structure and alleles. PfMSP6 is a dimorphic antigen consisting of the allele classes HB3 and Dd2, which differ at several polymorphisms in the N-terminal domain.

affinity chromatography (nickel column), followed by anion-exchange chromatography (MonoQ column). After purification, antigen folding was verified by circular dichroism spectroscopy.

Indirect ELISAs

Indirect ELISAs were used to detect any naturally generated anti-PfMSP6 antibodies. All ELISAs were conducted in the research labs of the MIGIA cohort study in Iquitos, Peru (See Figure 2). 96-well plates were coated at a recombinant antigen concentration of 50 ng/well, using only one recombinant antigen in each experiment. 100µL of sera diluted 1:100 in phosphate buffer was added to each well, followed by multiple washes and the addition of horse-radish peroxidase (HRP) linked mouse anti-human secondary antibodies. After the addition of HRP substrate, each reaction was stopped and each plate was read at 450nm using an ELISA plate reader. An optical density (OD) was obtained for each reaction. Each serum sample was tested against both antigens and was run in duplicate, using the average of the OD values for analysis.

Negative controls consisted of serum from patients living in the city of Iquitos who have not traveled out of the city and have no record or recollection of having *P. falciparum* malaria. Positive controls were serial dilutions of a positive pool consisting of individuals known to have strong anti-*P. falciparum* antibody responses. Unfortunately, the positive pool that had been established for the antigen PfMSP3 did not function for PfMSP6. As a result, antibody levels in patient serum samples could not be quantified. Samples were instead assigned values of "positive" if the average OD value was greater than the OD values of the negative controls or "negative" if the average OD value was less than the OD values of the negative controls.

Results and Discussion

Serum from a total of 243 Peruvian patients was tested against the N-terminus of the PfMSP6 allele types HB3 and Dd2. Of these 243 patients, all had serum extracted during *P. falciparum* infection. Figure 1 indicates the results of ELISAs using time of infection serum samples. A total of 79 patients responded as positive, constituting 32.5% of the patients tested. Compared to other MSPs, particularly PfMSP3, this response appears low. However, unlike other MSPs, anti-PfMSP6 antibodies seem to cross-protect against allele types. Of the 79 positive responders, 49 showed a positive response against both allele classes, even though only one infection occurred. The potential of cross-protection among anti-PfMSP6 antibodies warrants further investigation of PfMSP6 as a malaria vaccine candidate.

Of the 79 positive responders, 56 patients had blood drawn at regular intervals after *P. falciparum* infection. These intervals consisted of one week, two weeks, three weeks, one month, and two months post-infection. Figure 2 indicates the results of ELISAs using post-infection serum samples. Of the 56 patients tested, 5 showed a positive response at least one week after infection. The longest positive response detected lasted one month post-infection. The low longevity of anti-PfMSP6 antibodies was not entirely unexpected since this is commonly seen with antibodies generated against other MSPs.

The results of this preliminary study suggest that PfMSP6 is not very immunogenic. Despite this, when antibodies are generated, they appear to cross-protect between allele types. Since cross-protection is uncommon among antibodies generated against MSPs, PfMSP6 should not be overlooked as a vaccine candidate. The authors intend to elaborate on this research in the future to provide sufficient information

regarding the immunogenicity and vaccine potential of PfMSP6.

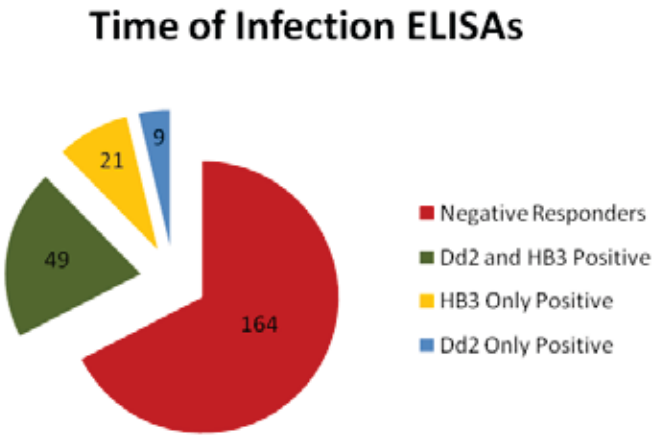


Figure 5. Results of time of infection ELISAs. Serum extracted from *P. falciparum* infected patients during infection was tested against recombinant PfMSP6 N-terminal domains of allele classes HB3 and Dd2.

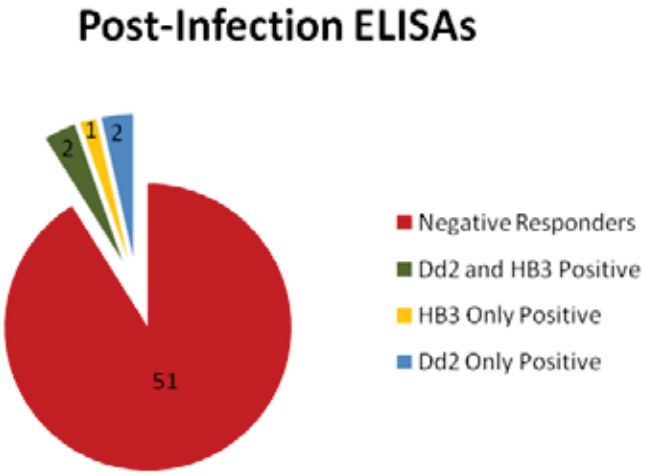


Figure 6. Results of post-infection ELISAs. Serum extracted from *P. falciparum* infected patients post-infection was tested against recombinant PfMSP6 N-terminal domains of allele classes HB3 and Dd2.

Acknowledgements

The authors would like to thank the Sparkman Center for Global Health and the UAB Framework Program for Global Health. Thanks are also due to Eva Clark, the MIGIA project team, and the participants within the MIGIA cohort study.

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short report

Polymer Waveguides for Explosives Detection

Rena L. Hammer, William F. Sherwood, Medhat S. Farahat and David E. Nikles

Abstract

Spectroscopic ellipsometry detected changes in refractive index for films of either polycarbonate, poly(1-vinylimidazole) or poly(2-vinyl-4,6-diamino-1,3,5-triazine-co-styrene) on silicon wafers when 4-nitrotoluene entered the films. The changes in refractive index were much more than adequate ($\Delta n > 0.003$) for use in an explosives sensor based on a Mach Zender interferometer. In the case of the poly(1-vinylimidazole) and the poly(2-vinyl-4,6-diamino-1,3,5-triazine-co-styrene) the refractive index changed after only 4 seconds exposure to a saturated atmosphere of 4-nitrotoluene vapor. These results suggest we can build a very sensitive, fast detector for TNT based explosives.

Spectroscopic ellipsometry detected changes in refractive index for films of either polycarbonate, poly(1-vinylimidazole) or poly(2-vinyl-4,6-diamino-1,3,5-triazine-co-styrene) on silicon wafers when 4-nitrotoluene entered the films. The changes in refractive index were much more than adequate ($\Delta n > 0.003$) for use in an explosives sensor based on a Mach Zender interferometer. In the case of the poly(1-vinylimidazole) and the poly(2-vinyl-4,6-diamino-1,3,5-triazine-co-styrene) the refractive index changed after only 4 seconds exposure to a saturated atmosphere of 4-nitrotoluene vapor. These results suggest we can build a very sensitive, fast detector for TNT based explosives.

Introduction

In the early 20th century the U. S. has faced numerous military and homeland security threats. One pernicious problem has been how to detect explosive devices, particularly improvised explosive devices. This is the objective of our research.

Figure 1. Schematic of a Mach Zender interferometer consisting of a polymeric waveguide with two optical paths. The laser beam enters from the left and leaves to the right

Our approach is to use a polymeric waveguide in the form of a Mach Zender interferometer, figure 1, as a sensitive, selective detector of the volatile components of explosives. The laser beam enters from the left and is split into two beams, one traveling the upper path, while the other travels the lower path. The optical path length for light is the physical path length times the refractive index of the medium. If the two optical path lengths are exactly the same, then the two beams will be in phase when they recombine, constructively interfere and give a high amplitude output. If the optical paths differ by $\lambda/2$ then the two beams will be out of phase and they will destructively interfere to give a low amplitude output.

To use the Mach Zender interferometer as a sensor, the polymer will be one that specifically absorbs the volatile components of an explosive. One leg of the Mach Zender interferometer will be exposed to the environment so that it can absorb any analyte in the gas phase, while the other leg will be encapsulated so as to not be able to absorb the analyte. The head space above TNT filled land mines contains 0.35 to 9.7 pg/mL 1,3-dinitrobenzene and 0.28 to 1.4 pg/mL 2,4-dinitrotoluene. 4-Nitrotoluene is also present and since we had this on our shelf, we chose to use 4-nitrotoluene as our analyte. When the analyte enters the polymer there will be an increase in the refractive index for one leg of the interferometer, causing a phase lag in the laser light traveling through that leg, relative to the other leg. When the beams are recombined this phase lag would result destructive interference and the intensity of the light leaving the interferometer would decrease. If the device used a He-Ne laser (λ = 632.8 nm) and the interferometer had a path length of 100 microns, then we would require a change in refractive index of only 0.003 to go from highest output amplitude (constructive interference) to zero amplitude (destructive interference).

In this first report we describe results that confirm our ability to see adequately large changes in refractive index. We also report new polymers directed at solving the problem of specificity.

Experimental

Materials. All reagents and solvents were purchased from Aldrich or from Fischer Scientific, unless otherwise noted. 2-Vinyl-4,6-diamino-1,3,5-triazine (VDAT) was purchased from TCI. Poly(1-vinylimidazole) was purchased from SelectiveTechnologies Inc.. Flint, MI.

Synthesis of poly(2-vinyl-4,6-diamino-1,3,5-triazine-co-styrene) (PVDAT-co-PS). PVDAT-co-PS was prepared using a free radical polymerization of VDAT and styrene as described by Chen and Sun. The VDAT content was 20 mole percent and the reaction was run at 80°C in DMSO using AIBN as the initiator to give a random copolymer.

A 3% solution of polycarbonate was made using 0.93 g polycarbonate beads and 29.18 g cyclohexanone. The solution was heated and then shaken using the wrist-action shaker in

order to ensure that the polycarbonate completely dissolved. A 2.8% solution of 4-nitrotoluene was made using 0.74 g 4-ntirololene and 25.43 g cyclohexanone. From the 3% solution of polycarbonate and 2.8% solution of 4-nitrotoluene six different solutions were made. Each solution contained a different percentage of 4-nitrotoluene by weight (0%, 5.8%, 11.0%, 14.8%, 20.3%, 25.2%). Each solution weighed approximately 1.76g. Films were made of each solution on silicon wafers using a spin coater set at 2900 rpm for 120 seconds. The films were allowed to dry at room temperature overnight. Films that were made from the solutions that contained the lower amounts of 4-nitrotoluene had a blue color, while the films containing higher amounts of 4-nitrotoluene had a brown tint.

A two or three weight percent solution of poly(1-vinylimidazole) was spin coated onto silicon wafers by first spinning at 200 rpm for 15 s, followed by spinning at 500 rpm for another 15 s, then at 1000 rpm for 20 s, 2000 rpm for 20 s and finally at 3000 rpm for 20 s. The films were further dried at 70°C for one hour.

A three weight percent solution of PVDAT-co-PS in DMF was spin coated onto silicon wafers by first spinning at 200 rpm for 15 s, followed by spinning at 500 rpm for another 15 s, then at 1000 rpm for 20 s, 2000 rpm for 20 s and finally at 3000 rpm for 20 s. The films were further dried at 70°C for one hour.

Instrumentation.
The refractive index for the polymer films was determined using a J. A. Woollem Co. variable angle spectroscopic ellipsometer. For each film values of ψ and δ were determined for wavelengths from 300 to 1,000 nm at angles of 60, 65, 70, 75 and 80°. The data was fit to a model having a polymer film on a silicon wafer. The curve of n(λ) was constrained to a Cauchy model. The result was curves of n(λ) and k(λ).

Results and Discussion
Our first task was to determine whether polymer films containing 4-nitrotoluene would show an adequate change in refractive index (Δn > 0.003). In figure 2 are plots of refractive index as a function of wavelength for polycarbonate films with and without added 4-nitrotoluene. The curve with added 4-nitrotoluene has a higher refractive index than the one with no 4-nitrotoluene. Through most of the spectral range the difference in refractive index was about 0.02, a factor of six greater than what is required to get a signal in the waveguide.

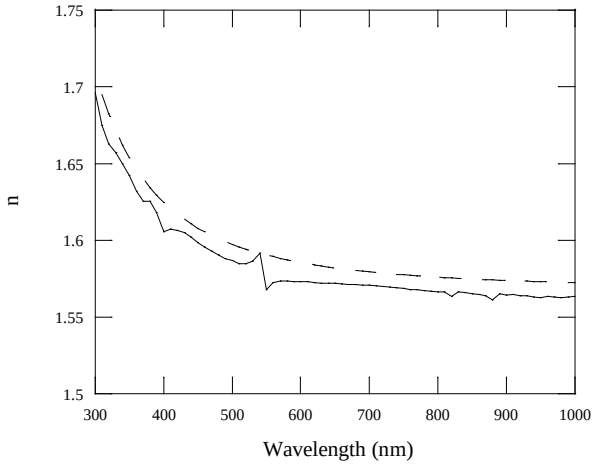
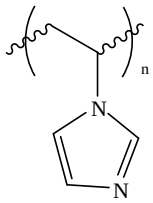


Figure 2. Plots of refractive index (n) as a function of wavelength for polymer carbonate films with no 4-nitrotoluene (solid curve) and with 4-nitrotoluene (dashed curve).

Our next task was to find new polymers with high affinity for the nitroaromatic molecules that are components of the vapor above explosives containing TNT. Since nitroaromatics are electron poor, we sought polymers with electron-rich functional groups or with functional groups that can hydrogen bond with the nitro groups. Our first candidate was poly(1-vinylimidazole) having electron-rich imidazole groups. A film was exposed to 4-nitrotoluene for only 4 seconds. The plots in figure 3 show a very large increase in refractive index after exposure. This suggests we could build a sensor with a fast response (seconds) and is very sensitive to nitroaromatic vapors.



Poly(1-vinylimidazole)

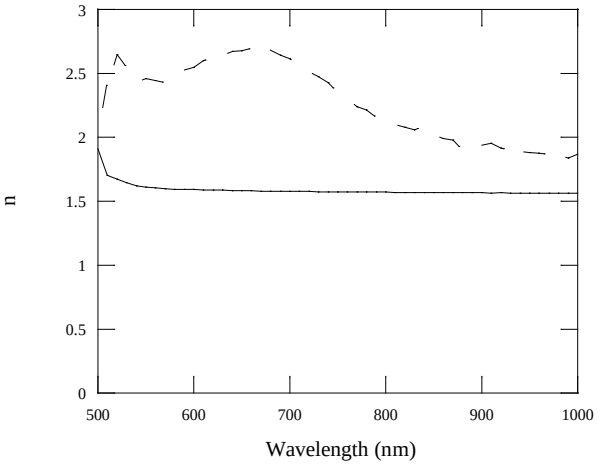
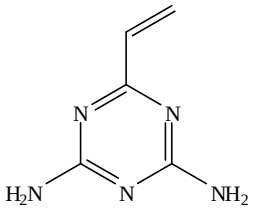


Figure 3. The change in refractive index of a polyvinylimidazole polymer film before (solid curve) and after (dashed curve) a four second exposure to a saturated vapor of 4-nitrotoluene.



2-vinyl-4,6-diamino-1,3,5-triazine (VDAT)

Poly(2-vinyl-4,6-diamino-1,3,5-tria-zine-co-styrene) (PVDAT-co-PS) was prepared using a free radical polymerization giving a random copolymer of styrene and 2-vinyl-4,6-diamino-1,3,5-triazine (VDAT). We expected the VDAT group would have a strong affinity for 4-nitrotoluene due to hydrogen bonding between the amino group on the polymer chain and the nitro groups. In the random copolymer used here the VDAT content was 20 mole percent. The plots in figure 4 show that this copolymer also responded very quickly to a saturated atmosphere of 4-nitrotoluene. After only four seconds exposure there was a measurable increase in refractive index. The increase was not as great of the case for poly(1-vinylimidazole). Perhaps this was because there are relatively fewer triazine functional groups in PVDAT-co-PS than imidazole groups in poly(1-vinylimidazole).

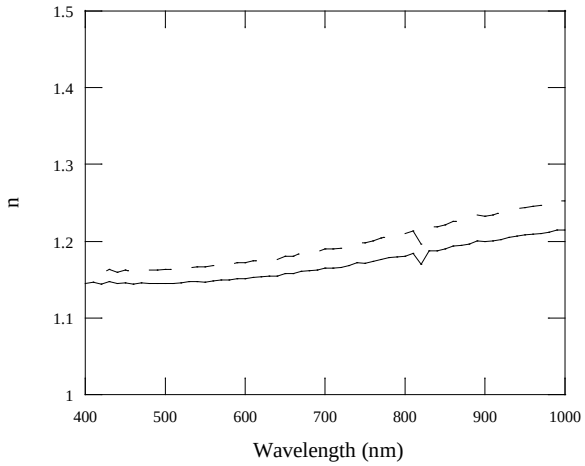


Figure 4. The change in refractive index of a PVDAT-co-PS film before (solid curve) and after (dashed curve) a four second exposure to a saturated vapor of 4-nitrotoluene.

Conclusions

We have shown that when 4-nitrotoluene enters polymer films the refractive index increases by an amount more that sufficient for detection by a sensor based on a Mach Zender interferometer. Furthermore the response can be fast, on a timescale of seconds. This is a very encouraging first step. However in order to realize a working sensor we must demonstrate that the absorption, leading to a refractive index change, is specific to the desired analyte and not to any other volatile organic specious that would raise the refractive index. To this end we look to building on the affinity of nitroaromatic species to electron rich functional groups. We will also use a molecular imprinting approach to further enhance specificity.

Acknowledgements

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faculty interview: biology

The Importance of Echinoderms: *An Interview with Dr. Stephen Watts*

Marine Biology, Kim Trawick

Recently, I had the chance to interview Dr. Stephen Watts about his position at UAB, his passion for research, and his current research. Dr. Watts is a world-renowned echinoderm biologist, who has specialized in marine ecology, biochemistry, and nutrition. He has been a professor and a research scientist at UAB since 1987. In 2000 he was also made the Chair of the Biology Graduate department, and has since assisted in making the Biology graduate department one of the largest on campus. From the beginning of his career at UAB, he has been responsible for teaching Undergraduate Cell Biology. During this interview, Dr. Watts talked about how he got into research, how he came to UAB, and also gave some advice to undergraduate students interested in working in research facilities at UAB.

Q) How did you get started in research?

A) I got into research my last year at Auburn. I heard about other students that were involved in research, and I also had TAs who were in research and they enjoyed it. So, it sounded like something that might be fun to try.

Q) What did you want to do before you started research?

A) I did not know. My parents wanted me to go into medical school, but I don't like sick people.

Q) So did you know that you wanted to do marine science?

A) I loved marine science. I grew up on marine science. We were scuba divers in high school. We used to dive and see all the neat animals. That was a period of time when there were lots of TV shows that promoted marine science, like Jacques Cousteau. There were lots of specials coming on at that time. There was a media love affair with the ocean at that point. So marine science sounded like something good to do, though we didn't have a clue of what a job in marine science would be.

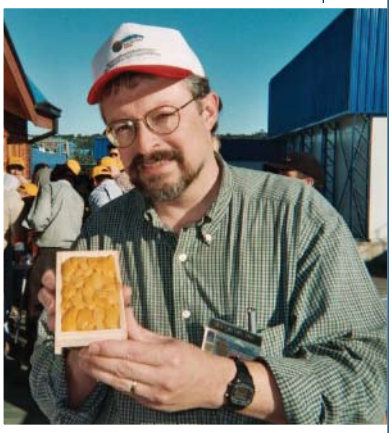
Q) What was your first research experience like?

A) I was working with William Mason. He was a well-known biologist at Auburn. He was well known for being hardcore. I had taken invertebrate zoology from him, and had done well in that class. So, I went to work with him and one of his Ph.D. students. We worked with the Tartigrades, water bears. It was systematic work, where I had to identify them based on their structures. It was the most wonderful thing I had ever done because I got to engage in science and think. We were doing new things, innovative things, we were using our minds, using the classes that we had taken, and I thought it was just phenomenal. It was very exciting to me to participate in research.

Q) Where did you go after Auburn?

A) At that point in time, I didn't think I had the ability to participate in research. I looked at Master's students that TA'd my class, and I thought these guys must be brilliant. I put them on a huge pedestal, and I didn't even think I could get to that point, but I was willing to make an attempt. So, I looked into Marine Science programs, and found the University of South Florida. I found one of the faculty, Dr. John Lawrence, who worked with echinoderms. I like echinoderms, particularly sand dollars because we used to collect a lot of sand dollars on our scuba diving trips. I thought it was a neat animal.

We were doing new things, innovative things, we were using our minds, using the classes that we had taken, and I thought it was just phenomenal. It was very exciting to me to participate in research.



Q) What did you do a USF?

A) I went to visit Dr. Lawrence. He indicated that he would be willing to accept me, and I was to start off as a behavioral ecologist. In my first term there, I took a course from him called physiological ecology, which had a laboratory with it. That laboratory was designed so that the students would participate in a project that they could publish. That course was so exciting to me, learning about how the physical environment affects the physiology and biochemistry of the animals that live in those environments. You could understand how the animals were affected by understanding how they effected in their cells and biochemistry, and it all

came together. You could explain ecological concepts based on biochemical concepts. I really enjoyed that type of cross-disciplinarian approach.

Q) Did you stay at USF to get your Ph.D.?

A) Only after I got my Masters did I feel that I had the ability to go on to get a Ph.D. I still did not know what I wanted to do for a job, but I enjoyed the science. I stayed and worked with sea stars and how their reproductive cycles changed seasonally, and I also worked with sea urchins as side projects.

Q) When did you decide what your career goals were?

A) About one year before I finished my Ph.D. I finally recognized that I could work at that particular level because I had good training and a very good committee. It was a very rigorous Ph.D. program, but it helped prepare me for the future. I graduated with 9 publications, which was pretty good for a Masters and Ph.D. After that I went straight on to a post-doctoral position.

Q) Where did you do your post-doc?

A) I was split between the University of New Hampshire and the State University of Utrecht in the Netherlands. I went to New Hampshire, and we worked on spermatogenesis in sea stars. I was looking at the hormonal systems, steroids in particular, involved in spermatogenesis.

Q) What did you gain from your time in New Hampshire?

A) I learned how to write grants. I was funded by the two people that hired me, and some of the money came from the Netherlands.

Q) What did you do in the Netherlands?

A) I worked with Peter Voogt, who was a world-renowned steroid technologist. We learned how to do the work in steroids with sea stars.

A) I knew that I would have to go into professorship somewhere, and I started to apply for jobs. I applied to all Marine Science centers. UAB was the only school without a marine science center. When I came to UAB, I didn't come to get a job. I came for the interview because my family was nearby. Once I got here, I saw that UAB had changed. When I grew up, UAB was an equivalent of a junior college and most of the faculty only had Masters degree. When I came back for a job, it was a very different university that had a lot to offer. They were very generous, and they were trying to make the university more of a research-based school. I really enjoyed it here, and I decided to take the position in '87.

Q) What was your initial position?

A) I taught Cell Biology, Introductory Biology, and a graduate course, but primarily my job was research. We initially continued with sea star spermatogenesis, but overtime I worked with Dr. George Cline and he got us involved in aquaculture. We understood that funding is required, and it is much easier to get funding when you work with a species of economic value. Working with tilapia in aquaculture opened up new sources of funding.

Q) How did you get involved with sea urchin research?

A) We started with the sea urchins at USF during my Masters program. In the early '90s, we saw the importance of sea urchins on the world markets and went to NOAA to get funding to research this. In the mid-90s, NOAA came back to us and agreed to fund us because they started to see the problems with sea urchins on the world market. We started with some aspects of nutrition and basic culture husbandry. There were a lot of engineering components, as we continued to study the biology of husbandry. In the late '90s, we shifted more heavily to the nutrition component recognizing that you can't house urchins in-shore without knowing what to feed them.

Q) What is your current research on?

If you can help guide those students in a particular direction, you can affect their outcome. That's important. I saw that we could do things that would enhance the students' ability to go out and get jobs.

Q) After your post-doc, what did you want to do as your career?

A) The nutrition of aquatic organisms, particularly those who have economic value or those that have value as biomedical models, including sea urchins and zebrafish.

Talk to the faculty; get to know the faculty in the classes they are taking. Don't be strangers to the faculty. If I don't know a student's name by the end of the term then they didn't do their job. I can only do so much because there are so many, but if they come to me and they talk to me I will get to know them and I can help direct them to other areas.

Q) How do you foresee your research impacting the scientific community?

A) Animal models have come under increased scrutiny for use in testing. In the old days, there was no thought about what type of animal was used. Now there is a shift to move away from using complex vertebrates, and perhaps use other models that are easier to and are cheaper to use. We knew that it is far cheaper to fish and sea urchins as models rather than rats and mice, but we have to make sure that we are developing the model adequately so that they can be used.

Q) When did you become the director of the Biology Graduate Department?

A) That would be almost 9 years ago.

Q) Why did you want to do that?

A) From day one, I was interested in the graduate department. When I got here, we had 6 graduate students. When I took over, we had almost 30 graduate students, and today we have 58 registered graduate students. So, we have almost doubled the number of graduate students. My interest in graduate students comes from several things. Number one, my former mentor was a graduate program director, and I saw that he had a huge impact on the lives and futures of students. If you can help guide those students in a particular direction, you can affect their outcome. That's important. I saw that we could do things that would enhance the students' ability to go out and get jobs.

Q) What is your favorite part about being the graduate director?

A) Talking to lots of students, and seeing different areas of research. I like talking and interacting with the students.

Q) How do you feel about teaching the undergraduate students?

A) I very much enjoy teaching the undergrads, but it is not as much fun as it used to be because of the class sizes. I don't get the opportunity to interact with the students with such large classes. I miss knowing and being able to help all of my students, but this is part of becoming a large department. But I really enjoy watching the undergraduates because you get to watch them step up. You can take someone with very little confidence, and make them realize that they are much smarter than they think they are.

Q) Do you have any advice for undergraduates that want to get into research?

A) Talk to their advisor first to make sure they have the grades to do it, and then determine what area of the coursework they like best. Talk to the faculty; get to know the faculty in the classes they are taking. Don't be strangers to the faculty. If I don't know a student's name by the end of the term then they didn't do their job. I can only do so much because there are so many, but if they come to me and they talk to me I will get to know them and I can help direct them to other areas.

Collagen mRNA Expression in Human Fetal Lung Fibroblasts Treated with Varying Concentrations of Ascorbic Acid, PGE2, and TGF-β1

Dixon Dorand

Abstract:

Previous experiments have demonstrated that ascorbic acid contributes to the growth of collagen chains. In this study, we have examined the mRNA expression of collagen in Human Fetal Lung (HFL) cells treated with different concentrations of AsA, PGE2, and TGF-β1. The mRNA expression of collagen chains I α 1, I α 2, III α 1, VI α 1, VI α 2, and VI α 3 were all measured using the Taqman PCR method. The results of these studies showed that: 1) AsA modulates collagen gene expression having the greatest effect on Collagen Type III α 1. 2) TGF-β1 increased collagen gene expression, but not identically for all alpha chains for each different collagen type. 3) PGE2 inhibits collagen expression, but not identically for all collagen chains with the greatest effect seen in Collagen Type I α 1. 4) When TGF-β1 and PGE2 were both added to the cell culture mRNA expression was significantly increased showing that TGF-β1 has the dominant role in collagen mRNA expression, although the mechanism for this action is unknown. The reproducibility of the assay should be assessed. It can be concluded that PGE2 may differentially regulate collagen gene expression.

Introduction:

The formation of collagen rich scar tissue is a basic biological response to both internal and external wounds. The degree of scarring is determined by the severity of the wound and the intensity of the cellular response. Understanding the mechanisms behind the formation of excessive scarring is an important part of understanding a variety of diseases. Chronic Obstructive Pulmonary Disease (COPD) is a disease state characterized by airflow limitation that is not fully reversible. It is a progressive disease usually caused by cigarette smoking, but can be caused by other long term irritants such as pollution, dust, or other noxious gases. The airflow limitation is both progressive and associated with an abnormal inflammatory response of the lungs leading to fibrosis. It causes a variety of symptoms including coughing with excessive mucus production, wheezing, shortness of breath, and tightening of the chest. COPD results in less air flowing in and out of the pulmonary system for a variety of reasons. First, the elasticity of the bronchi, bronchioles, and alveolar sacs loose their elasticity. Second, the thin epithelium of the alveoli are often destroyed resulting in inadequate gas exchange. Third, the walls of the bronchi are thickened due to inflammation. Lastly, the airways are clogged due to the overproduction of mucus. The most common course of treatment for COPD is the use of bronchodilators or inhaled glucocorticosteriods. Bronchodilators relieve obstruction by relaxing the muscles controlling the airways; they can either be short or long acting. Glucocorticosteriods reduce airway inflammation. Both treatments are given as inhalants (12).

To understand how to treat COPD you first must understand the general concepts behind the formation of the associated collagen rich scars. Individual collagen chains are synthesized on membrane bound ribosomes and injected into the lumen of the endoplasmic reticulum as larger precursors (pro-alpha chains). Procollagen is formed when selected prolines and lysines are hydroxylated. Each pro-a chain then combines with two others to form a hydrogen bonded, triple strain helical molecule known as procollagen. After secretion of propeptides of fibrillar procollagen molecules are removed by specific proteolytic enzymes outside the cell. The function of propeptides is to guide the intracellular formation of the tristranded collagen molecules. The removal of the procollagen prevents intracellular formation of large collagen fibrils which is harmful to the cell. Fibril formation is driven by the collagen molecules tendency to self-assemble. After fibril formation they are greatly strengthened by the formation of covalent cross-links between lysine residues of the constituent collagen molecules. Understanding the mechanisms behind collagen formation is important because disrupting any of the formative steps will inhibit collagen production.

Collagen is an integral part of the extracellular matrix that surrounds fibroblasts. There are several different collagen chains with a varying number of helical alpha chains that form its structure. While the roles of some collagen chains are widely understood different collagen chains have different roles throughout the body, specifically in would healing. The different alpha chains derive their stability from hydrogen bonding between molecules. Gylcine must be every third amino acid in the chain, and the structure usually follows the pattern Gly – X – Y where X is usually praline and Y is usually 4-Hyp.

Among the eleven collagen strains there are five groups of collagen. Fibril-forming (fibrillar) collagen includes types I, II, III, V, and XI. Type I fibrillar collagen is found in a variety of places including the bone, skin, tendons, ligaments, cornea, and internal organs. It accounts for over 90% of the body's collagen. Type III fibrillar collagen is found in the skin, blood vessels, and internal organs. Fibrillar collagen is secreted into the extra cellular space where it assembles into higher order polymers called fibrils. They further aggregate into larger bands called collagen fibers. Fibril-associated collagen includes types IX and XII that decorate the surface of the collagen fibers. Network forming collagen includes types IV and VII. Type IV constitutes a major part of the mature basal lamina as a felt-like sheet, but it has structural

and biosynthetic properties that drastically differ from those of collagen I and III. Unlike collagen I and III, collagen VI is not degraded by metalloproteinases. Even though its functions are not fully understood it is suspected to form an integral part of the anchoring filaments for collagen I fibers and the basement membrane. Additionally, it may have a significant role in cell-binding and have protease inhibitor function (13). Collagen VI also differentiates itself from other collagen types because its large N- and C-propeptides are not removed via proteolytic processing to form mature collagen. Lastly, collagen VI α 3 has a length twice that of the other two alpha chains (3).

Cells mediate collagen fibril formation mechanically and chemically. Fibroblasts mechanically alter the collagen they secrete by pushing it into compact sheets that are then drawn out into cables. Fibroblasts generate long-range order in the extra cellular matrix to create things like tough, dense layers of connective tissue that sheathe and bind together most organs. The diversity of collagen presents a variety of challenges to biomedical research because the role of collagen type and each individual alpha chain must be assessed to understand its mechanism of action.

There are a variety biological factors that regulate collagen production both *in vivo* and *in vitro*. Vitamin C, also known as ascorbic acid (AsA), is obtained from fruits and vegetables. AsA is a water soluble vitamin that plays an integral role in collagen synthesis such as in the bone, cartilage, and gums. It is also an antioxidant, aids in detoxification, and improves iron absorption. A lack of AsA results in scurvy causing degeneration of skin, teeth, and blood vessels. It also results in general weakness, delayed wound healing, and impaired immunity (5). AsA is required for the hydroxylation of proline and lysine in collagen via the enzyme ascorbate. Proline is important because its derivative 4(R)-L-hydroxyproline (4-Hyp) plays an essential role in the folding of collagen and the maintenance of its structure. The addition of 4-Hyp makes the collagen molecule stable and it raises the melting temperature by 28°C in a 10 peptide molecule. Scurvy results when AsA is absent leading to collagen instability and connective tissue problems (5,8).

Prostaglandins are eiconsanoids, 20 carbon long molecules, which are made by most vertebrate cell types and have a wide variety of biological activities. Their main role is in the regulation of pain and inflammatory responses, contraction of smooth muscles, and regulation body temperature (1). PGE2 is a central lipid mediator in inflammation and pain. Evidence suggests that adult fibroblasts treated with PGE2 show collagen I inhibition in vitro. PGE_2 transmits its signal through several different G-protein coupled receptors. The four receptors, E1-E4, use a variety of mechanisms: EP1 signals through G_q, by inducing increased intracellular Ca^{2+}; EP2 and EP4 signal through G_s, by activating adenylate cyclase which increases intracellular cAMP; and EP3 primarily signals through G_i, has variants that mediate multiple signaling pathways leading to decreased cAMP (9,14). Protein kinase A (PKA), a proven cAMP effector, and its activation alters cellular function by one of two ways. It either directly phosphorylates transcription factors or it indirectly modulates other signaling pathways. PGE2 has been shown to inhibit fibroblast proliferation and collagen expression in patient-derived normal adult lung fibroblasts via E prostanoid-2 receptor (EP2) and cAMP signaling (9). Increased cAMP, due to PGE2, also mediates the activity in embryonic fibroblasts via the EP2 receptor (7). Even 2 fold changes in cAMP concentration were effective at suppressing collagen production in cultured HFL fibroblasts (2).

TGF-β1 is especially important in wound healing by stimulating a transformation of fibroblasts to myofibroblasts. It promotes the formation of collagen-rich scar tissue to give additional strength to a healing wound. TGF-β1 is a pleiotrophic cytokine that induces the extra cellular matrix expression and inhibits growth of vascular smooth muscle cells. Evidence suggests adult fibroblasts treated with TGF-β1 have increased collagen gene expression in vitro. TGF-β1 stimulates the production of collagen types I and III in human corpus cavernosum smooth muscle cells (HCCSMC). Exogenous TGF-β1 also resulted in detectable levels of collagen types X and VI. However, the induced collagen growth by TGF-β1 was suppressed by PGE1. These data further show the role of prostaglandins in the regulation of collagen synthesis in the corpus cavernosum. Excess collagen growth in HCCSMC did lead to fibrosis (11). The exact mechanism of increased collagen is not known.

The purpose of this study is to determine the role of AsA, PGE2, and TGF- β1 individually and when combined. In this study, we have examined the mRNA expression of collagen in HFL cells treated with different concentrations of AsA, PGE2, and TGF-β1. Based on previous studies, it was hypothesized that AsA would increase collagen mRNA expression. Furthermore, it was hypothesized that PGE2 would inhibit collagen mRNA expression and TGF-B1 would increase collagen mRNA expression. This study is unique because it deals with how AsA, PGE2, and TGF-β1 regulate three different types of collagen and their various alpha chains. Type VI collagen is not clearly understood so comparing its regulation to other collagen types is a necessary step to determining its role in wound healing. Determining mRNA expression is an important step to understanding collagen synthesis because it is one of the earliest steps in protein production.

Materials and Methods:

Cell Culture:

The human fetal lung (HFL) fibroblasts were plated in DMEM, 10% fetal bovine serum (FBS), and 1% fungizone. Dulbecco's Modified Eagle's Medium (DMEM), and fetal bovine serum (FBS) were from Invitrogen Life Technologies (Grand Island, NY). The cells were treated with 10 ug/mL of Ascorbic Acid (AsA) and grown for 48 hours in a 37°C incubator. The cells

were then treated with 100 pM TGF-β1, and 1 uM PGE2, and a mixture of TGF-β1 (100 pM) & PGE2 (1 uM). The re-treated cells were then incubated for another 24 hours. All of the cells used were between passage 8 and 15.

Extraction & RNA Harvest:
The cells were then extracted using Trizol from Invitrogen Life Technologies (Cat. No. 15596-026). The Trizol extraction method maintains the structure of RNA while disrupting cells and dissolving its cellular components. Chloroform was added, followed by centrifugation which separated the solution into an aqueous upper phase and an organic lower phase. RNA was exclusively in the aqueous phase and was recovered by precipitation with isopropyl alcohol. In order to check the concentration of RNA the isolated RNA had an A_{260}/A_{280} ratio of >1.8 in TE (10). Once the concentration of RNA was known the sample was then treated with DNAse. The concentration and purity of RNA determined the amount that needed to be added to the 10x buffer, DNAse, and RNAse free water. This treatment assured that the only nucleic acids present in the sample were RNA.

Taqman PCR:
Samples were then treated with reverse transcriptase and run on a Mini-cycler using a protocol for high reverse transcription. Samples were then stored at 4°C until being plated in 96-well plates. Collagen primers and probes were then added to their respective column for all six alpha chains tested. Taqman PCR was then performed to obtain cycle threshold. Each cDNA sample was run in duplicate for every PCR. The values were then calculated using the following formulas. Amount of target, normalized to endogenous reference and relative to a calibrator is given by: $2^{-\Delta\Delta CT}$
Derivation of Formula, Exponential Amplification of PCR Xn = Xo x (1+Ex)n
Xn = number of target molecules at cycle n
Xo = initial number of target molecules
EX = efficiency of target amplification
n = number of cycles
Cycle Threshold indicates fractional cycle number at which amount of target reaches a fixed threshold:
XT=Xox(1+EX)CT1x=KX
XT = threshold number of target molecules
CT,X = threshold cycle for target amplification
KX = constant

Results:
When HFL fibroblasts were treated with AsA, an increase in collagen production was found for all alpha chains tested. The most significant increase was found in collagen III α 1 (29.20), followed by II α 2 (10.17), VI α 3 (8.49), II α 1 (8.43), VI α 2 (5.20), then VI α 1 (5.26) (Fig. 1). Even the small increase in VI α 1 shows double the amount produced in non treated HFL fibroblasts.

When HFL fibroblasts were treated with TGF- β1, an increase in collagen production was found in all but one of the collagen strains tested. The largest increase in expression was found in collagen III α 1 (20.32), then I α 1 (18.53), VI α 1 (3.77), I α 2 (3.32), and VI α 2 (2.67) (Fig. 2). However, collagen VI α 3 actually showed a reduction in mRNA expression (0.87). These values are set with 1.00 representing HFL fibroblasts treated only with AsA. For example, in collagen III α 1 the addition of TGF-β1 resulted in an 18.53 fold increase in addition to the 8.43 increase caused by AsA.

PGE2 inhibition was found in four of the six alpha chains tested. The inhibitory effects were the largest in collagen VI α 3 (0.20), followed by III α 1 (0.27), VI α 2 (0.36), and I α 2 (0.47). An increase in mRNA expression was found in collagen VI α 1 (10.44) followed by I α 1 (2.21) (Fig. 3). Collagen VI α 1 shows a significant increase mRNA expression compared to the inhibitory effects shown by VI α 3. The mRNA increase or decrease was compared to cells treated with AsA as the control value of 1.00.

When both PGE2 and TGF- β1were both given to HFL fibroblasts an increase in collagen production was found.
The most significant percentage increase in collagen mRNA expression was found in type VI collagen. The VI α 1 (508.51%), followed by VI α 2 (428.90), VI α 3 (372. show a large increase compared to the other collagen types tested. Increase in mRNA expression was also found for collagen I α 2 (295.82%), followed by III α 1 (52.16%), then I α 1 (41.06%) (Fig. 4).

Effect of AsA on mRNA expression in HFL Fibroblasts:

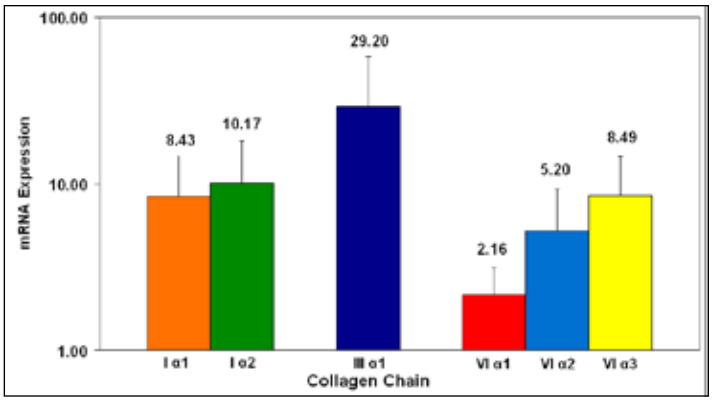
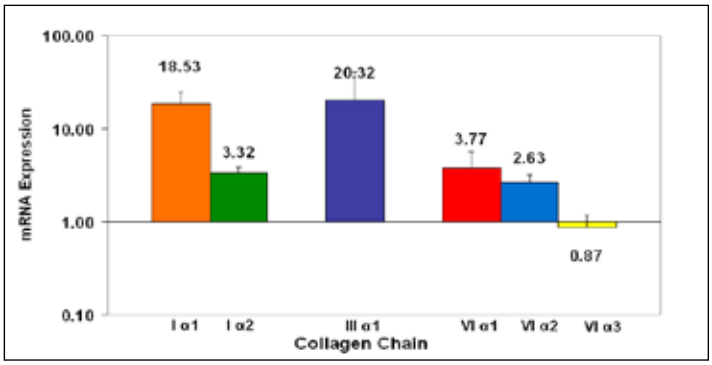


Figure 1. The role of AsA in HFL was tested alone to ensure that it induced collagen formation. AsA increased mRNA collagen expression for all experimental chains tested. The effect was the greatest on collagen III α 1.

Effect of TGF-β1 on HFL Fibroblasts:

Figure 2. (top of next page) TGF-β1 resulted in increased production of all collagen chains except collagen VI α 3.



Effect of PGE2 on HFL fibroblasts:

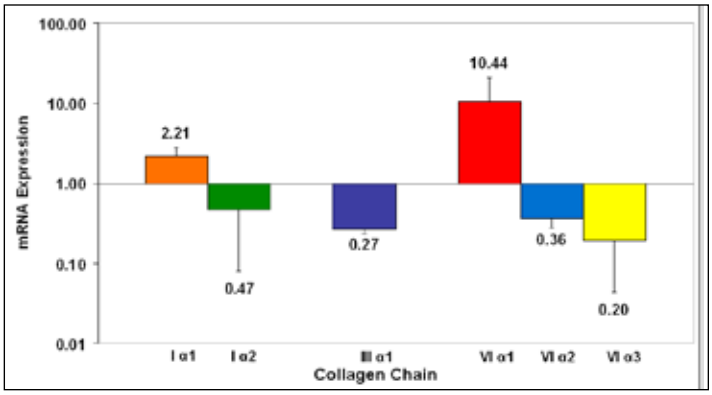


Figure 3. PGE2 inhibited collagen mRNA production in at least one alpha chain of each collagen molecule. The largest effect was on collagen VI α 3. However, collagen I α 1 and collagen VI α 1 were both increased with treatment.

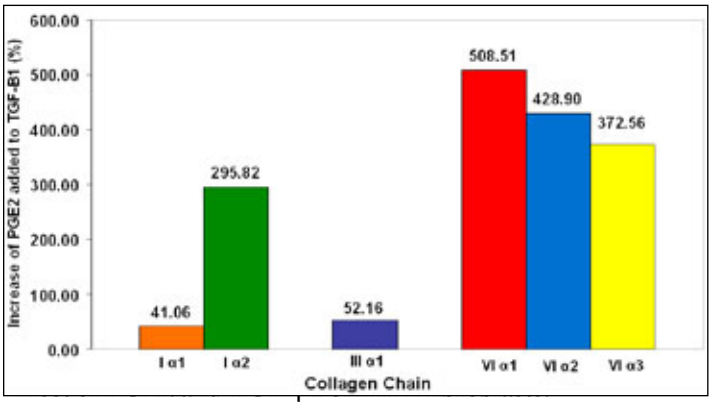


Figure 4. Simultaneous dosage of HFL fibroblasts with both PGE2 and TGF-β1 led to a dramatic increase in collagen mRNA production. Collagen VI showed the most significant expression.

Discussion:

The results of this study indicate that AsA induces collagen transcription in HFL fibroblasts. It was hypothesized that AsA would increase collagen mRNA expression, and AsA had the greatest effect on Collagen Type III α 1 (Fig. 1). These results suggest that AsA plays an integral role in the formation of the collagenous extra cellular matrix as procollagen is transformed to collagen. The important factor to determine is the proper dosage of AsA for cell culture. Previous cell culture techniques provided data that 10 ug/mL of AsA was the optimal concentration for experimental purposes (12). However, more analysis is needed to understand the definitive role of AsA in the formation of the extracellular matrix. In an earlier study the recommended AsA concentration was between 25-50 ug/mL and used a concentration of 20% FBS (4). Tests with other collagen strains are necessary to determine if AsA induces collagen mRNA production in all fibrillar collagen chains. In addition, fibril-associated, network forming, and transmembrane collagens should be tested to see if similar results are obtained. The viability of AsA may have affected results of the experiments as it has a half life of only a few hours. It may have been more effective to add fresh AsA to the medium each twelve hours to ensure that AsA activity was maintained throughout the experiment.

In a study by Chan, et al, AsA did stimulate collagen I and II synthesis reaching a maximum at day two (6). AsA maintained a high specific rate of production until day 10 and afterwards collagen production declined. In addition, the study found after only a 10 hour treatment of AsA deficient cells induced collagen synthesis. AsA increased collagen mRNA expression in collagen I α 1, I α 2, and III α 1. However, the increase in collagen mRNA (2-3 fold) did not correspond to the increased collagen protein synthesis (6-7 fold). These data show that there are transcriptional modifications as well as post-translational modifications that account for the increase of collagen expression in 2-year old human child fibroblasts (6). These data show that there may be additional factors to AsA induced collagen expression that are beyond the scope of the current study. In order to definitively conclude AsA's role in inducing collagen expression in all of the alpha chains tested additional information on the amount of protein produced needs to be assessed. Currently, it can be observed that AsA increased collagen expression differentially in the six alpha chains tested.

From the present study it can be concluded that TGF-β1 increased collagen gene expression, but not identically for all alpha chains for each different collagen type (Fig. 2). mRNA expression in collagen I and III chains was induced. These data show a similar effect to the previous experiments with HCCSMC which show increased collagen I and III production (11). More experiments need to be performed before conclusive observations can be made on the role of TGF-β1 in HFL fibroblasts. Not only does the mRNA need to be measured, but the protein levels should also be tested using Western blot analysis. The differential result from collagen VI is not definitive. Collagen VI is not included in the four main categories of collagen and its role is not as intricately understood. Its inclusion in the experiment was to obtain experimental data to be used in further experiments to further understand its role in the formation of the extra cellular membrane.

At present there is evidence suggesting that PGE2 inhibits collagen expression, but not identically for all collagen chains. The greatest effect was seen in collagen VI α 3 (Fig. 3). The differential regulation of collagen I alpha chains does not provide enough analysis to determine if PGE2 does or does not inhibit expression of collagen in HFL fibroblasts. The mRNA expression of collagen I α 2 is inhibited but collagen I α 1 mRNA is induced. Physiologically, the inhibition of collagen I α 2 mRNA may be reason enough to believe that PGE2 does inhibit collagen production. If one of the integral alpha chains of collagen I is missing then it would be logical that it could not form its triple helix structure effectively. However, further analysis using Western blot techniques is necessary to draw any concrete conclusions as to the amount of collagen I expressed in HFL fibroblasts treated with PGE2. A similar argument can be made for collagen VI α 1, but still without a definitive conclusion. It can be concluded that PGE2 may differentially regulate collagen gene expression.

When TGF-β1 and PGE2 were both added to the cell culture mRNA expression was increased showing that when the two factors are added together they produce a more pronounced effect. It cannot be determined if TGF-β1 has a dominant role in collagen mRNA expression because the mechanism for this action with PGE2 is unknown. Prostaglandins have a wide variety of roles in modulating the body and especially different receptors. Even though PGE1 was shown to inhibit the effects of TGF-β1 in HCCSMC, PGE2 uses different EP receptors for its mechanism of action (11,14). Furthermore, it cannot be assumed that TGF-β1 has the same effect on two different cell types. In order to understand the induced collagen mRNA in HFL fibroblasts expression protein analysis should be performed to see if post translational modifications inhibit such a pronounced induction of collagen production.

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Correlation Between Hypertension and the Progression of Alzheimer's Disease

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Abstract

In Alzheimer's disease (AD) amyloid beta (Aβ) plaques accumulate in the brain parenchyma, or cortex as well as in the cerebral vasculature. This increase in Aβ accumulation in blood vessels causes cerebral amyloid angiopathy (CAA) and, possibly, hemorrhagic stroke. Changes in cerebral blood pressure and blood flow, due to aging or hypertension, likely lead to the cognitive dysfunction (dementia) that is associated with AD. This study aimed to determine if prolonged hypertension in transgenic (Tg) AD model mice causes cerebral hypoperfusion, or insufficient blood flow through the brain, which in turn leads to cognitive impairment and, possibly, increased Aβ deposition. Tg AD model mice were made hypertensive and compared to non-hypertensive Tg AD mice. Blood pressure and cerebral blood flow were measured, and behavioral tests were performed to analyze the cognitive changes in the animals. The amount of amyloid deposition, glial activation, and changes in blood vessel density/integrity were measured. The results show that prolonged mid-life hypertension in Tg AD model mice significantly increased behavioral deficits, elevated Aβ deposition, and increased glial activation compared to non-hypertensive Tg AD mice. Furthermore, there was an increase in dysfunctional vessels in the plaque-deposit rich areas in the brain of hypertensive mice compared to non-hypertensive animals. Together the data indicate that early treatment of hypertension could reduce the incidence and severity of Alzheimer's disease.

Introduction

In the elderly, Alzheimer's disease (AD) is the most common form of dementia. The two pathologies that characterize the disease are the presence of large numbers of intracellular neurofibrillary tangles (NFTs) and extracellular neuritic plaques in the brain (e.g., Braak and Braak, 1991; 1998; Selkoe, 2001). Neurofibrillary tangles consist of hyperphosphorylated, twisted filaments of the cytoskeletal protein tau, whereas plaques are primarily made up of amyloid β (Aβ [Selkoe, 2001; Dickson and Vickers, 2002]), a 39-43 amino acid long peptide derived from the proteolytic processing of the amyloid precursor protein (APP [Selkoe, 2001). When APP is sequentially cleaved by the β-secretase and γ-secretase, one of the resulting breakdown products is Aβ, in contrast, initial cleavage by α-secretase (in the middle of the Aβ sequence) leads to production of APPs-α and the C83 peptide (Selkoe, 2001).

Most cases of AD are sporadic, however approximately 5 % of AD cases are familial (Selkoe, 2001), these cases are related to mutations in the genes for APP, and presenilin 1 and 2 (PS1 and PS2 [Selkoe, 2001]). Transgenic mice expressing mutated human AD genes offer a powerful model to study the role of Aβ in the development of pathology (e.g., Duff and Suleman, 2004; McGowan et al, 2006).

Hypertension is a risk factor for stroke and vascular dementia, and the incidence of these diseases grows with increasing blood pressure. Recent studies have shown that hypertension is also a risk factor for Alzheimer's disease (AD). AD is associated with the accumulation of amyloid beta (Aβ) in plaques in the brain parenchyma but also with substantial amyloid beta deposition in the cerebral vasculature leading to cerebral amyloid angiopathy and hemorrhagic stroke. Furthermore, it has been demonstrated that AD patients have cerebral hypoperfusion and cerebral hypoactivity. Clearly, cerebrovascular disease is not the sole cause of AD, but mounting evidence indicates that changes in cerebral blood flow do contribute to the cognitive dysfunction associated with AD. The brain controls cerebral blood pressure and blood flow, but with long-standing hypertension and increasing age this regulation is altered. This change results in modifications in the cerebral circulation as sustained hypoperfusion impacts the aging process to induce augmented pathology. The relation between blood pressure and cognitive function and dementia has, in recent years, been researched extensively (e.g., De la Torre, 2004). Some studies have shown an inverse association between blood pressure and the prevalence of dementia and Alzheimer's disease, whereas other studies have yielded mixed results that largely depend on the age at which blood pressure is measured and the time interval between blood pressure and outcome assessments (Qiu et al, 2005). Some studies suggest that midlife high blood pressure is a risk factor for late-life cognitive impairment and dementia, and that low diastolic pressure and very high systolic pressure in older adults may be associated with subsequent development of dementia and Alzheimer's disease (Qiu et al, 2005; Skoog and Gustafson, 2006). Our preliminary studies indicate that hypertensive Tg AD model mice exhibit increased amyloid depositions compared to normotensive Tg AD mice. These results have led to the hypothesis that sustained hypertension will lead to decreased functional hyperemia in the brain which in turn contributes to brain hypoperfusion and adds to the cognitive impairments of AD. Further, decreased blood flow will concurrently lead to increased amyloid beta deposition in the brain due to decreased clearance of Aβ. Thus, the following studies are designed to test the hypothesis that long-term untreated hypertension will lead to perturbed vascular function, and increased cognitive dysfunction and pathology in AD model mice and that antihypertensive treatment of hypertensive Tg AD model mice will attenuate cerebral hypoperfusion

and significantly decrease cognitive deficits and amyloid beta deposition in the brain.

Materials and Methods

Animals:

In our study we used APP-DI (Davis et al., 2004) transgenic AD female mice (10/group). At six months of age Alzet minipumps containing human Angiotensin II (130 μg/day) or saline were implanted s.c. under anesthesia with isoflurane. The mice were treated (and, thus, were hypertensive) for two months. All mice were maintained in accordance with Institutional Animal Care and Use Committee (IACUC) regulations at the University of Alabama at Birmingham.

Behavioral analysis:

After seven weeks of hypertension, the mice were subjected to a battery of behavioral tests for hippocampus-related spatial memory. To rule out differences in anxiety or stress levels between animals, the open field and elevated plus maze were used as basic measures; to analyze cognitive deficits, we tested the mice in the water maze task (Liu et al, 2002) and the Barnes maze (Barnes, 1979) these tests were performed in the UAB Behavioral Core).

The *water maze apparatus* and procedure previously described in detail, uses a blue plastic pool, 120 cm in diameter and a see-through round platform, 10 cm in diameter, located 1.0 cm below the water surface (Liu et al, 2002). During day 1 through 5 of the testing period, the mice are trained to find the hidden platform that is kept in a constant position throughout these 5 days. Four trials are run per mouse each day so that all starting positions are equally used (in a random order). The mice are given 90 s to find the platform and 10 s to stay on the platform. The inter-trial interval is approximately 10 min. Learning of the task is evaluated by recording the swimming speed, latency to find the platform, path length, and percentage of trials each animal finds the platform.

The *Barnes maze* that is used has a design similar to the one developed by Barnes (1979) for rats but it has been adapted for mice testing. The raised (120 cm above the floor), round platform has a diameter of 140cm, and the escape holes are distributed along the rim of the platform. Only the correct escape hole, one of the many holes around the rim, has the escape cage attached under it. The mice are trained to escape from the open platform (the start position is in the middle of the platform) to the by finding this specific cage. An entry into an incorrect hole is deemed a reference memory error, and an entry into a hole the mouse has previously examined, is a working memory error. The mice are removed from the maze after escaping or after 4 minutes has passed, whichever comes first. An entry into the cage is defined as the head entering the escape hole. Learning of the task is evaluated by recording the latency to find the correct escape hole, the path length, and percentage of trials each animal finds the correct hole. Four trials are run per day per mouse; the learning of the maze takes an average of 5 days.

Blood pressure measurements:

Blood pressure was measured every two weeks starting from 1 week prior to the infusion, using indirect, tail-cuff blood pressure measurements supported by the Hatteras blood pressure measurement apparatus.

Blood flow measurements:

The head of the animals were fixed into a stereotaxic frame, the apex of the skull being exposed, to allow laser-Doppler flowmetry (LDF) of the cerebral blood flow (CBF). A hole was drilled in the skull above the middle cerebral artery. The laser Doppler probe (BIOPAC; LDF100c unit with TSD144 probe) was placed stereotaxically above this location (which corresponds closely to the expected region of maximal change in the flow response). The LDF signal, systemic arterial blood pressure, and heart rate were recorded continuously while simultaneously being processed on a PC (see figure; changes following phenylephrine injection). We recorded CBF before, during, and after a 5 sec whisker stimulation to measure the functional hyperemia response (i.e., the cerebral autoregulation; Rosengarten et al, 2005).

Histological analysis:

Aβ deposition, inflammation and blood vessel density and size were quantified in the mice. At 8 months of age (following the behavioral, blood pressure and blood flow analyses described above), the animals were sacrificed. The mice were transcardially perfused with 0.9 % saline followed by 4% paraformaldehyde in 0.1 M Na-phosphate buffer. The brains were removed and placed in the fixative for 2 hours, then transferred to a 30% sucrose solution; the brains are kept in this solution overnight on a shaker table. Six series of (1 in 6) coronal sections (35 μm) were cut through the brain using a sliding, freezing microtome. One half of the first series of sections were stained with cresyl violet, the other half - with an antibody against human Aβ using the W0-2 antibody (mouse anti-humanA□β4-10). One half of the second series were immunohistochemically stained for GFAP (mouse anti-GFAP; Sigma), whereas the other half were stained for CD11b (rat anti-mouseCD11b; Serotec), a marker of microglia. One half of the fourth series is stained for glucose transporter1 (Glut-1, rabbit anti-Glut1; Chemicon).

In short, the sections were rinsed overnight in Tris-buffered saline (TBS), then the series of sections were transferred to a solution containing the primary antibody, the solution consisting of TBS with 0.5 % Triton X-100 added (TBS-T). Following incubation in this solution for 18 h on a shaker table at room temperature (20oC) in the dark, the sections

are rinsed in TBS-T and transferred to the solution containing the appropriate secondary antibody (at 1:500). After two hours, the sections are rinsed with TBS-T and transferred to a solution containing mouse ExtrAvidin® (Sigma), following another rinse the sections are incubated for approximately 3 min with Ni-enhanced DAB. Following rinsing in 0.1 M phosphate buffer (pH 7.4), all sections are mounted on slides and coverslipped with DPX and inspected on a microscope using brightfield illumination.

Quantification of Aβ deposits:
The appropriate areas of the brain were digitized using a Olympus DP70 digital camera, and the images were converted to grey scale. To avoid changes in lighting, which could affect measurements, all images were acquired in one session. The area covered by Aβ (in the W0-2 stained material) was measured using the ScionImage (NIH; Frederick, MD, USA) program (Kadish et al. 2002). These measurements were done in triplicate (i.e., at three rostrocaudal levels of the appropriate cortical areas and arteries; Kadish et al., 2002) to minimize variability. The percentage of area covered by Aβ (i.e., the Aβ load) is the area occupied by Aβ divided by the total area of brain measured. Data were analyzed by ANOVA (SPSS version 10.0), and post-hoc tests (Tukey and Scheffe) were carried out to determine the source of a significant main effect or interaction.

Results

Angiotensin II treatment:
The treatment with Ang II for two months using Alzet minipumps resulted in a significant increase in blood pressure (Figure 1), sham treatment (i.e., saline) did not affect blood pressure. General health and body weight was not affected by Ang II treatment. The blood pressure of Ang II treated mice was significantly higher by twelve days after implantation of the Alzet mini-pumps, and stayed significantly higher during the full infusion period.

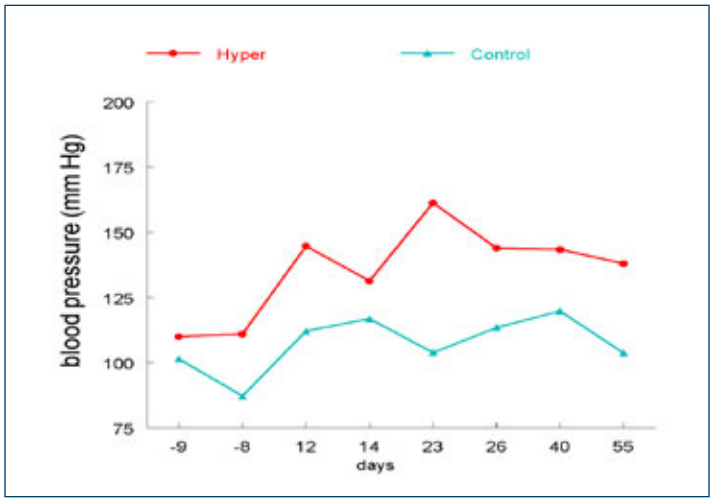

Fig. 1 Graph showing the difference in blood pressure between the Angiotensin II treated and control mice. Notice the significant increase in blood pressure in the Angiotensin II treated mice after implantation of the Alzet mini-pumps.

Behavioral test analysis:
Ang II treated mice had a longer escape latency in the Barnes maze (Figure 2), compared to the timings of the saline-treated, control animals. Similarly, in the water maze the Ang II treated mice had a longer escape latency than the control mice (Figure 2).

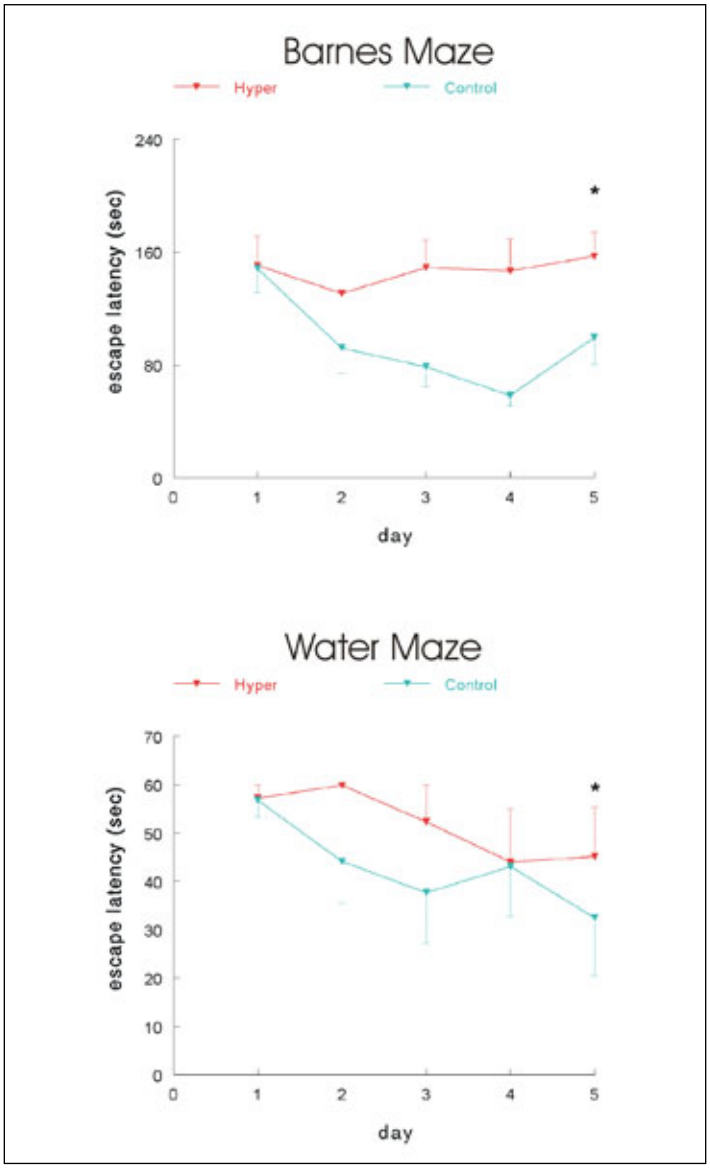

Fig. 2 Graphs showing the learning curves in the water maze (A) and the Barnes maze (B) of Angiotensin II treated and control mice over a five day testing period. Note the significant learning deficit in Angiotensin II treated mice.

Laser Doppler blood flow measurement:
The analysis of laser Doppler blood flow graphs shows that Ang II treated animals took a longer time to respond to the stimulus and also had significantly slower recovery time to return to the baseline blood flow level (Figure 3), i.e., they had

a significantly impaired hyperemia response, in comparison to the response of the control animals.

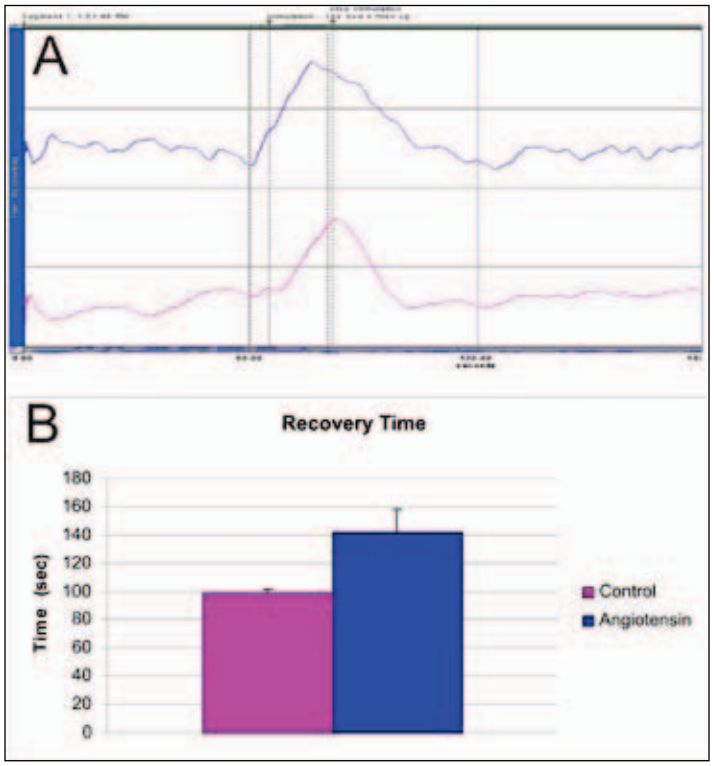

Fig. 3 **A:** graph showing the laser Doppler measurement of blood flow in somatosensory cortex of Angiotensin II treated and control mice. **B**: comparison between recovery times after whisker stimulation for Angiotensin II treated and control mice.

Pathology:
In contrast to the sham treatment, the Ang II treatment increased the amount of Aβ deposition significantly, 0.89±0.10 and 2.92±0.33 ($P<0.05$) respectively (Figure 4), especially in the hippocampus. Further, the number of blood vessels in the thalamus that show CAA significantly increased however, no changes are present in cortical arterioles.

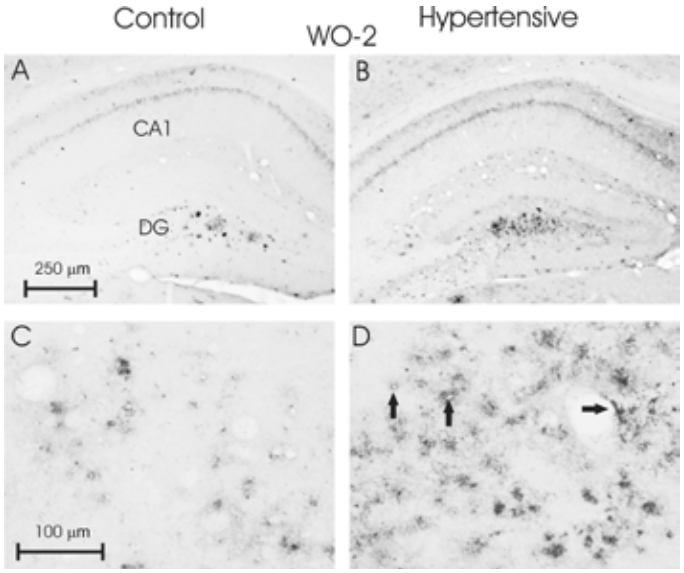

Fig. 4 (bottom of last column) Four photomicrographs showing Aβ stained sections of the dorsal hippocampus (A, B) and the ventrobasal complex of the thalamus (C, D), A and C from Angiotensin II treated mice, B and D from Control mice. Arrows in panel D shows amyloid beta deposition in blood vessel walls. CA1 – CA1 field of the hippocampus, DG – dentate gyrus.

There was significant increase in density of staining for GFAP (Figure 5). In the astrocytes there are increased amyloid depositions in the Ang II treated mice in comparison to the depositions in the control mice, indicating an elevated inflammation state in hypertensive mice. Blood vessel density did change slightly in the hippocampus (Figure 5), and there are similar changes in the thalamus (results not shown). More interestingly, both the hippocampus and thalamus show an increase in dysfunctional vessels in the plaque-deposit areas (Figure 5).

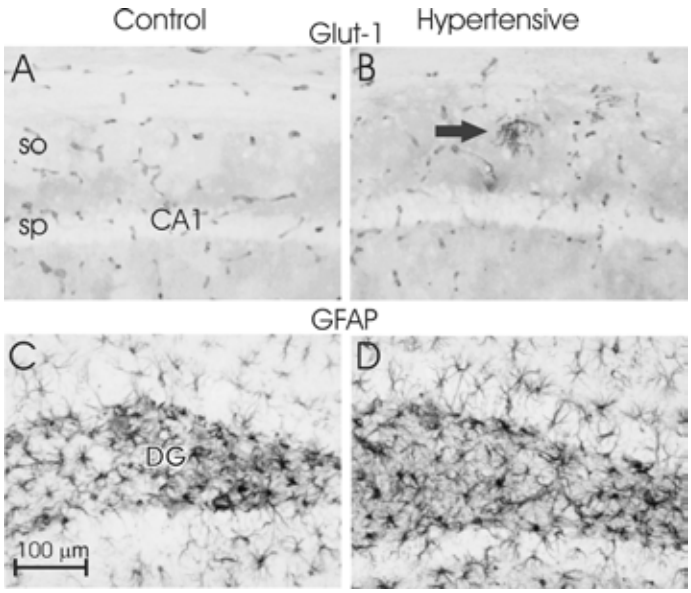

Fig. 5 Four photomicrographs showing Glut1 (blood vessels) and GFAP (astrocytes) stained sections of the dorsal hippocampus left side from Angiotensin II treated mice, right side from Control mice. Arrow in panel B shows abnormal blood vessel in the brain of hypertensive mouse. so – stratum oriens, sp – stratum pyramidale.

Discussion
The results of these studies demonstrate that mid-term hypertension (6 – 8 months of age) in AD model mice significantly increased Aβ deposition in the hippocampal formation. Furthermore, the hypertensive animals showed a significant decrease in learning abilities and memory capacity. Due to the impaired cerebral hyperemia there is decreased cerebral blood flow which leads to increased amyloid deposition and greater glial activation; in turn, these events lead to cognitive deficits. Therefore, maintenance of normal blood pressure is critically important for brain neuronal function.

The relationship between blood pressure, cognitive function, and dementia has, in recent years, been researched extensively (e.g., De la Torre, 2004). Most studies suggest that midlife high blood pressure is a risk factor for late-life cognitive impairment and dementia, and that low diastolic pressure and very high systolic pressure in older adults may be associated with subsequent development of dementia and Alzheimer's disease (Qiu et al, 2005; Skoog and Gustafson, 2006). Observational studies and randomised clinical trials have provided evidence for a protective effect of antihypertensive therapy against dementia and stroke-related cognitive decline (Savaskan, 2005). Atherosclerosis resulting from long-standing hypertension, and cerebral hypoperfusion secondary to severe atherosclerosis (and to low blood pressure) may be major biological pathways linking both high blood pressure in midlife and low blood pressure in late-life to cognitive decline and dementia. Our data confirm that hypertension increases learning and memory deficits in AD model mice.

In our current studies, we have used the APPSwDI Tg mouse line (Davis et al., 2004), these mice develop pathology relatively rapidly, the first parenchymal plaques appear approximately at 3 months of age, furthermore at that age several arteries are already loaded with Aβ. With increasing age both the cortical and the blood vessel Aβ load significantly rise. Our data indicate that midlife hypertension (i.e., from two months of age) significantly increases amyloid deposition in both the parenchyma and in blood vessel walls. One of the two characteristic pathological hallmarks of human AD is the presence of neuritic plaques (Braak and Braak, 1991). Neuritic plaques have a dense core of aggregated Aβ peptides (Braak and Braak, 1991; 1998; Selkoe, 2001). It has been suggested that these deposits of extracellular Aβ originate from direct secretion of Aβ followed by extracellular accumulation. Alternatively, this protein would first accumulate intracellularly, followed by cell-death and subsequent release of the accumulated Aβ (e.g., Gouras et al., 2000; Selkoe, 2001). Presently, it is still unclear how the extracellular deposition of Aβ and the progression in AD neuropathology are related to the increased production of APP and Aβ.

Most neuritic plaques are surrounded by activated glial cells (e.g., Akiyama et al., 2000; Rogers and Lue, 2001). Similarly, plaques in AD-model mice are quite often accompanied by activated glial cells, both astrocytes and microglia (e.g., Bondolfi et al., 2002). However, the role of activated microglia is unclear; on one hand they could protect the brain by removing Aβ , on the other hand they secrete inflammatory cytokines and generate NO, and can thus damage and kill bystander neurons (Akiyama et al., 2000). Their role in the uptake of Aβ is disputed, however, with some groups seeming to show clearance of Aβ by microglia (e.g., Rogers and Lue, 2001), while others show that microglia do not seem to take up Aβ (Stalder et al., 2000). Most of the plaques in our animals are surrounded by activated glial cells and likewise, most CAA deposits are associated with both activated microglia and activated astrocytes.

Together the data indicate that early treatment of hypertension could reduce the incidence and severity of Alzheimer's disease.

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Extracellular ATP Activates Multiple Calcium Signaling Pathways in Osteoblasts

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Abstract

Mechanical stimulation of osteoblasts (bone forming cells) increases extracellular release of ATP and leads to an increase in intracellular calcium $[Ca^{+2}]_i$. This ultimately activates downstream targets such as Calmodulin Kinase (CaMK) and leads to an increase in osteoblast proliferation and bone formation. The role of CaMKII in regulating osteoblast proliferation in response to ATP is unclear. The purpose of this study is to determine the specific molecular mechanisms that are involved in the osteoblasts response to extracellular ATP treatment. To examine the effects of extracellular ATP on osteoblast proliferation, we treated MC3T3-E1 (murine clonal osteogenic cell line) with (100 uM) ATP for 24 hours. Here we show that ATP treatment caused an 86.7% increase in osteoblast proliferation. Furthermore, ATP treatment resulted in a 40% increase in the protein levels of Cyclin D1, which is known to be a critical mediator of cell growth and proliferation. Mechanistically, we demonstrate that ATP treatment (10 minutes) decreases the activation of CaMKII (75.4%) and increases the phosphorylation of ATF-2 and CREB (95.9%, 94.4%, respectively). The overexpression of CaMKII in MC3T3-E1 osteoblasts resulted in a 98.2% decrease in the ATF-2 activation and a 76.9% increase in the phosphorylation of CREB in response to ATP treatment. These results describe a novel signaling pathway in response to ATP that involves CaMKII, ATF-2, and CREB.

Introduction

Osteoblast cells are bone-forming cells that are essential for maintaining balance in the dynamic nature of bone formation[1]. Bone remodeling involves two main characteristics: resorbtion of bone by osteoclasts and formation of bone by osteoblasts[1]. In this study, the main focus is osteoblast proliferation and its response to extracellular ATP.

Mechanical stimulation, such as fluid shear stress or strain, of osteoblast cells is known to cause an increase in extracellular release of ATP[2]. This extracellular release of ATP plays an important role in bone remodeling because of its involvement in calcium signaling pathways[3]. The oscillations of intracellular calcium levels caused by the extracellular ATP in response to mechanical loading influence the activation of downstream targets involved in gene expression, osteoblast proliferation, and bone formation[4].

Moreover, in regards to the calcium signaling pathway, extracellular ATP is known to bind to two types of purinergic receptors, including the P2X ligand-gated ion channels and the P2Y G-protein coupled receptors[2]. There are seven known P2X subtypes and eight known P2Y subtypes[5]. The $P2Y_2$ receptor specifically could function as "switches" for bone formation[5]. For P2X receptors, ATP is the agonist that binds to the receptor to open the gate and allow extracellular calcium entry into the cell[1]. In respect to P2Y receptors, ATP activates the G-protein to activate phospholipase C, which produces inositol triphosphate[1]. The inositol triphosphate then allows the release of intracellular calcium stores from the endoplasmic reticulum and mitochondria[6].

In addition to the regulation of intracellular calcium through the purinergic receptors and its ability to activate downstream proteins, Ca^{+2}/CaM-dependent protein kinase II also regulates intracellular calcium and the phosphorylation of downstream molecules[7]. First, four calcium ions bind to calmodulin, a calcium binding protein, which induces conformational change[1]. Then, the activated calmodulin binds to calmodulin Kinase II (CaMKII), allowing the autophosphorylation of CaMKII[1]. Because of its ability to autophosphorylate, CaMKII is able to remain activated even after intracellular calcium levels decrease[1]. CaMKII is essential in bone growth because it is a multimeric serine/theronine kinase that plays an important role in calcium signaling pathway as a transducer, leading to activation of downstream proteins and osteoblast proliferation and differentiation[8].

Some of the downstream proteins include the following that were studied in this experiment: cyclin D1, ATF-2 and CREB. Activating Transcription factor (ATF) and cAMP response element binding (CREB) are a family of stress-responsive transcription factors[9]. As part of the Mitogen-Activated Protein Kinase (MAPK) pathway, p38 kinase and/or c-Jun N-terminal kinase (JNK) phosphorylates ATF-2, which ultimately activates various gene targets such as c*yclin D1*[9]. The protein Cyclin D1 is an important mediator of cell growth and proliferation, regulated by ATF-2[9]. Therefore, the activation of this calcium signaling pathway by extracellular ATP can eventually result in osteoblast proliferation and bone growth.

Method

Cell Culture

MC3T3-E1 GFP and MC3T3-E1 overexpressed CaMKII osteoblast cells were grown in α-MEM media containing phenol red (10% BSA, 1% P/S) for 72 hours. Cells were seeded at density of 100,000 cells/well. Media was changed to serum-free media 24 hours before treatment to starve the cells.

Cell Count
MC3T3-E1 (density of 30,000 cells/well) osteoblasts were grown in α-MEM media containing phenol red (10% BSA, 1% P/S) for 24 hours. Media was changed to serum-free media 24 hours before treatment. ATP (100uM) was added to cells for 24 hours, and cell count was conducted on these cells as well as control cells.

Intracellular Calcium Measurement
MC3T3-E1 parent cells were grown in α-MEM media containing phenol red (10% BSA, 1% P/S) for 24 hours on sterile coverslips. Cells were then washed with media containing no phenol red to remove all traces of phenol red containing media. Cells were loaded with Fluo-4 AM dye (10uM) and placed in incubator for 45 minutes. Then the coverslips were placed in new culture dish and washed with media containing no phenol red to remove any non-specific dye associations. Once the coverslip was placed under the microscope, a baseline was established. After the first ten frames, 10uM ATP was added.

Cell Treatment: Osteoblast Response to ATP Treatment Studies
MC3T3-E1 GFP and MC3T3-E1 overexpressed CaMKII osteoblast cells were treated with ATP (100uM) for 10 minutes. Prior to treatment, cells were starved for 24 hours in serum-free media. To stop the treatment, the media was removed from the wells. Protein was extracted using lysis buffer with protease inhibitor and phosphatase inhibitor.

Western Blot Analysis and Densitometries
Protein extracted from cells was separated using 10% SDS-PAGE. Proteins were transferred to immobilon P-PVDF membrane. Any non-specific binding was blocked with blocking solution. The following primary antibodies were probed for: Cyclin D1, p-ATF2, p-CREB, and p-CaMKII. To quantify the levels of protein expression, densitometry was conducted using the ImageJ program.

Immunohistochemistry
After treating MC3T3-E1 osteoblast cells (cell density of 25,000 cells/well) with ATP for 10 minutes, cells were fixed with 4% formaldehyde. Then cells were treated with Triton X-100. Cells were blocked in 5% BSA for 1 hour, and then primary antibody p-ATF2 was added (1:50 dilution) overnight in a humidity chamber at 4°C. After incubating the biotin-conjugated secondary antibody (1:750 dilution) for 30 minutes, a 1:20 dilution of avidin biotin enzyme reagent was added for 30 minutes. Cells for control and ATP treated cells were stained brown with peroxidase substrate (DAB) for 8 minutes and 30 seconds and pictures of slides were taken (200X).

Results
Extracellular ATP treatment increases osteoblast proliferation
Extracellular ATP treatment for 24 hours on MC3T3-E1 osteoblast cells caused an 86.7% increase in proliferation when compared to the control.

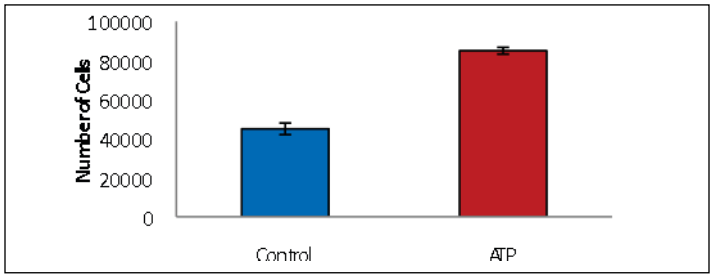
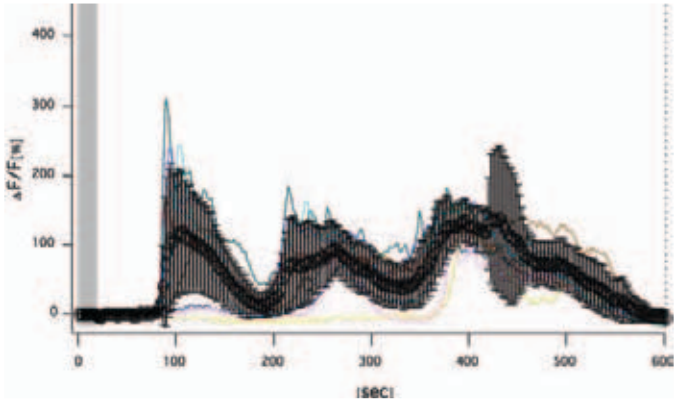


Figure 1: **Extracellular ATP treatment increases osteoblast proliferation.** MC3T3-E1 cells were treated with 100uM ATP for 24 hours. Cells were then counted. Values represent the mean ± SD of ATP treated cells compared to control. The study was performed once in triplicate.

Extracellular ATP increases intracellular calcium
Calcium imaging shows an increase in intracellular calcium when stimulated with 10uM ATP. Imaging also shows variations in relative levels of intracellular calcium release from one cell to another. Based on the ΔF/F graph, calcium release follows an oscillatory pattern, resulting in the rise and fall of calcium levels within the cell.



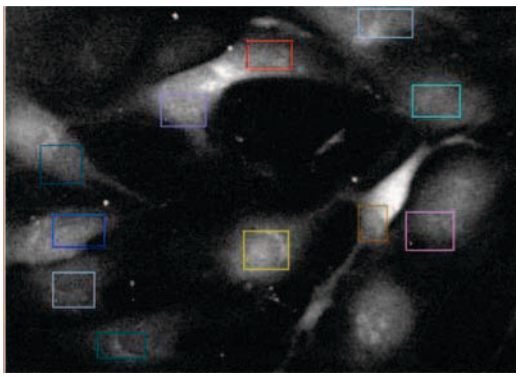


Figure 2: **Extracellular ATP increases intracellular calcium.** MC3T3-E1 osteoblasts were grown on coverslips and treated with 10uM ATP. All traces of media containing phenol red were removed before loading the dye. Fluo-4 AM (10uM)

dye was used for calcium imaging. Values represent the mean ± SD of ATP treated cells. The study was performed two times.

Extracellular ATP increases Cyclin D1 protein levels
Extracellular ATP treatment in MC3T3-E1 osteoblast cells led to a 40% increase in cyclin D1 protein levels compared to the control.

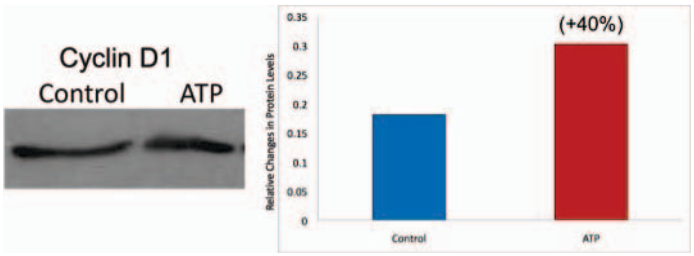
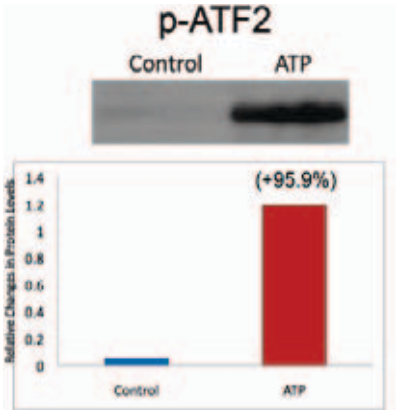
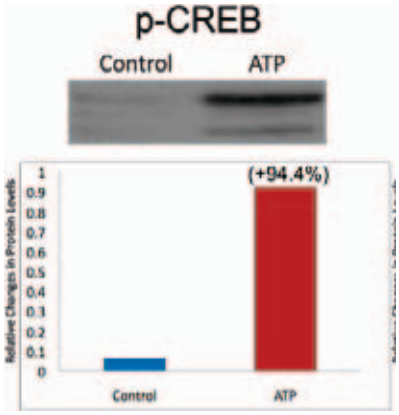


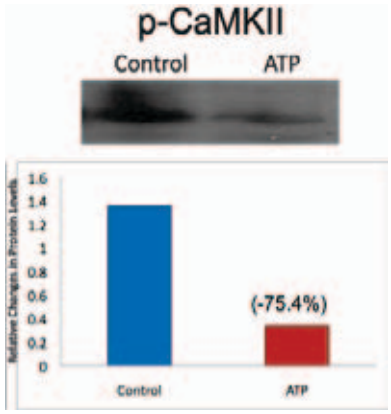
Figure 3: **Extracellular ATP increases Cyclin D1 protein levels.** MC3T3-E1 osteoblast cells were cultured in serum-free media for 24 hours before treatment. Cells were treated with ATP (100uM) for 10 minutes. Proteins were extracted and separated by SDS-PAGE. Immunoblots were developed using antibody against Cyclin D1. The ImageJ program was used to quantify changes in protein levels. The study was performed once.

Extracellular ATP increases the activation of calcium downstream molecules
Extracellular ATP treatment (10 minutes) in MC3T3-E1 osteoblast cells increased the phosphorylation of ATF-2 95.9% and increased the phosphorylation of CREB 94.4%. However, the same treatment caused a 75.4% decrease in the activation of CaMKII.

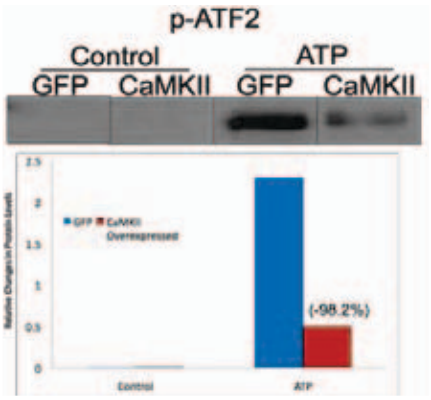
Figure 4: (next column) **Extracellular ATP increases the activation of calcium downstream molecules.** MC3T3-E1 osteoblast cells were cultured in serum-free media for 24 hours before treatment. Cells were treated with ATP (100uM) for 10 minutes. Proteins were extracted and separated by SDS-PAGE. Immunoblots were developed using antibodies against p-ATF2, p-CREB, and p-CaMKII. The ImageJ program was used to quantify changes in protein levels. The study was performed once.







ATF2 activation in response to extracellular ATP is CaMKII-dependent
In overexpressed CaMKII in MC3T3-E1 osteoblast cells, the activation of ATF-2 decreased (98.2%) and increased activation of CREB (76.9%).



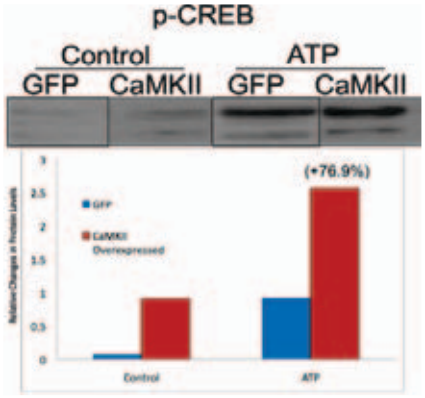


Figure 5: (bottom of page 41) **ATF2 activation in response to extracellular ATP is CaMKII-dependent.** Overexpressed CaMKII in MC3T3-E1 osteoblast cells were cultured in serum-free media for 24 hours before treatment. Cells were treated with ATP (100uM) for 10 minutes. Proteins were extracted and separated by SDS-PAGE. Immunoblots were developed using antibodies against p-ATF2 and p-CREB. The ImageJ program was used to quantify changes in protein levels. The study was performed once.

Extracellular ATP increases nuclear translocation of p-ATF2
Immunohistochemistry was done on MC3T3-E1 osteoblast cells, looking for translocation of p-ATF2. Looking at the image of the control sample, the protein p-ATF2 is mainly located in the cytoplasm and surrounds the nucleus. In regards to the ATP treated osteoblast cells, nuclear translocation is observed because dark staining is visible in the nucleus as well as slight staining in the cytoplasm.

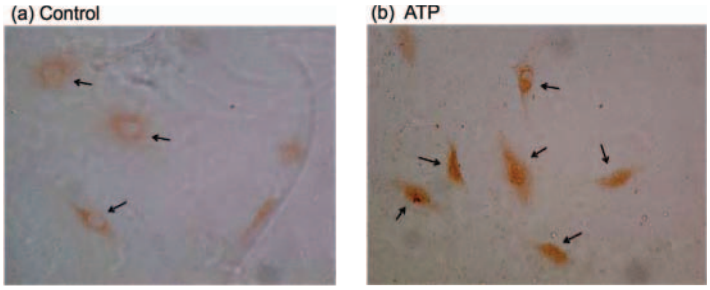


Figure 6: **Extracellular ATP increases nuclear translocation of p-ATF2.** MC3T3-E1 osteoblast cells were cultured on glass coverslips (25,000 cells/well) and changed to serum-free media 24 hours before treatment. Cells were treated with 100uM ATP (bottom panel) for 10 minutes and fixed with 4% formaldehyde. Primary antibody p-ATF2 was added (1:50 dilution). Cells were stained (brown), indicated by arrows. Photographs were taken with Nikon microscope (200X). The study was performed once. (a) Control (b) 100uM ATP

Discussion
The cell count experiment showed an increase in the number of osteoblast cells, demonstrating the direct affects of ATP on osteoblast proliferation. Therefore, when extracellular ATP is released as a result of mechanical stimulation, bone growth is also affected. The calcium imaging study showed that intracellular calcium levels increase in a cyclic pattern caused by the stimulation of extracellular ATP. This cyclic or wave pattern further suggests that extracellular calcium levels are strictly regulated. This regulation could occur through the purinergic receptors (P2X and P2Y) and through the activation of calmodulin and CaMK.

Based on the immunoblots and relative changes in protein levels, the phosphorylation of ATF2 seems to be CaMKII-dependent because in MC3T3-E1 osteoblast cells, p-ATF2 was upregulated, and in overexpressed CaMKII MC3T3-E1 osteoblast cells, p-ATF2 was downregulated. With p-CREB, an upregulation was seen when CaMKII was increased and decreased, meaning other factors might be controlling the phosphorylation of CREB in addition to CaMKII. To check the translocation of p-ATF2, immunohistochemistry was done on osteoblast cells. Based on the images, nuclear translocation is observed, meaning extracellular ATP treatment has the ability to influence the activation of ATF2 through the calcium signaling pathway. The data suggests this novel signaling pathway involving CaMKII and ATF2.

For future studies, more downstream proteins can be tested to determine if extracellular ATP influences their activation. Also, to determine if a specific purinergic receptor, P2X or P2Y, regulates the phosphorylation of downstream targets, a study can be done that inhibits one or more of these receptors. Another study that can be performed involves the nuclear factor of activated T cells (NFAT), a transcription factor that is regulated by calcium levels[10]. Specifically, the affects of extracellular ATP on the activation of NFAT can be tested.

Conclusion
ATP stimulates osteoblast differentiation through a novel signaling pathway that involves CaMKII, ATF2, and CREB.

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Movement Patterns of the Common Periwinkle *Cenchritis muricatus* in the Rocky Supralittoral Zone of San Salvador, Bahamas

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Research Mentors: James McClintock, Ken Marion, and Robert Angus

Abstract

Directional movement patterns of adults of the littorinid periwinkle Cenchritis (Tectarius) muricatus were documented over a five and half day period in May 2008 at low, mid, and upper supralittoral (the region above the intertidal zone that is wet only by occasional sea spray) sites on the northwestern rocky coast of San Salvador, Bahamas. Daytime surface temperatures on the rocky substrata were extreme ranging from 24 to 48 ˚C. Snails at all three sites generally moved only short distances (approximate mean = 1 cm/hr). Nonetheless, snails at the mid-level site moved significantly greater distances than did snails at either the low or upper sites. There was a significant increase in distances moved following a rain event (approximate mean = 2 cm/hr). Moreover, at upper and lower sites, snails moved greater distances during the night than during the day. These findings suggest that snails increase the distances they move when surface substrata temperatures are low due to rainfall or a reduction in exposure to sunlight (night). This likely reflects a mechanism to reduce physiological stress due to desiccation as mucus is lost during locomotion. Selection for increased movements during these periods may be valuable in terms of meeting nutritional requirements as C. muricatus grazes on microalgae that occur on the rocky substrata.

Introduction

Supralittoral habitats of the Caribbean islands are well known to be characterized as physically stressed environments. Extreme tropical temperatures exert considerable desiccation stress on gastropods and other marine organisms that occupy this zone (Little 1989; McMahon and Britton 1991; Gochfeld and Minton 2001). For example, daytime temperatures in rocky supralittoral habitats of San Salvador, Bahamas may attain temperatures as high as 48 or even 50 ˚C (McClintock et al. 2007; present study). These supralittoral habitats are also rarely wetted even during extreme high tides, although they may periodically receive spray during strong storm winds or coastal surge events associated with hurricanes. To further exacerbate the situation the Caribbean region is characterized by intense periods of aridity which impose further desiccation stress on supralittoral marine life.

Gastropods that occur in supralittoral habitats possess a variety of mechanisms to mitigate the effects of thermal and desiccation stress including aspects of shell morphology such as pigmentation, small size, degree of ornamentation, and possession of an operculum (Vermeij 1973, Heath 1975, Britton 1995). Moreover, gastropods that occur in these regions may produce mucus seals that secure them tightly against the substrate (McMahon and Britton 1991) and possess behaviors that facility the occupation of shaded microhabitats (Garrity and Levings 1984).

Cenchritis muricatus (Linnaeus, 1758) is the most common gastropod in the supralittoral zone of the rocky shores in the Caribbean (Lewis 1960; Fraenkel 1968; Lang et al. 1998; Minton and Gochfeld 2001; Gochfeld and Minton 2001; Emson et al. 2002). Several studies have shown that *C. muricatus* generally move limited distances (Lang et. al. 1968; Burgett et al. 1987; McClintock et al. 2007). It has been suggested that individuals move directionally toward the sea during night hours when air temperatures are less extreme (Kaplan 1988). In contrast, Gochfeld and Minton (2001) noted a lack of directional movement and found that movements were not correlated with diurnal, tidal, or lunar cycles.

In the present study we further investigated the behavioral ecology of *Cenchritis muricatus* in order to determine whether there were differences in movement patterns (distances) during day and night periods at three discrete levels of the rocky supralittoral zone. Moreover, an opportunistic rain day event allowed an evaluation of whether individuals move greater distances during periods of substrate wetness.

Materials and Methods

The study site was located on the north shore of the island of San Salvador approximately 1 km west of the Gerace Research Centre (24° 3' 0", -74° 31' 0"). The site was comprised of weathered, deeply pitted, limestone. There was very little vegetation growing on the study site, however, the upper two thirds of the transect did contain *Rhachichallis americana*, *Strumpfia maritime*, and *Borricchia aborescens* (Cornel and Cornell 1982).

In order to examine the movement patterns of *C. muricatus*, three study sites were established at locations 2.1, 3.1, and 4.4 m above mean lower low water (MLLW). These sampling sites were chosen so as to represent supralittoral heights that were employed in a previous study to examine movement patterns in this gastropod (McClintock et al. 2007). A 50 x 50 cm quadrat (divided into 10 x 10 cm sectors with fine nylon line) was placed with its lower edge parallel to the shore line on to the substratum, and its location on the rocky substrata permanently marked using finger nail polish. This allowed the same exact site to be resampled during the study.

At each of the three sample sites, 15 snails were haphazardly selected and marked within the quadrant. Snail marking consisted of using super glue to attach a 3 x 3 mm piece of numbered plastic to the apex region of each snail shell. In addition, a small dot of red fingernail polish was placed on the shell apex in order to facilitate relocation of each individual on the rocky substrata .

The initial locations of each of the 15 snails within each sample site quadrant were recorded. Subsequent positions of each snail were recorded at 12 hour intervals over a five and half day period (eleven 12 hour periods). Measurements were recorded in the early morning and late evening so as to allow an evaluation of distances of movement during day versus night hours. At each 12 hr interval, surface substrata temperatures were recorded at each site using a thermometer

Distances moved by each snail when occupying the quadrant were based on its position relative to its most recent position (12 hr earlier). Once snails moved outside of the quadrant, each subsequent measurement of its distance moved was based on the distance from its position to the corner of the quadrant nearest its location.

Statistical Analyses

An Analysis of Variance (ANOVA) was used to compare the mean distances snails moved over 12 and 24 hour periods at the three supralittoral sites. A Tukey Post Hoc test was employed to conduct pair-wise comparisons. Prior to analysis with ANOVA and Tukey Post Hoc tests, all data was normalized using a log transformation.

Results

Snails at the mid–level site moved significantly greater distances per 12 hr than did snails at the high ($P < 0.01$) or the low site ($P = 0.05$) (Fig. 1). There was no significant ($P > 0.05$) difference between the distances moved by snails at the high and low site.

A rain event occurred on day 1. As such, we restricted our comparison of day versus night movements to full days that occurred on days 2, 3, 4 and 5 of our study. When 12 hr periods of movement coincident with night were compared to those during daylight, at the upper and lower sites snails moved significantly ($P < 0.05$) (Fig. 2). There was no significant difference ($P > 0.05$) between day and night distances moved at the mid-level site.

We compared distances moved on a day by day basis (24 hr periods) for the five full days of our study to evaluate the impact of the rain event that occurred during our study. Shown in Figure 3 are distances moved per day which varied significantly from one another (ANOVA: $P < 0.001$). Pairwise analysis indicated that distances moved during day 1 (rain event) were significantly ($P < 0.05$) greater than days 3, 4 and 5. While there was no difference between day 1 and 2, day 2 was significantly ($P < 0.05$) greater than day 4.

Mean substrate surface morning and evening temperatures were 38.5 °C± 4.8 °C (n = 6) and 27.0 °C± 2.4 °C (n =5), respectively.

Discussion

Similar to other studies our qualitative observations indicated that *Cenchritis muricatus* occurred in high abundance in the supralittoral zone of San Salvador, Bahamas (Lewis 1960, Frenkel 1968, Lang et al. 1998, McClintock et al. 2007). Our experimental results indicated that snails at low, mid and high level study sites moved on average short distances (approximately 1 cm per hr). These limited movements are similar to what has been reported in other studies with *C. muricatus* and likely reflect the extreme conditions under which they live (Lang et al. 1968, Burgett et al. 1987, McClintock et al. 2007). While distances of movement were generally low, we did detect significant differences between our suppralittorial sites. *Cenchritis muricatus* moved greater distances at the mid-level infralittoral site (3.1 m above MLLW). The basis for this pattern could be related to several factors. Snails at the high site may move less than those at the mid site due to the increased stress associated with heat on the upper reaches of this zone. In contrast, snails at the low site may move less than mid-level snails because their food source, microalgae, is likely to occur at greater density closer to the sea. Thus, they would need to graze less to acquire a similar amount of nutrition. Mid-level snails may move further to optimize grazing under a thermal stress regime that facilitates greater movement than the upper site. A number of studies have suggested that heat stress is an important factor in the restricting movement behaviors *C. muricatus* (McMahon 1990, Britton 1995, Lang et al. 1998).

Contrary to previous studies (Lang et al. 1998, Gochfeld and Minton 2001, Emerson et al. 2002, McClintock et al. 2007), our study revealed significant differences between night and day distances of movement for two of the three sites examined (low and upper site). For snails in the upper site it may be advantageous to reduce dramatically (6 fold decrease) their movements during the day to avoid desiccation stress. We found morning temperatures (taken when the study sites were fully exposed to the sun) were almost twice those of evening temperatures (taken when the study sites were shaded). In contrast, snails at the lower site may have the luxury of meeting their nutritional needs during night time hours and thus avoiding movements during the day time. This would allow them to avoid day time heat stress and potential increased susceptibility to predation (as locomotion inhibits firm attachment to the substrate). Snails at the mid-level site did not move more at night than during the day, but moved

greater distances overall than snails at low or high sites suggesting the demands of nutrition and heat stress outweigh the benefits of reducing their day motion.

Although there was only a single day of rain during the study, its impact on the movements of snails when compared to more typical dry days was dramatic. We found that during the rain day when the substrata was wet, snails moved on average 2 to 3 times greater distances than on dry days. This supports previous observations that *C. muricats* moves greater distances when substrates are moist from storms (Gochfeld and Minton 2001; Emson et al. 2002). Interestingly, snails on day 4 of our study moved the shortest distance overall and this coincided with the hottest day of the five and half day study. Temperatures reached 48 °C on this day. That snails moved the greatest distance during a period of rain and the shortest distance on the hottest day reflects the tremendous heat related stress that *C. muricatus* must cope with in the infralittoral habitats San Salvador. The present study, and those of others on movements in this species, would suggest that heat stress is the single most important factor governing the movement patterns of *C. muricatus* in the Caribbean.

Acknowledgements
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Figure Legends

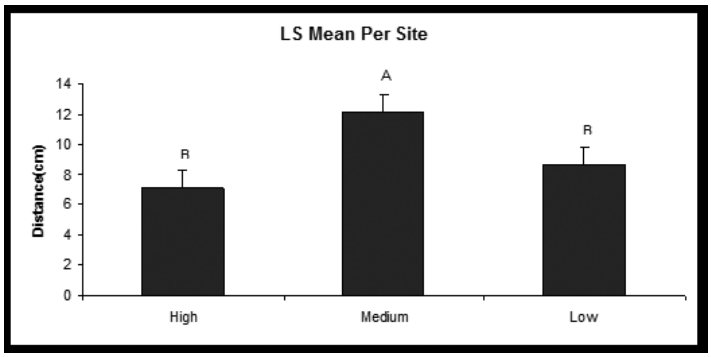

Figure 1: Least squares (LS) mean ± 1 SE for 12 hr incremental distances over a five and half day period for the littorinid gastropod *Cenchritis muricatus* at three supralittoral sites (2.1, 3.1 4.4 m above MLLW) on San Salvador, Bahamas. Different capital letters on top of bars indicate statistically significant differences.

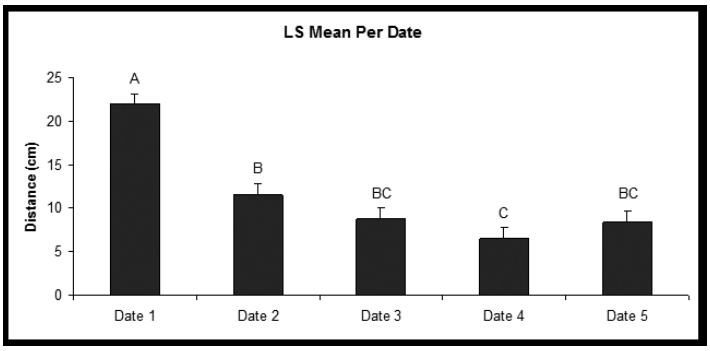

Figure 2: Least squares mean ± 1 SE during the day (open bars) and night (closed bars) over a five day period for the littorinid gastropod *Cenchritis muricatus* at three supralittoral sites (2.1, 3.1 4.4 m above MLLW) on San Salvador Island, Bahamas. Asterisks indicate significant differences.

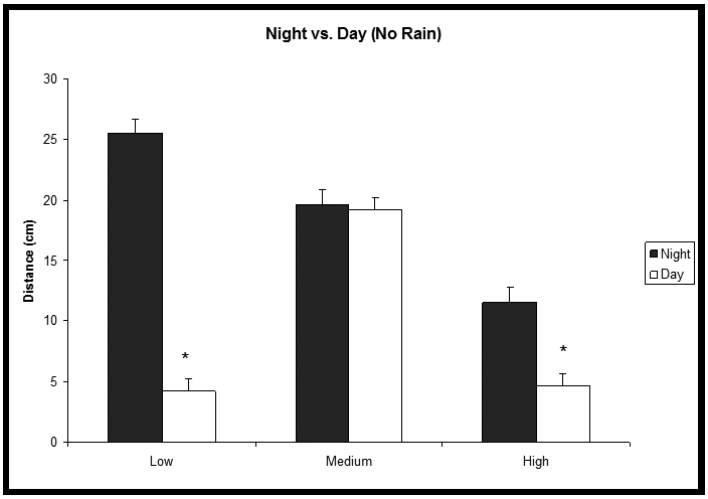

Figure 3: Least squares mean ± 1 SE distances moved over five days (24 hr) in the littorinid gastropod *Cenchritis muricatus* for all sites combined. Day 6 is not presented as it was sampled only in the morning hours. Different capital letters on top of bars indicate statistically significant differences.

faculty interview

The Fight Against HIV: *An Interview with Dr. Michael Saag*

Infectious Diseases, Matt Morton

One wouldn't have thought it was a cold, gloomy morning upon meeting Michael Saag, M.D.—his optimism and energy filled the second-floor conference room of the Bevill Biomedical Research Building as we prepared to begin the interview. It was this same energy, this passion for research obvious to those who have heard him speak, which interested me in Dr. Saag's journey from undergraduate to Director of UAB Center for AIDS Research (CFAR).

As an undergraduate, he began work at Tulane with a pharmacy group studying the effects of Aspirin. Later, during medical school in Louisville, his research focused more on neurology and mapping the feline jaw-opening reflex. When asked about his diverse research experiences, Dr. Saag felt that "what's important as an undergraduate is to focus on gaining a lot of different experiences—really going broad; don't sit up in a certain mindset because your mind is likely to change multiple times in the course of a career path, especially through the educational process… Even though we all want to plan our careers, you can't, because a lot of it's serendipity, a lot of it's opportunity, and a lot of it is that as we all go through the education experience, we're learning, we're growing, and we're thinking in new ways."

In 1981, Saag was drawn to UAB for two reasons. At the time, his belief that he would pursue cardiology led him to choose a residency at UAB for their reputation in cardiology. Also, his wife was from Birmingham. By 1988, Saag and others had started the 1917 Clinic with a mission to mix HIV/AIDS patient care and research as a way of testing new therapies. Between 1999 and 2004, they converted the old patient database into an electronic medical record, making it possible to answer the "routine [questions] in practice like, 'if I use this drug does it perform the same way in practice as it did in the clinical trials?'" They also study unusual side effects and practice patterns for current therapies, but according to Saag, "the limitation now is basically imagination and time. If we can think of a question and we have the time to answer it, it'll get answered. It's like a little laboratory, it's an informatics laboratory."

With such a functional laboratory at his fingertips, my next question for Dr. Saag was how such information is applied in the real world. Information from the Clinic helps drive changes not only in clinical practice situations, but also in the policies affecting HIV/AIDS patient care and research.

"For best practices, we've defined how certain drugs work in practice versus how they work in the clinical trials. That's an important set of data. Recently we were part of a major study that helped define when to start Anti-Retroviral Therapy (ART) by pooling cohort data together. We're part of several groups like that, one of which I run in the United States called CNICS, or the CFAR Network of Integrated Clinical Systems, where we pool data from nine other centers from around the country. In the study of several thousand patients who started ART, we found that those who waited did worse. So the notion was to start treatment a little bit early."

"Something we've done policy-wise is shown cost of care. Cost of care analysis has informed congress on how to fund HIV care in the United States; what it said is that HIV clinics can't make money on their own and need government programs to help provide service to patients. Another thing we've done was a simple analysis of mortality, based on when patients start treatment. We've found that, if people wait until they have more advanced disease, their mortality in 10 years is 50%, whereas if they start earlier it's only about 5%. Moreover, the problem is, and this is where the policy comes in, that the majority of patients we have seen showing up for care show up very late and the only way to find those patients earlier is to test earlier. The proof of this is that the only group of patients we've seen come into care earlier are pregnant women. And the reason for that is that they're getting tested routinely for HIV. Data like that coming from a clinic like ours contributed to a discussion in 2006 with the CDC about changing its recommendations for universal opt-out testing so that every person, as I say, who is sexually active or has every thought about being sexually active should be tested for HIV."

"A final part of our mission is to perform medical education so that physicians, nurse practitioners, and physicians assistants can continue their education. Standing up in front of audiences a lot of times we're using data from our own clinic when we make points, so there's no question that it's having impact."

Next, we discussed the future focus of HIV/AIDS research. First, Dr. Saag addressed the groundbreaking research happening at UAB.

"In the big picture of AIDS research on our campus, the first and foremost thing we all want to do is find a cure. While that is not talked about very much, there are people here who are working on the concept of the virus going latent in cells. It's the latently infected cells that are the barrier to cure, so trying to focus on that in the laboratory and then thinking of new thera-

peutic interventions that might dig into that is very important."

In contrast to the laboratory work, much of Dr. Saag's other research at UAB is in finding new therapeutic interventions and exploring factors that affect treatment. For instance, the effects of depression on a patient's success taking medication, as well as the effect of HIV infection on the aging process are two interesting topics of research currently being studied.
Dr. Saag works with groups from around the world on various other research initiatives.

"We've been doing a lot of work in Africa, working particularly out of Lusaka, Zambia with the PEPFAR program, which sends funding from the U.S. to pay for medication. We now have 190,000 patients we've enrolled into care over the last five years, which is remarkable, and that's just in Lusaka."
Just as interesting as the work being completed in Zambia is the way the information from that study is being used in Alabama to make a local impact. With the Zambama project, Saag and his colleagues are taking the best practices they've learned in Zambia and applying them back to Alabama.

"We always had this almost condescending paternalistic view of us going in with white coats and 'S's' on our shirts to save the world. Well, we have a lot to learn, as well. As far as stigma against HIV, people not getting tested, and people not having access to care, believe it or not there are not a whole lot of differences between rural Alabama in some areas and Lusaka, Zambia. They also have a lot of stigma in Lusaka, but because the problem was so enormous there—up to 1 in 4-5 people in their entire country between the ages of 20 and 45 being infected with HIV—they figured they better do something about this, so they did. They've done a lot of really interesting things about reducing stigma, getting testing for patients and individuals, and getting them into care. We're using a lot of those best practices and starting to study how well they work in areas of Alabama where there are pockets of high concentrations of HIV infected people who don't know their status. If they don't know then they show up late and if they show up late they have a higher rate of dying, so if we find them early and get them into care, we'll save lives."

In the interest of HIV/AIDS prevention, Dr. Saag also shared with me his feelings on abstinence-only sex education.
"It's poor. It's ill informed. It makes the person who gives the message feel good, but the question is, does it have any impact? I'm not trying to say that people's intentions are wrong when they adopt an abstinence only education approach and I'm sure for a certain segment of people at risk it works, but, in my experience over 25 years of dealing with this epidemic, it's only touching maybe 10-15% of people and for 85% of people that message goes totally onto deaf ears. It's not that people are evil, or mean, or wrong, or anything, it just basically means that people are people and that the normal, innate, biologic drive for people to want to have sex is in the same part of the brain that drives eating. We have to understand how to help people manage their lives in a way that puts them at less risk for not just HIV, but other sexually transmitted diseases, as well."

Finally, I asked Dr. Saag what advice he would give to undergraduates considering research activities both now and later as a career?

"My advice would be three things. The first is to keep your options open and to not be afraid of exploring opportunities that feel outside of your comfort zone. The only way you're going to know what you like and what you don't like is by going out and doing something. The creative director at Actor's Theater of Louisville had a great commentary where he said, 'I want everybody to come to all the plays and have an experience.' If you walk away saying, 'I didn't like that play' or you go to a play you didn't like, he goes, 'Okay, at least now you know what you don't like,' and I think that's kind of the right attitude."

...keep your options open and to not be afraid of exploring opportunities that feel outside of your comfort zone. The only way you're going to know what you like and what you don't like is by going out and doing something.

"The second thing is not to stress too much over having a game plan. Everybody feels like they need to have a roadmap for success. 'I'm going to do this,' 'I'm going to do that'... and that you have to have your career path planned out. A lot of the career path is serendipity, a lot of it is opportunity, a lot of it is discovering down deep what you like and what you don't like. You've got to go do it. You've got to live life."

"The third thing is to have fun. Life should be a joy; it should be a great experience. When you're in the lab immerse yourself in it. Get excited by the science. Get excited by the discovery. Get excited by the people you're around. Then you can sort out what it is you like and what you don't like later."

When asked if he had any final comments, Dr. Saag simply replied, "I would like to see football season last all year. That would be good."

faculty interview: chemistry

Dr. David Graves: Exploring the Chemistry of Nucleic Acids

Chemistry, Drew Buie

Dr. David Graves has spent 25 years investigating the interactions of small molecules with nucleic acids and trying to answer the basic questions -- what, why, how, and what of it. As chair of the department of chemistry, Dr. Graves has worked hard to facilitate an increase in research as well as increasing undergraduate research experience.

Q How did you become interested in research?

A) I was an undergraduate chemistry major and found that working in a research laboratory was both interesting and an excellent way to earn extra money. I worked part-time in the UAB Department of Obstetrics and Gynecology (OBGYN) in the endocrine research laboratory. I became interested in research by working on projects determining steroid hormone levels in two types of patients: women trying to become pregnant or women who were pregnant and having various health problems with their pregnancies. My job was to determine the concentrations of different types of estrogens (E1, E2, E3, E4) and monitoring how the concentration levels of these estrogens change over gestational periods. One can use these estrogen levels to monitor the well being of both the mother and fetus. As an undergraduate, I found it intriguing that such a small molecule like esterdiol could have such a profound impact on so many organ systems in the body. The steroid binds to a protein called an estrogen receptor. The estrogen receptor protein then directs the expression of discrete genes ensembles throughout the body. It was the initial concept that something as small as a particular estrogen molecule could cause vast changes in the numerous organ systems through its controlled interactions with nucleic acids. What was most intriguing was that the estrogen receptor protein was highly selective in selecting its DNA target(s). The selectivity at the molecular level and the forces driving these interactions formed the foundation of the next four decades of my scientific interests.

For my graduate studies, I was admitted to the Ph.D. program in the UAB Department of Biochemistry research, and worked in the Laboratory of Molecular Biology under the direction of Drs. Lee and Dr. Lemone Yielding. In my Ph.D. research, I was able to extend my research interests in targeting nucleic acids in a variety of ways. My Ph.D. research was more focused toward the biochemistry of cancer and effects of DNA damage and repair on cancer. My graduate research consisted of development of novel compounds that would selectively target nucleic acids, and, through my research under their direction, I became more and more entrenched in studying the molecular interactions of the small molecules with nucleic acids. For this reason, I chose to do my postdoctoral work at the University of Rochester working with Professor Thomas Krugh in the Department of Chemistry in the area of biophysical chemistry of

nucleic acids. While at the University of Rochester, my research focused on the structure, stability, and sequence-selective nature of the interactions of numerous anticancer agents with their target DNAs. After four years at the University of Rochester in the Krugh Laboratory, I joined the faculty in the Department of Chemistry at the University of Mississippi. As a new tenure track assistant professor, I continued to examine the interactions of anticancer agents with nucleic acids.

Of the vast number of drugs that are currently in use as chemotherapeutic agents in the treatment of cancer, roughly 50% have DNA as their biological target. We need to gain a basic understanding of how these agents exert their biological activity if we are to improve their activity in their evolution to the next generation of drugs - the discovery of new compounds that will target nucleic acids. Our research focuses primarily on the biophysical characterization of the interactions of parent compounds that are currently used to treat cancer to discern why and how selected anticancer agents targeted DNA and triggered apoptosis in cancer cells. Research efforts focused on the structural and thermodynamic origins of sequence selectivity. The rational design of new DNA targeted drugs requires a thorough understanding of the binding mechanisms of existing compounds that bind to DNA with unique types of specificity.

Q) How long have you been at UAB and what persuaded you to come here?

A) After 20 years in the Department of Chemistry at the University of Mississippi, I returned to UAB in 2003 as the Chair of the Department of Chemistry. I feel that one of the primary reasons for my selection for this position at UAB was due to my strong background in biomedically related research in drug discovery. Since my arrival at UAB, my goal has been to enhance the Department of Chemistry's research efforts in areas of drug discovery, biophysical chemistry, structural biology, and computational chemistry. We have had significant successes in hiring outstanding new faculty and building state-of-the-art infrastructure in these areas. Through these enhancements, we have had considerable success in promoting extensive collaborative research ties between the Department of Chemistry and numerous departments and research centers within the UAB biomedical research community.

Q) What advice would you give to undergraduates who are considering research activities both now and later as a career?

As an undergraduate researcher, you will gain valuable experience in what science is all about: how it is conducted from the lab bench to the actual publication of manuscripts and dissemination of knowledge to the rest of the scientific community. In reference to career goals, it provides you with a unique experience to enhance your resume for any field you wish to pursue, including medicine, dentistry, optometry, and any other health profession.

A) One of the most enjoyable and beneficial experiences you will ever have as an undergraduate is that of getting involved in a research laboratory. In reality, this is why you are at UAB, to take advantage of its world-class research environment. It is the perfect opportunity for you to grow both in your academic and career goals. As an undergraduate, you will have very strong research mentoring, and we want you to succeed; we are going to be putting you on projects that will be exciting, productive, and significant. As an undergraduate researcher, you will gain valuable experience in what science is all about: how it is conducted from the lab bench to the actual publication of manuscripts and dissemination of knowledge to the rest of the scientific community. In reference to career goals, it provides you with a unique experience to enhance your resume for any field you wish to pursue, including medicine, dentistry, optometry, and any other health profession. Students wishing to continue postgraduate degrees in research will go on to graduate schools. Undergraduate research will help you to select and make you highly competitive for top research institutions for your advanced degrees. Having a research experience on your resume allows you to stand out in a crowd. In my experience here at UAB, I have found the undergraduate chemistry majors at UAB to be highly motivated, focused, and very successful in achieving their goals upon graduation. Those students who take advantage of the research opportunities are very successful in their future careers in their postgraduate studies in medical and graduate schools.

Cocaine Contamination of Currency in Birmingham AL

Jeremy R. Felix, Rena Hammer, and Elizabeth A. Gardner

Abstract:

Numerous studies have shown that a high percentage of currency is contaminated with nanogram to nearly milligram volumes of illicit drugs, especially cocaine. A study was conducted in the Birmingham Alabama area to determine the percentage of cocaine on dollar bills, as well the advantages and disadvantages of two extraction methods—a chloroform extraction and a chloroform extraction with an acid/base wash (chloroform/acid/base extraction). Fluorescence was examined as a potential presumptive test for cocaine on the currency. The objective was to develop a baseline determination of cocaine concentration on bills in Birmingham, a simple but adequate methods of analysis, and equally adequate methods of extraction. Two sets of twenty $1 bills were collected from local Regions (set 1) and Wachovia (set 2) banks and subjected to a chloroform extraction and an chloroform/acid/base extraction; the results were 65% and 32% positive for set 1 and 65% and 35% positive for set 2 for a chloroform and chloroform/acid/base extraction, respectively. Analysis of the data confirms that the chloroform/acid/base extraction results in a cleaner GC/MS spectrum, is better to run on the GC column, but yields less intensity in chromatogram peaks due to sample loss through the multi-step cleaning process. Fluorescence was not found to be an accurate predictor for the presence of cocaine on dollar bills using a 254 nm Uv source.

Introduction:

It has been shown through numerous studies that a high percentage of both US and international currency are contaminated with cocaine and other controlled substances.[1] After the publication of such reports, the US courts have ruled that police sniffing dogs can no longer be used in order to apprehend a suspect or confiscate what is thought to be drug related money.[2] However, internationally the ruling is much different. The UK allows mass spectrometric analysis of cocaine contaminated bills to be used as supporting evidence in court cases, and the technique of mass spectrometric analysis to determine cocaine contamination has been accepted by the UK Court of Appeals.[3]

The most obvious source of direct contamination is from the handling of large sums of cash at drug deals and the use of rolled up bills to snort cocaine.[4] Other sources of contamination include money counting machines at banks, which have been shown to contain dust with significantly high proportions of cocaine in the mixture.[5] Other research following these results also has shown that both counting machines as well as hand counting transfers nanogram amounts of cocaine to European banknotes.[6]

US currency paper is made up of a special mixture of 75% cotton and 25% linen, with red and blue synthetic fibers distributed throughout.[3] UK sterling banknotes are also made up of cotton and linen, so it is reasonable that both should exhibit similar qualities in the ageing of the bill, as well as the transfer of cocaine to the bills.[7] Studies have shown that through time the fibers of UK banknotes change, forming cavities in the fibers that could potentially trap contaminates, including cocaine crystals. Others have shown that the inks of the bills, grease, and chemical binding can play a role in the capture of cocaine on currency.[6] No research could be found on the possible contamination of bills through the passage of currency through wallets and purses, however the characteristic cavities seen in bills does not eliminate the possibility for controlled substance transfer in wallets and purses. Whether such transfer, if it occurs, yields detectible amounts is unknown.

Because of the ability of bills to pick up cocaine and other drugs from many different sources, the variable of age and wear of the bill, and the unknown amount of cocaine commonly picked up by any specific bill, it is very hard to determine prosecutable amounts of cocaine for currency and even establish a low variance baseline to distinguish background from contamination.[8] Because of this, research has been ongoing in different countries of the world to try to create baselines for bills in specific areas of the country, states, and even cities.

Research on different methods of analyzing bills and the extraction of cocaine from the bills is also an important consideration in cocaine detection. Several methods have been developed or modified to analyze currency for cocaine contamination. Research labs have used tandem mass spectrometry (MS-MS) for the analysis of currency in which volatile substances on the bills are ionized between heat plates and pulled through the mass spectrometer.[9] A somewhat more time consuming method is to run a GC/MS of prepared samples. This GC/MS method has also been used in research labs for the determination of cocaine on currency, and often follows a purifying step involving solid phase extraction (SPE).

GC-MS-MS methods as well as $Ru(bpy)_3^{2+}$ ECL combined with cell electrophoresis (CE) have been developed as non-destructive methods in opposition to the destructive methods of tandem mass spectroscopy, where the part of the bill passed

through the heated plates during analysis is incinerated.[10] Nondestructive methods are preferred by the scientific community, especially in crime labs where the preservation of evidence is a priority. The high sensitivity, fast analysis times, and non-destructive methods claimed by the researchers for each method gives researchers and forensic toxicologists options with results similar to those used in tandem mass spectroscopy without any destructive measures being taken.

A final concern in the analysis of currency for illicit drugs is the extraction methods employed. An Esteve-Turrillas et al.[11] study on a non-destructive method of cocaine determination on bills also employs a suitable, friendly, and non-destructive solvent extraction as well. It was determined that while cocaine is highly soluble in chlorinated solvents, such as chloroform, such solvents have the potential to destroy security marks and holographic strips on currency. Acetonitrile, on the other hand, is less destructive to currency, but also removes large amounts of other solid compounds with the cocaine. It was finally determined that methanol was the least destructive method and sufficient for the removal of cocaine from currency. Although these tests were performed on UK banknotes, the similar design of US and UK currency would most likely yield similar results in each of these solvents.

In this experiment, a simple yet effective way of extraction, analysis, and cocaine determination was employed. Analysis of paper currency in the Birmingham Alabama area by gas chromatography mass spectroscopy (GC/MS) was chosen due to the readily available access to a GC/MS in most research and crime labs, as well as methods previously used in earlier research, as noted above. Twenty $1 bills were collected from a local Wachovia branch and subjected to an initial methanol extraction to remove the cocaine, yet preserve the bills. The extraction was divided so that both a chloroform extraction and a chloroform/acid/base extraction could be used in the determination of cocaine on the currency.

The GC/MS work for the collected twenty $1 bills was preceded by a UV fluorescence test of the bills under a 254 nm handheld UV light. Labino, the maker of a UV lamp for controlled substance fluorescence, has documented that cocaine of at least 87% pure fluoresces under their UV light source.[12] According to Mercolini, cocaine has an excitation wavelength of 230 nm and emission at 314 nm,[13] and thus the twenty $1 bills collected were tested to see if fluorescence is an indicator of the presence of cocaine on currency.

A chloroform/acid/base extraction of cocaine works by taking advantage of the ability to change the form of cocaine by adjusting the pH above or below the cocaine pKa value of 8.6.[14] By first placing the extracted contents of the currency in an acidic environment and adding chloroform, all cocaine in the sample should be in its hydrochloride form and stay in the water layer due to its high solubility; however, most organic impurities will go into the chloroform layer. Removing the chloroform layer will remove most of the organic impurities from the sample. The sample is then made basic, turning the cocaine in the sample into the free-base form.[15] Cocaine will then be soluble in the chloroform layer and insoluble in the water layer. Any salt impurities left in the sample will be dissolved in the water layer. By once again removing the chloroform layer and keeping it for analysis, a much cleaner sample containing cocaine can be run on the GC/MS.

Methods:

Materials: methanol, Fisher Scientific; Hampton, NH: Lot#001162; sodium carbonate, Acros; Geel, Belgium: Lot #B0122733; 12.1 N HCl, Fisher Scientific; Hampton, NH: Lot #065373; chloroform, Aldrich; Milwaukee, WI: Lot #JU 04354CU; chloroform, Sigma Aldrich; St. Louis, MO: Lot#09767JH; exempt cocaine standard, Cerilliant Round Rock, TX: Lot #FC032007-01A

The GC/MS analysis was performed on an Agilent Technologies 6890N Network GC System with an auto sampler. The GC was interfaced with an Agilent Technologies 5975 MSD utilizing a splitless capillary inlet and a HP 5 column (30m x 0.250mm).

Collection of Currency:

Twenty $1 bills were collected from a Wachovia bank located in Birmingham Alabama. Each bill was collected and stored in a separate plastic bag at room temperature until analyzed.

UV Fluorescence:

An UV fluorescence test was performed under a 254 nm short wave UV light to check for a correspondence between currency fluorescence and cocaine concentration on the bill. The presence of fluorescence was recorded.

Positive and Negative Controls

A cocaine control was run at the beginning of each set of 20 samples. A blank was injected between each sample to check for carry-over from the previous run.

Methanol Extraction of Currency

Each bill was crumpled and placed into a 20 mL glass vial and 10 mL of methanol was added, covering the entire bill. They were allowed to soak overnight and were sonicated for 10 minutes in a FS20H Fisher Scientific Sonicator before removal of methanol to test tubes.

For the second set of bills, the methanol extraction was divided evenly into two test tubes per bill in order to perform a chloroform extraction and chloroform/acid/base extraction with equally concentrated samples. The methanol was then evaporated to dryness on a N-EVAP112 Nitrogen Evaporator.

Method 1 Water/Chloroform Extraction
For the first set of 20 one dollar bills, the dried residue was dissolved in deionized 1 ml water and extracted in methanol. After GC/MS analysis, the samples were again dried and then underwent a chloroform/acid/base extraction.

Method 2 Chloroform Extraction: (second set of bills only)
One ml of chloroform was added to the dry residue in the test tube and sufficiently agitated. The chloroform was pipetted into 1 ml GC vials and analyzed on the GC/MS.

Method 3 Chloroform Extraction with Acid/Base Wash (chloroform/acid/base):
One mL of deionized water was added to the test tube followed by enough drops of 12.1 N HCl to make the pH < 3.00. One milliliter of chloroform was then added to the test tube. The chloroform layer was removed as organic waste, and the pH brought to > 12.00 by the addition of saturated sodium carbonate. One mL of chloroform was then added to each the test tube, and the chloroform layer was removed and placed in GC/MS vials and analyzed by GC/MS.

Control Experiments:
All three extraction methods were performed on a Cerilliant exempt cocaine standard to first verify that all three methods were successful and to give a qualitative result of sample loss with each extraction method. From analysis of these results, it was decided that the chloroform extraction from the methanol residue was sufficient and that adding water before extracting into chloroform was not required.

Results and Discussion:
Cocaine Contamination of Currency:
The GC/MS method for cocaine was developed using the positive cocaine controls. The retention time of 9.845 ± 0.05 min. was determined and the characteristic peaks for cocaine, *m/z* 82, 182, and 303 were selected from the accepted literature values for cocaine.[16]

The presence of cocaine was determined by extracting the *m/z* 82, 182, and 303 from the mass spectra. The peaks resulting from the extracted ion chromatograms were much more easily distinguishable from the background noise and were determined as positives or negatives by the presence or absence of these peaks at 9.845 min. The extracted ion chromatograms (EIC) for a positive and a negative result are shown Figures 1 and 2.

In addition to the impurities in the mass spec. of the samples, there was considerable carry-over of the impurities from the chloroform extracted samples into the blank injection that followed each sample injection. While some of these impurities eluted during the retention time for cocaine, none of them were determined to be cocaine upon analysis (Fig. 3).

Sixty-five percent of the first set of the twenty $1 bills analyzed by Hammer using a water/chloroform extraction were contaminated by cocaine. When re-analyzed with a chloroform/acid/base extraction, it was determined that 6 out of 19 samples were contaminated with cocaine with one not being analyzed due to the presence of too many impurities to safely run on the GC/MS (Table 1). Comparing percentages of 65% to 31.6% shows that the chloroform/acid/base extraction method causes sample loss through the multi-step cleaning process. However, as seen in Figure 4 and Figure 5, the chloroform/acid/base extraction resulted in a cleaner GC/MS spectrum, eliminated problems of carryover of impurities into blank injections between cocaine sample runs, and is thus better to run on the GC/MS column.

In order to qualitatively look at the loss of sample by the three extraction methods, three 200 µl samples of a cocaine standard (1g/ml) were extracted by methods 1, 2, and 3. As illustrated in the chromatograms of the different extraction methods on the cocaine control (Figure 6), the water/chloroform extraction gave a slightly better yield than the straight extraction into chloroform, though with only one trial, this may not be a significant difference. However, the chloroform/acid/base extraction does show a significant loss of cocaine through the multiple steps of the extraction.

Another possible reason for the fewer positives between the water/chloroform and chloroform/acid/base extractions was the time between analyses. The chloroform/acid/base extraction was done approximately three months after the initial water/chloroform extraction.

A second set of twenty $1 bills were collected from a Wachovia bank as a follow-up on the previous results. In this experiment, 65% of the samples were positive for cocaine using the water/chloroform extraction method, and 35% of the samples were positive for cocaine using the chloroform/acid/base extraction method as seen in Table 2.

Interestingly, this data corresponds to the percentage results determined from the Hammer extraction and the chloroform/acid/base extraction performed on those samples. It also shows that the chloroform/acid/base extraction results in a cleaner GC/MS spectrum, eliminated problems of carryover of impurities into blank injections between cocaine sample runs, and is thus better to run on the GC/MS column as stated before.

As seen in Table 3, and compared to the data in Table 2, there was no correspondence between the GC/MS results and the fluorescence seen under the 254 nm UV light source. This is apparent in the fact that out of the 11 bills that had at least some fluorescence only 7 of them tested positive for cocaine, and out of the 9 bills that did not fluoresce 6 of them

tested positive for cocaine. Results are even less applicable to the less sensitive chloroform/acid/base extraction method in which the two heaviest fluorescing bills, bills 5 and 20, showed no cocaine present in the chloroform/acid/base extraction. This signifies that even the most heavily fluorescing bills did not have the highest cocaine concentration and that bills that did not fluoresce still contained cocaine in the less sensitive chloroform/acid/base extraction method.

The bills were once again submitted to the UV light test after methanol extraction of the contents on the bills, and no changes in fluorescence were observed.

Conclusions:
It was determined that the results from a chloroform extraction vs. chloroform/acid/base extraction could result in a 30% difference in bills testing positive for cocaine. However, the chloroform/acid/base extraction resulted in a cleaner GC/MS spectrum, is better to run on the GC column, but yields less intensity in chromatogram peaks due to sample loss through the multi-step cleaning process. Some factors that should be taken into consideration when interpreting these results are:

1. Each methanol extraction from the second set of bills was divided in half so that the methods could be compared. If the entire volume was analyzed, the chloroform/acid/base extraction may have proved as accurate as the chloroform extraction. This could be evaluated by adding a controlled amount of cocaine to a set of bills before analysis.

2. In the extraction experiments of the controls, the analyte was already in the base form and a chloroform extraction would be the most effective method for analyzing these samples. However, cocaine on the bills would be expected to be the salt form, as that is currently the most common form encountered in crime labs. A way to test this would be to spike clean bills with both the base and salt forms of cocaine.

It was also determined that UV fluorescence at 254 nm has no preliminary determination of cocaine presence on the currency and showed that the methanol extraction method to remove contaminants from the currency had no effect on whatever material was causing the fluorescence. Further work needs to be done if a method is to be developed. The final determination of percent cocaine on bills tested in the Birmingham Alabama area using GC/MS, chloroform extraction and chloroform/acid/base extraction, and an extracted ion chromatogram for final determination showed that 65% of the bills from the chloroform extraction were contaminated with cocaine, and that 35% showed cocaine contamination from the chloroform/acid/base extraction. This data is consistent with the analysis of Hammer and the follow up chloroform/acid/base extraction procedure.

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Endnotes

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Figure 1. Positive Cocaine Result in the Extracted Ion Chromatogram.

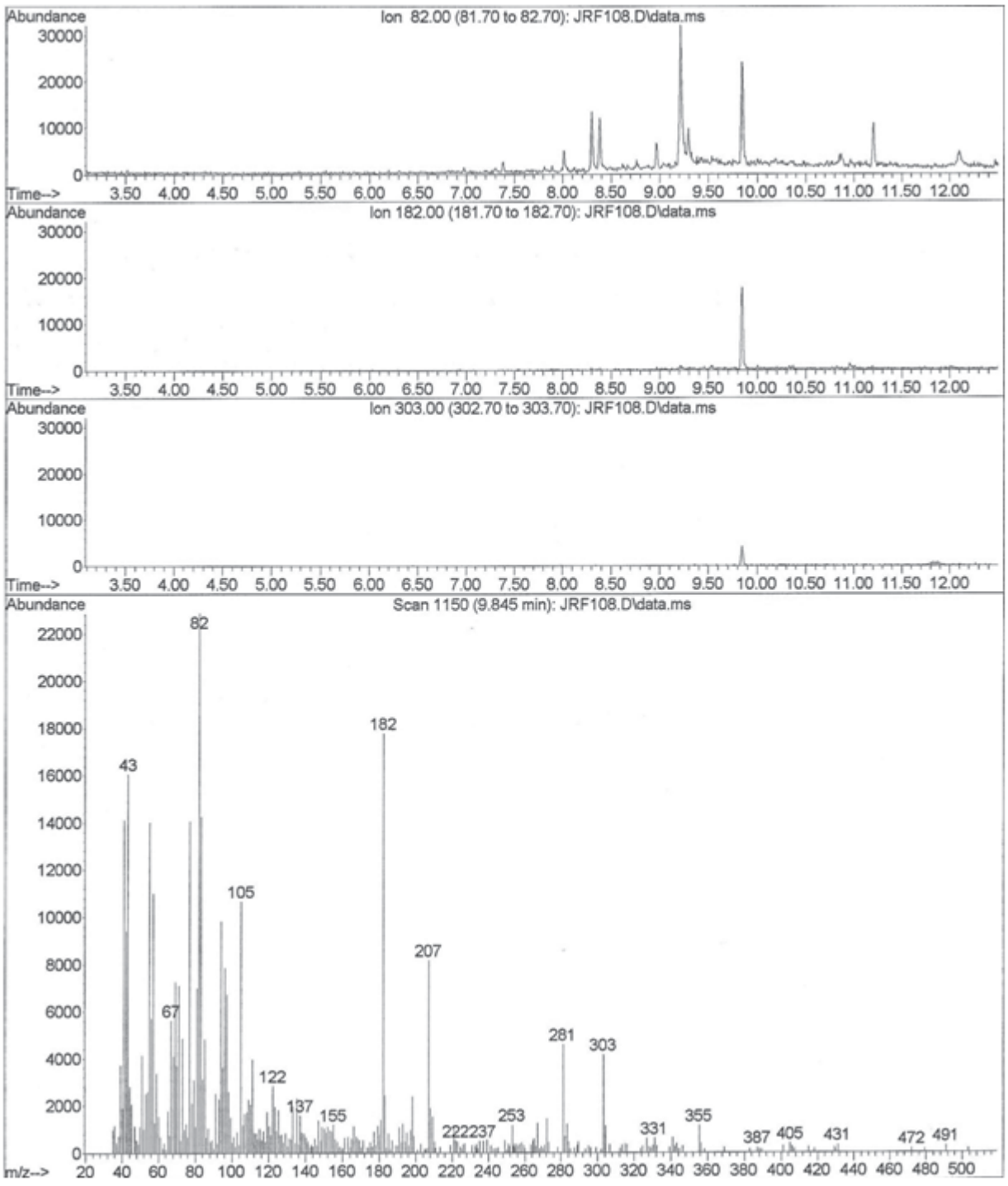

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Figure 2. Negative Cocaine Result in the Extracted Ion Chromatogram

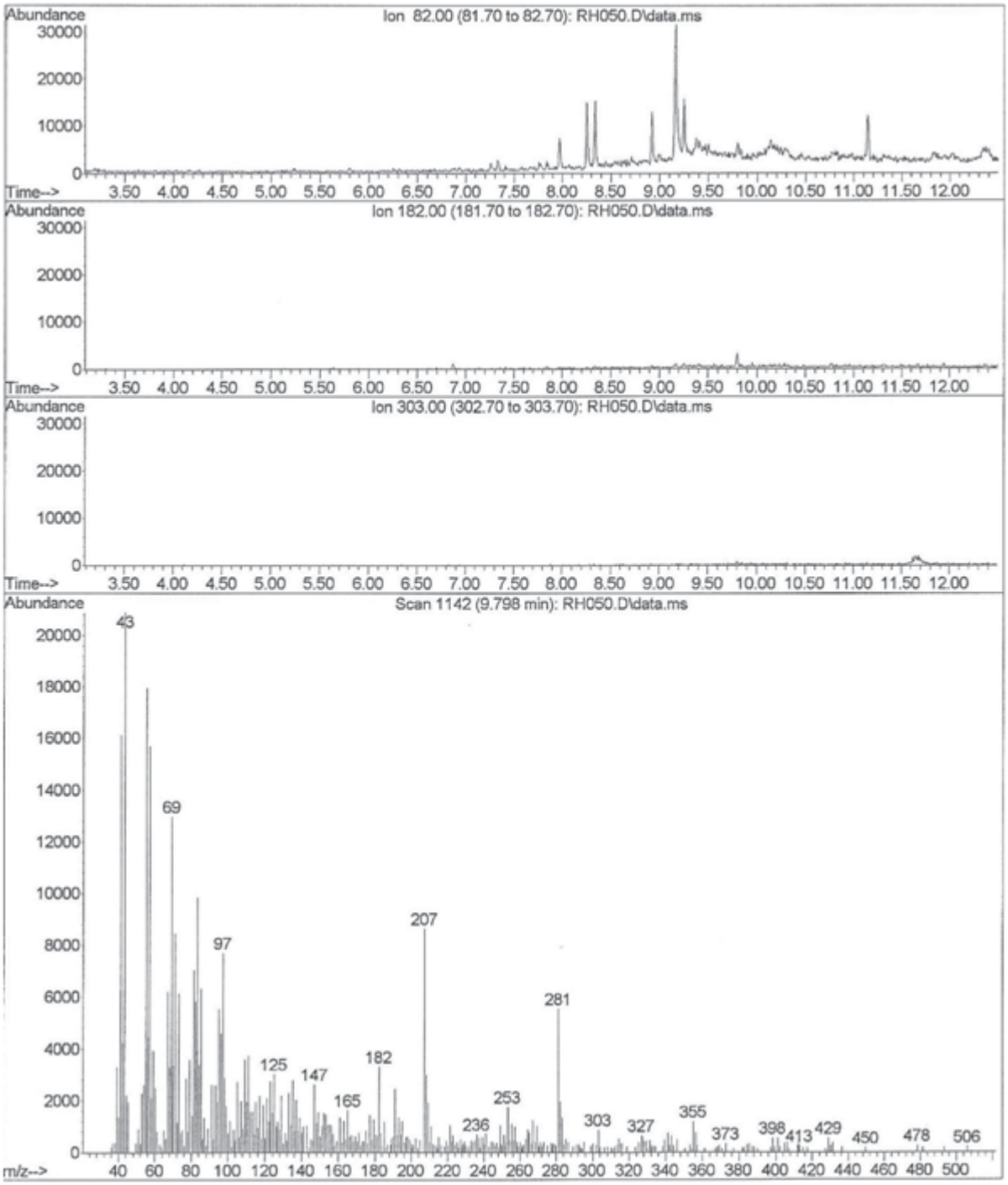

Figure 3. GC/MS of Chloroform Extraction Blank with Carry-Over at 9.839 min

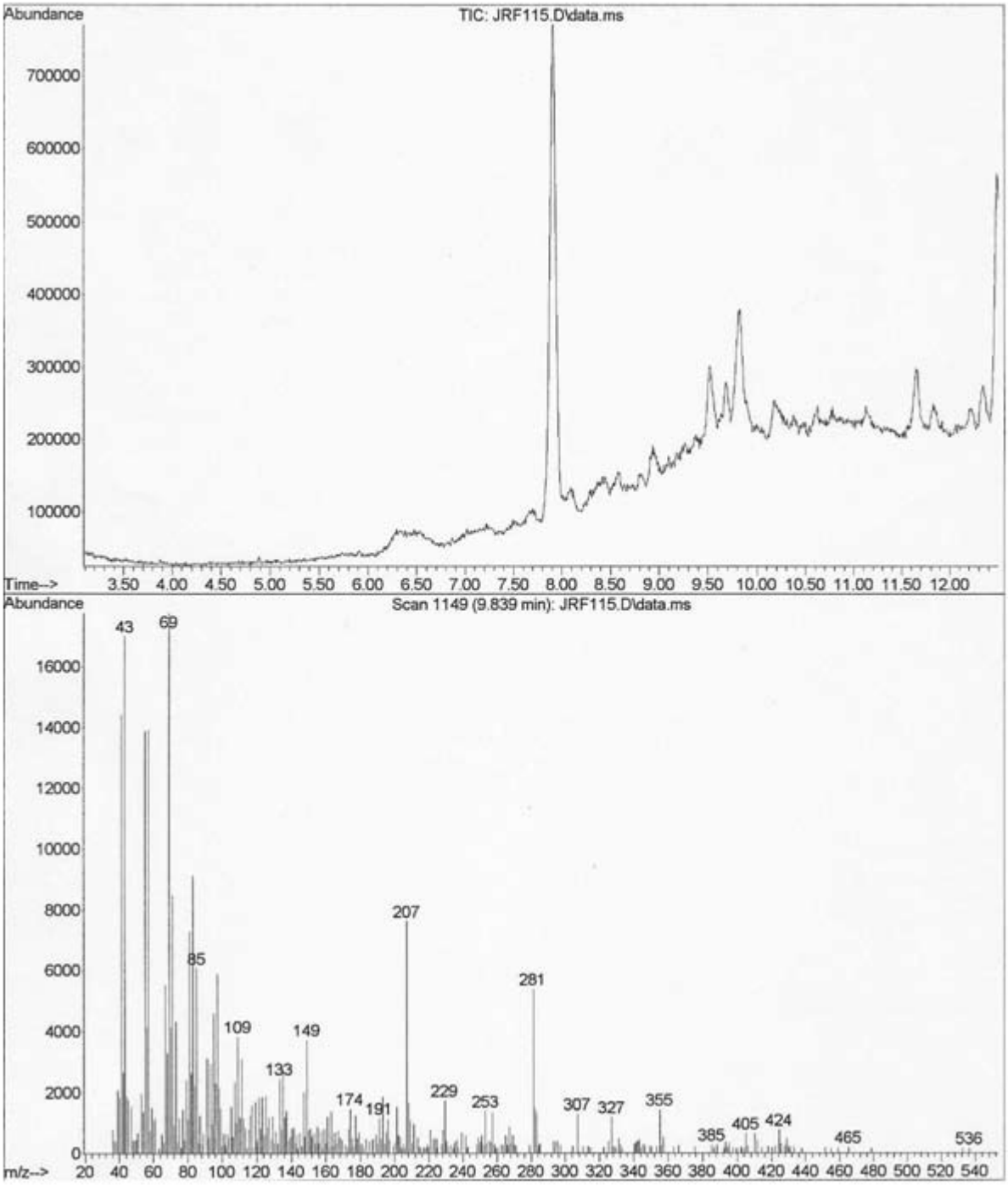

Figure 4. Chromatogram and Mass Spectrum from Chloroform Extraction (Positive for Cocaine)

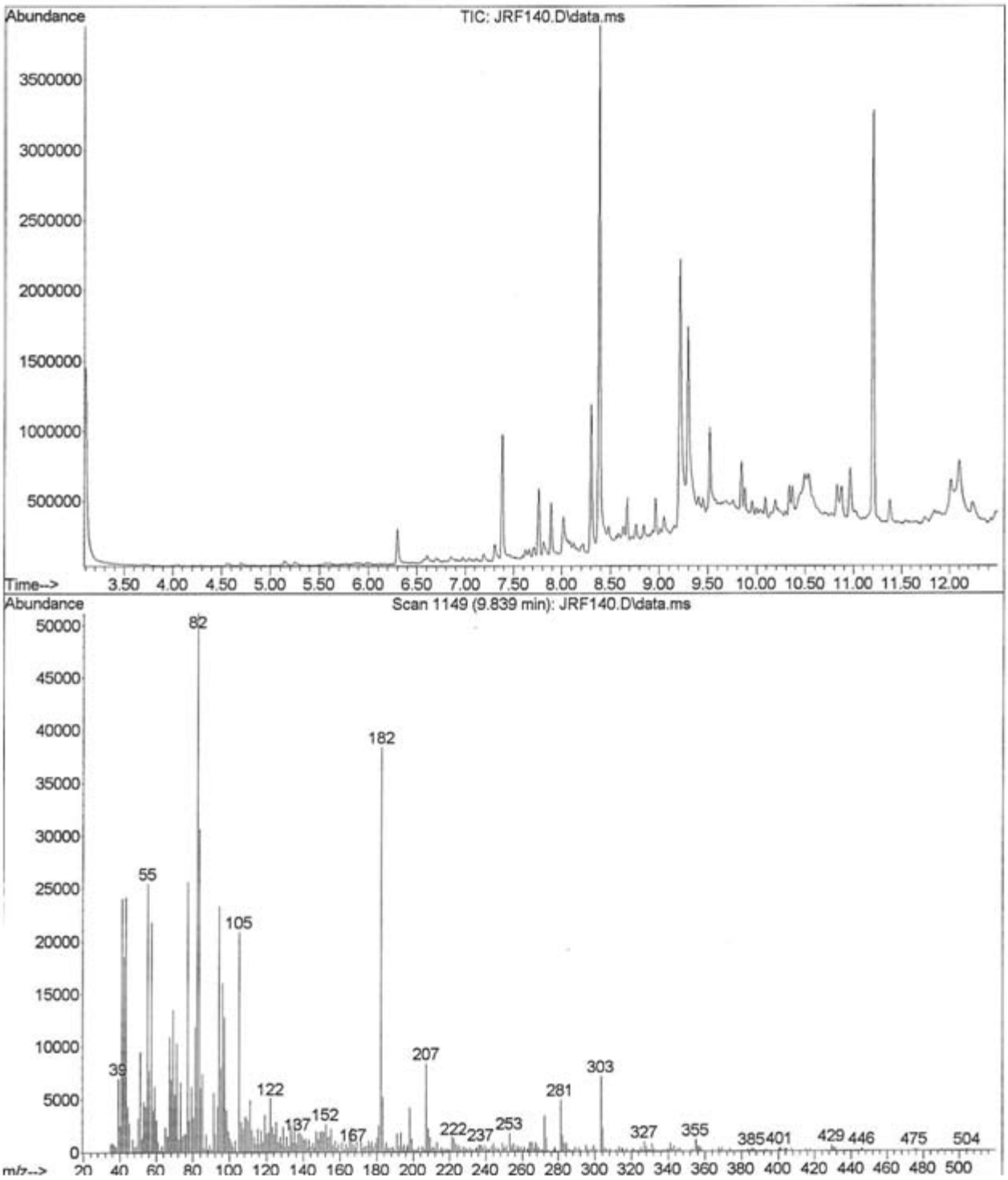

Figure 5. Chromatogram and Mass Spectrum from Chloroform/Acid/Base Extraction (Positive for Cocaine)

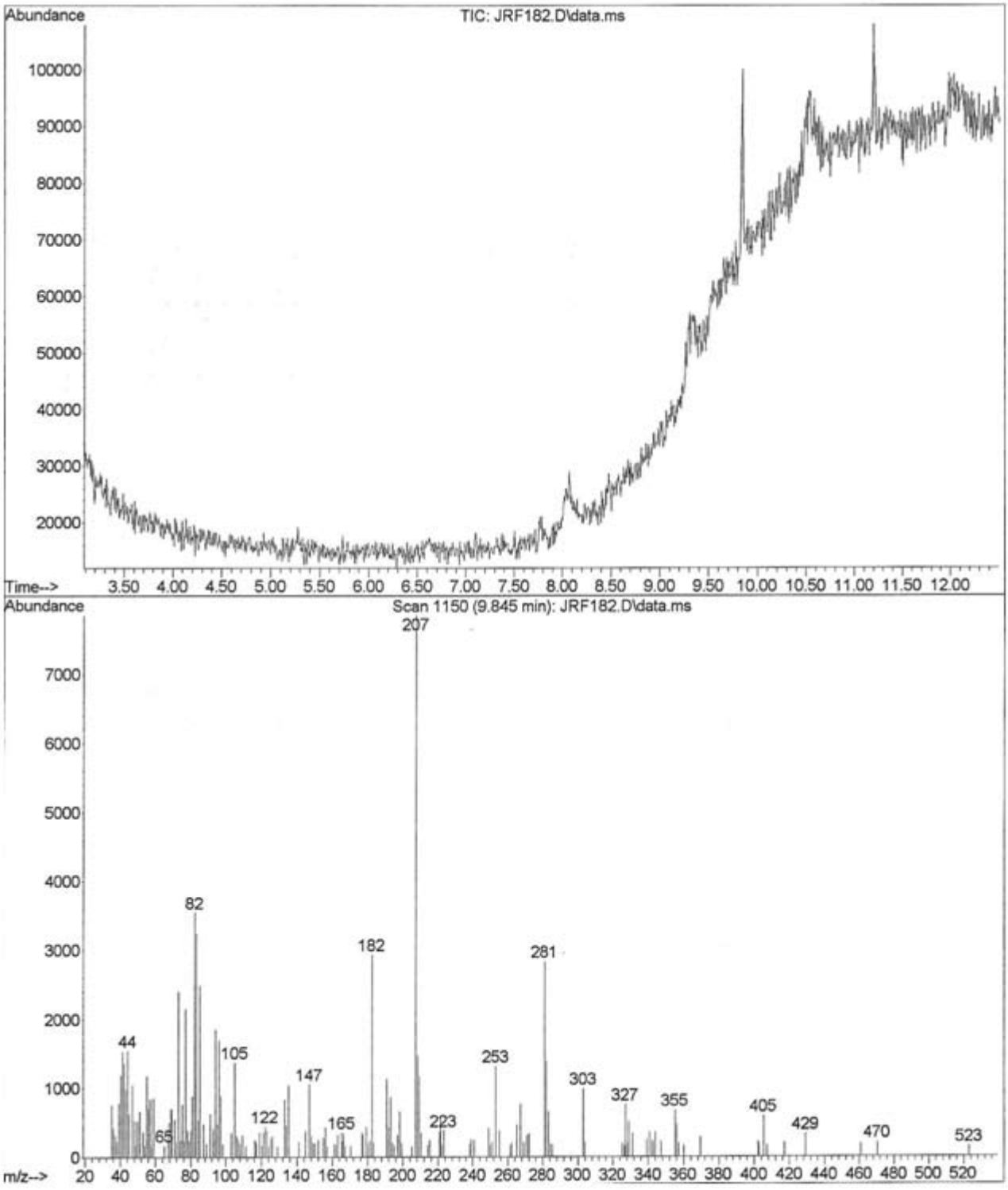

Figure 6. Chromatogram Overlay of Extraction Methods of Cocaine Standard

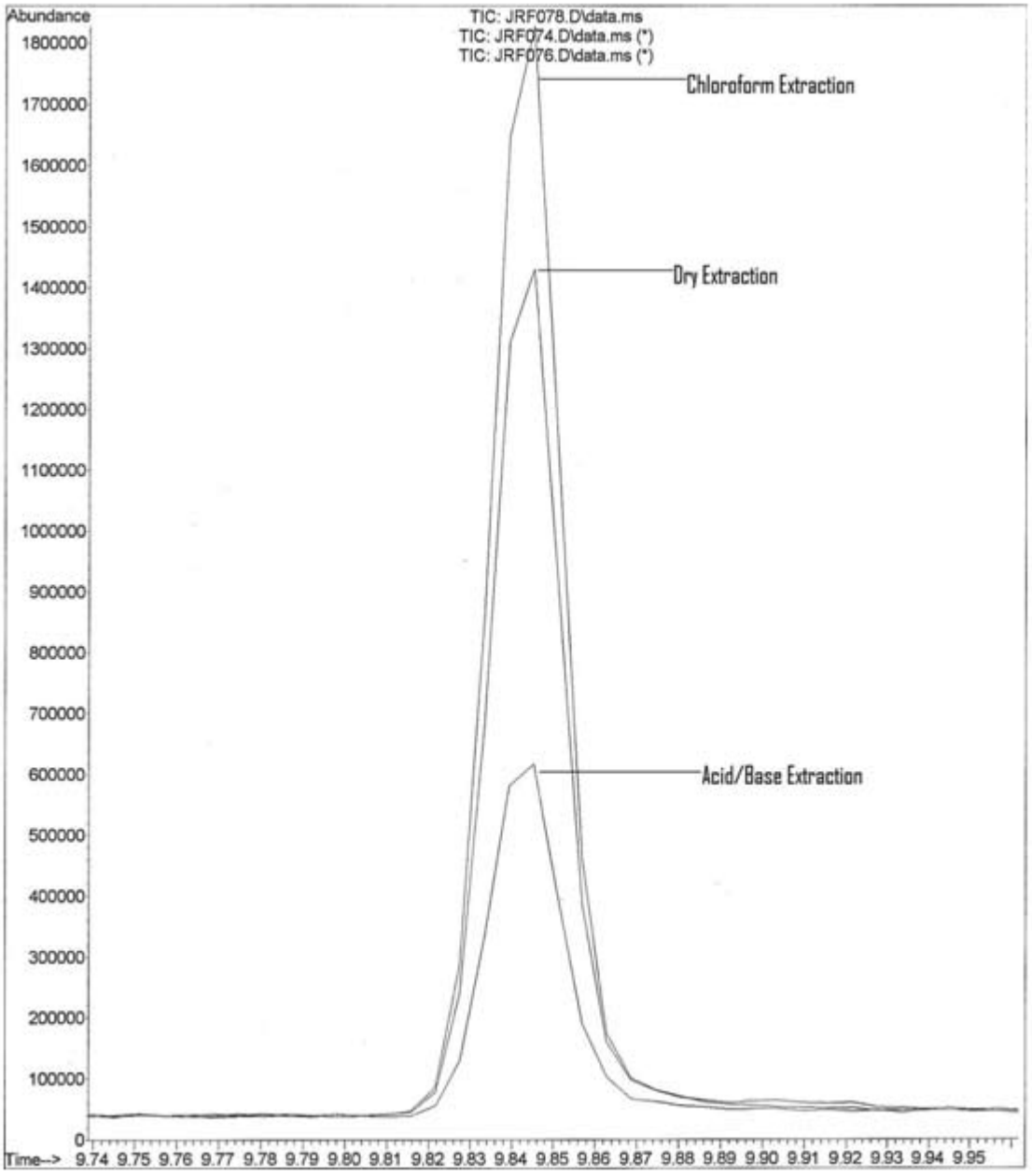

Table 1. Cocaine Analysis of Twenty $1 Bills from Hammer and A/B Extraction Using EIC Determination

Sample	Serial Number	Series	Cocaine Dry Extraction	Cocaine A/B Extraction
1	K31639841F	2003A	No	No
2	G74352837C	2003A	Yes	No
3	L65858869F	2001	Yes	Yes
4	C43331196A	2003A	Yes	Yes
5	F29900166I	2003A	Yes	No
6	E59694877B	2003A	Yes	Yes
7	L15588053P	2003A	No	No
8	F055151816A	2006	No	No
9	D42105175A	2003A	Yes	No
10	J79320301D	2003A	No	No
11	F52780916C	2003A	Yes	Yes
12	F48991587B	2006	No	No
13	A74411471B	2003A	Yes	Not Run
14	K65247169H	1999	Yes	Yes
15	F22886476G	2003A	Yes	No
16	H09465674C	2003	Yes	No
17	K89591350B	2003A	Yes	No
18	J57336529B	2003	Yes	Yes
19	K43708395F	2003A	No	No
20	I22572086L	1995	No	No

Table 2. Cocaine Analysis of Twenty $1 Bills Using EIC Determination

Sample	Serial Number	Series	Cocaine Dry Extraction	Cocaine A/B Extraction
1	G95919878D	2003A	No	No
2	E66746120A	2003A	Yes	Yes
3	KO1430953A	2006	No	No
4	F46387847J	2003A	Yes	Yes
5	L91961202K	2003A	Yes	No
6	H71011706A	2003A	No	No
7	F03840182K	2003A	Yes	No
8	F05931531A	2006	Yes	Yes
9	F83640498H	2003A	Yes	No
10	K01712161F	2003A	Yes	Yes
11	D09669487C	2003	Yes	No
12	G01967256I	2003A	No	No
13	E36503180A	2003A	No	No
14	D56775559C	2003A	Yes	Yes
15	H09295751A	2006	No	No
16	F54057570K	2003A	Yes	No
17	K19394902H	2003	Yes	Yes
18	L08800038P	2003A	Yes	Yes
19	B34225787C	2006	No	No
20	K53712767C	2003A	Yes	No

Table 3. UV Fluorescence at 254 nm of Currency Collected For Extraction

Sample	Serial Number	Fluorescence	Cocaine	Location On Bill
1	G95919878D	One Spot	No	Above "MER" on Back
2	E66746120A	Heavy	Yes	Around Entire Edge Front and Back. Solid Strip on Back Through "O" in Central *ONE*
3	KO1430953A	No	No	
4	F46387847J	No	Yes	
5	L91961202K	Entire Bill	Yes	Very Heavy on Entire Bill
6	H71011706A	One Slight Spot	No	Left Side of Bottom Serial Number
7	F03840182K	Slight Spots	Yes	Front Right Side of Bottom Left Hand 1, and above "N" in *ONE*
8	F05931531A	Two Small Spots	Yes	Bottom Left Back Edge
9	F83640498H	No	Yes	
10	K01712161F	No	Yes	
11	D09669487C	No	Yes	
12	G01967256I	Spots and Line	No	Front Bottom Left 1 and Edge. Strong Line on Bent Spot Diagonal of Bottom Left Hand 1
13	E36503180A	No	No	
14	D56775559C	No	Yes	
15	H09295751A	No	No	
16	F54057570K	Light	Yes	Slight Color on Front Left and Small Portion Top and Bottom Edge. Heavier and Solid Corresponding Back Side
17	K19394902H	No	Yes	
18	L08800038P	Big Spot	Yes	Spot Around Front Left Stamp. Large But Not Too Fluorescent
19	B34225787C	Spots	No	Two Spots on Left Front Edge in Middle. One Spot Top Edge Front Above *THE*
20	K53712767C	Almost Entire Bill	Yes	Heavy All Edges Both Sides and Big Spots Throughout Middle

CpG-Oligonucleotides Induce TLR9 Mediated Cellular Invasion in a Sequence and Structure Dependent Manner

Sonja C. Brooks[1], Jason S. Hudson[1], Katri S. Selander[2], and David E. Graves[1,2]

Abstract

The focus of this research is to discern the structural and biophysical features of small CpG containing deoxyoligonucleotides that have significant biological properties including the inducement of Toll-like receptor 9 (TLR9) in the mediation of cellular invasion. Cell invasion (metastasis) is a significant problem in the control and treatment of breast cancer. Recent research from our laboratory has demonstrated enhanced cellular invasion in the MDA-MB-231 breast cancer cells by ODN-M362, a 25-base single-stranded CpG-containing deoxyoligonucleotide. The mechanism(s) for this induction remain unknown; however, our studies reveal key insights into the structural and sequence requirements for DNA activation of this cellular invasion process. The deoxyoligonucleotides that are effective in eliciting an invasion response have been shown to adopt multiple structural motifs including stem-loops, hairpins, or duplex structures. Sequence modifications were designed to probe base sequence, structure, and stabilities that are required for initiating TLR-9 mediated cellular invasion. Our results demonstrate that these small deoxyoligonucleotides and their structures play a pivotal role as biological response modifiers in this invasion process. Hence, this research is focused on determining the relationship(s) between biological activity and secondary structure of the deoxyoligonucleotides. We have applied a number of biophysical methods including DSC, CD, and computational methods towards understanding the structure and stabilities of these deoxyoligonucleotides.

Introduction

Deoxyribonucleic acid (DNA) was characterized as the repository of genetic information in 1944. Since then, DNA has become a useful tool in identification and diagnostics, in the development of drugs to treat disease, and in gene therapy. More recently, researchers have found that DNA also plays an important biological role through involvement in cellular defense mechanisms and cellular immunity. In this paper, we investigate the interactions of oligodeoxynucleotides (ODNs) with Toll-like receptor 9 (TLR9), which have significant biological activity and play an important role in the control and treatment of breast cancer.

TLR9 is a mediator of the innate immune system that recognizes both microbial and vertebrate DNAs. Members of the Toll-like receptor (TLR) family all contain leucine rich repeat domains in the extracellular portion and an intracellular TIR (Toll-1L-1R) domain. There are at least ten members in the TLR family that can be divided into five subfamilies, one of which is TLR9 (including TLRs 7, 8 and 9). These proteins recognize pathogen derived RNAs and DNAs. Non-specific endocytosis of ODNs is required to activate antigen-presenting cells (APCs). Members of the TLR9 subfamily are expressed intracellularly in the endosomal-lysosomal compartment as opposed to other subfamilies that are bound to the cell surface. Upon recognition of vertebral or microbial DNA, TLR9 induces an inflammatory response mechanism (Wagner 2004).

Over the past decade, there have been a number of studies conducted to examine the effects of base sequence on the binding of ODNs to TLR9. It is commonly accepted in the literature that the ODN sequence must contain CpG nucleotides in order to bind to and activate TLR9. Rutz, et al, have demonstrated binding of CpG ODNs to TLR9 via surface plasmon resonance (SPR) biosensor technology (2004). A sequence-specific recognition of and response to a specific CpG containing ODNs by TLR9 has been reported in many studies (Bauer 2001). The ODN known as M362 is a 25-base long sequence that has been well characterized as a TLR9 agonist.

In this study, we propose that the activation of TLR9 is not only dependent on a specific CpG containing sequence, but also on the secondary structure of the ODN. ODN M362 contains a central 16-base long sequence that is self-complementary. This enables the ODN to adopt a hairpin conformation with dangling ends (four base overhang on the 5' end and five base overhang on the 3' end). In addition, the ODN may exist as a bimolecular duplex. We have performed a series of sequence modifications to determine if a particular secondary structure of the ODN is necessary to activate TLR9. Furthermore, we have applied several biophysical techniques, including differential scanning calorimetry (DSC) and circular dichroism (CD) spectrophotometry studies, in order to investigate the secondary structure of the ODN under various salt and pH conditions.

Methods

Sample Preparation:

Oligonucleotide sequences were purchased from Midland Reagents and used without further purification. They were prepared in Tris EDTA buffer at pH 7.5 for use in invasion assays. The oligonucleotides were prepared in 10 mM BPES buffer at varying sodium chloride concentrations and pH values for biophysical analysis.

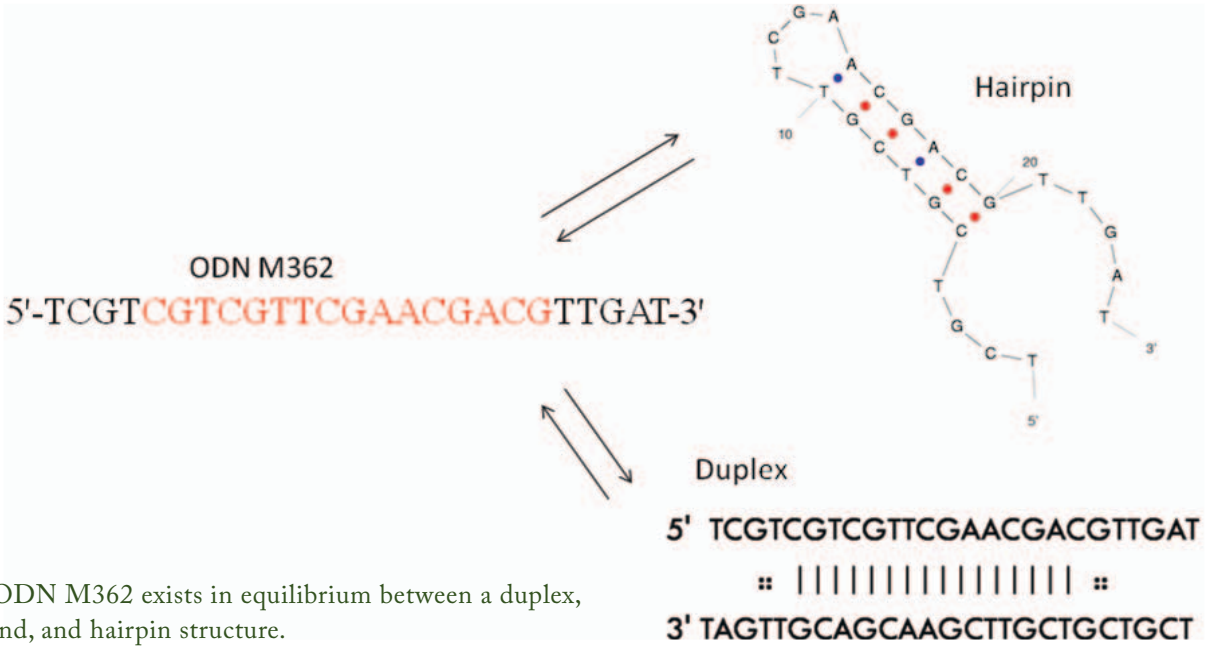

Figure 1. ODN M362 exists in equilibrium between a duplex, single strand, and hairpin structure.

Table 1. Sequence Variations of ODN M362.

Name	Length	Sequence
ODN M362	25	5'-TCGTCGTCGTTCGAACGACGTTGAT-3'
Truncated	16	5'-CGTCGTTCGAACGACG-3'
5' end	20	5'-TCGTCGTCGTTCGAACGACG-3'
3' end	21	5'-CGTCGTTCGAACGACGTTGAT-3'
Trunc + T	17	5'-CGTCGTTCTGAACGACG-3'
Trunc + TT	18	5'-CGTCGTTCTTGAACGACG-3'

Analysis of Structure and Stability of ODN M362:
The molecular modeling program MFOLD was used to predict structural features of ODNs that were shown to exert biological activity, including the induction of TLR9 mediated cellular invasion. This software is used to predict minimal energy structures of DNA and RNA oligonucleotides based on the base sequence, propensity for forming base pairs, base stacking, mismatches, and dangling ends. Using MFOLD, the most stable secondary structures for these oligodeoxynucleotides were determined and probed for biophysical stabilities of base pairing patterns within stems, loop structural and sequence features, and base pair mismatches within the hairpin stems. Results obtained from the secondary structure prediction allowed us to use rational design to incorporate subsequent changes into the base sequence to probe the effects of stem stability, loop sequence and size, and base pair mismatches within the stem on influencing the biological activity of these ODNs in the cell invasion. Accelrys Discovery Studio 2.0 was used to model the hairpin structures of the ODNs and to evaluate their energetic stabilities.

Cellular Invasion Assays:
MDA-MB-231 breast cancer cells were plated onto Matrigel matrices at a cell density of 1 x 10^4 cells per well in 500µL of culture medium. Oligonucleotide treatments containing a phosphorothioate backbone (PS) were added at a concentration of 5 µM. When noted, the oligonucleotides were left unmodified with a phosphodiester backbone (PD). A vehicle treatment of TE buffer was used as the negative control. The cells were allowed to invade for 22 hours after which the inserts were removed and stained with Hema 3 Stain set according to manufacturer recommendations. The number of invaded cells was counted microscopically at five preselected fields using a 40X objective. The results are given as mean ± sd, unless otherwise stated. Student's t test was used to calculate statistically significant differences between the various study groups.

DSC Studies:
DSC experiments were performed with a Microcal VP-DSC from 10°C to 90°C at a heating rate of 0.5°C/min against the appropriate buffer. All samples were prepared to 100µM and were degassed prior to use. At least five scans of buffer in the sample cell were run to acquire an adequate baseline, followed by at least five oligonucleotide melts.

Circular Dichroism Studies:
CD experiments were performed on an Aviv 400 CD spectropolarimeter using a 1 cm pathlength cell. Samples were prepared to 6 µM in 0.01 M sodium phosphate, 0.001 M disodium EDTA, and 100 mM NaCl (BPES) buffer at the designated pH. Data were collected from 215 to 320 nm at every 1 nm with a bandwidth of 1 nm. Time course experiments were monitored at 250 nm over 30 minutes. Spectra were corrected for buffer contributions, and the data were normalized to molar ellipticity (deg□cm^2□$decimol^{-2}$).

Results and Discussion
In order to discern the structural characteristics of the ODN

that are necessary to induce invasion in breast cancer cells, several sequence variations on the parent ODN M362 were performed. This ODN exists in equilibrium between a duplex with sixteen base pairs and a hairpin structure with six base pairs in the stem and four bases in the loop (**Figure 1**). The sequence modifications are summarized in **Table 1**.

The base sequence of the parent ODN M362 (25mer) was truncated to an entirely self-complementary 16mer ODN. This 16-mer can adopt both a hairpin and a duplex structure, as predicted by MFOLD. The sequence was further modified by introducing additional bases – T and TT – into the loop of the hairpin structure. The addition of the bases in the loop pushes the equilibrium to the hairpin structure, which is more likely to be the dominate species than a duplex with unpaired bases in the center of the sequence. This shift in equilibrium is demonstrated by DSC melting data. The first melt of the truncated 16mer in the absence of salt revealed two species in solution, with melting temperatures (T_m) of 32°C and 53°C (**Figure 2A**). This is indicative of two structures of the self-complementary ODN in solution, which supports the theory of equilibrium between a duplex and hairpin. The less stable structure in the absence of salt is the duplex, and the more stable is the hairpin. After the sample was cooled and melted again, the DSC profile revealed one melting transition at a T_m of approximately 52°C (**Figure 2B**). This corresponds to the annealing of the DNA into its most stable form, the hairpin structure. However, in the presence of salt, the duplex structure is stabilized more than the hairpin is. The DSC melting profile of the truncated 16mer in 100 mM NaCl indicated the presence of two structures, with T_m's of 39°C and 54°C (**Figure 2C**). The presence of salt stabilized the duplex structure by approximately seven degrees, and thereby altered the position of equilibrium between duplex and hairpin. Additional bases were inserted into the loop of the hairpin structure, in order to push the equilibrium to the hairpin. The DSC melting profile of the 17mer, which contains an additional T in the loop of the hairpin structure, revealed that this modification does favor the hairpin structure. The 17mer in 100 mM NaCl melted in a single transition, with a T_m of approximately 58°C (**Figure 2D**). Even in the presence of salt, this ODN does not adopt a stable duplex structure. The hairpin structure is further stabilized for the 18mer, with two additional T's in the loop. This is evidenced by the increase in melting temperatures determined by DSC, as summarized in **Table 2**.

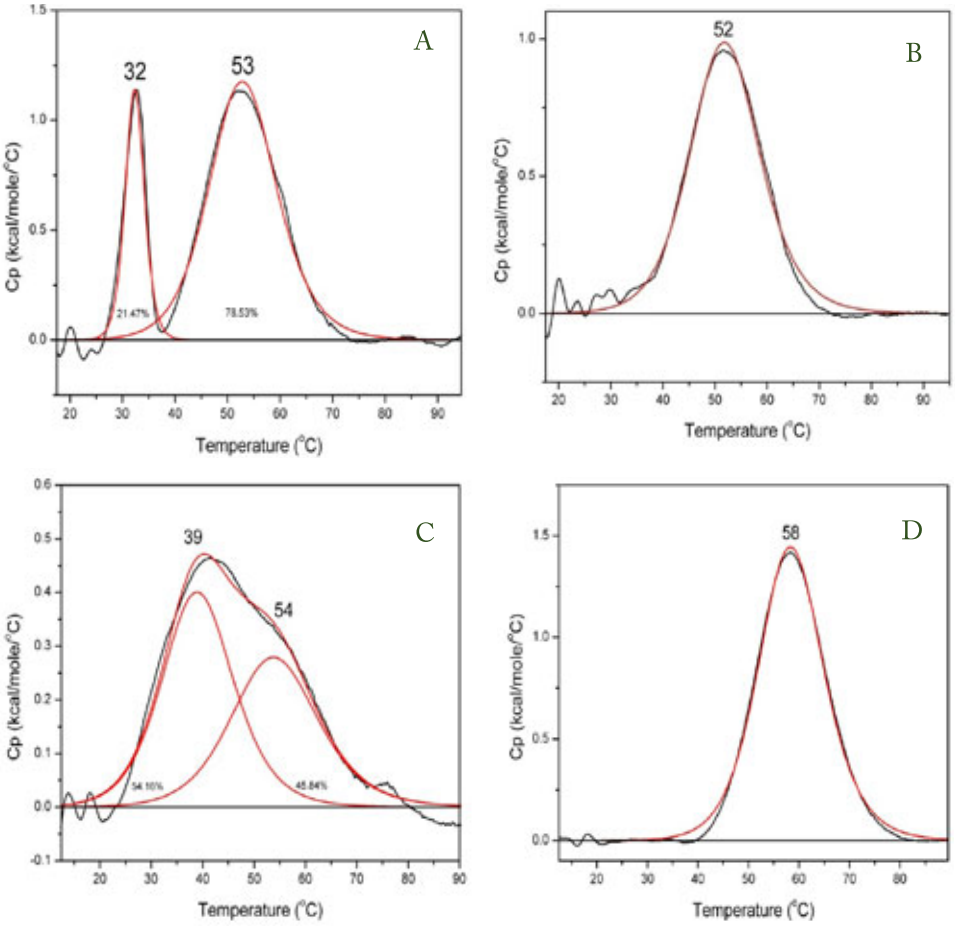

Figure 2. DSC melting profiles of 100 µM truncated 16mer (PS) (A: TE buffer, 1st melt; B: TE buffer, successive melts; C: 100 mM NaCl BPES buffer) and the 17mer (PS) with an additional T in the stem-loop (D: 100 mM NaCl BPES buffer).

Table 2. The melting temperature (T_m) of DNA hairpin structures in 100 mM NaCl BPES as determined by DSC.

Sequence	T_m (°C)
16mer (PS)	54.8 ± 0.53
17mer (PS)	58.15 ± 0.028
18mer (PS)	60.58 ± 0.038

The equilibrium between duplex and hairpin of the truncated 16mer was further evaluated by CD spectrophotometry. The change in the CD spectrum of the 16mer at pH 7 in the presence of 100 mM NaCl was monitored over an increase in temperature. The shift in the spectrum with increasing temperature resulted in two isoelliptical points (**Figure 3**). This is indicative of two species in solution, presumably the hairpin and the duplex. However, because TLR9 is expressed in the endosomal and lysosomal compartments, the interactions between TLR9 and the ODNs may take place in an acidic environment. Therefore, the CD spectrum of the 16mer at pH 5 was monitored over an increase in temperature. At low pH, there was no shift in the spectrum and no isoelliptical points were observed. This demonstrates that at pH 5, the equilib-

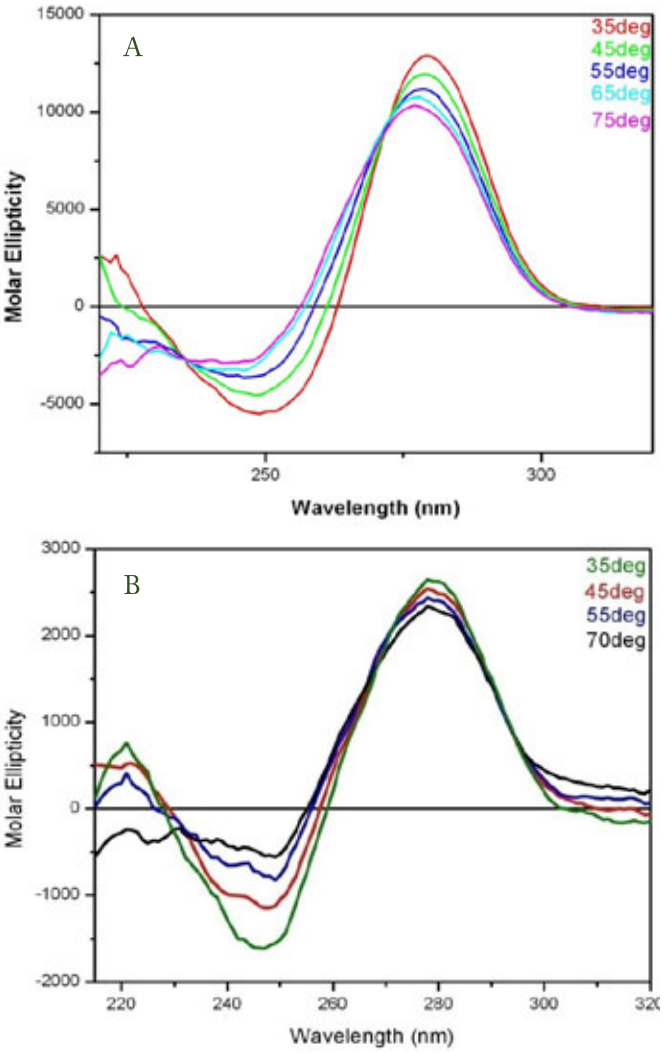

Figure 3. CD spectra of the truncated 16mer (PS) at pH 7 (A) and pH 5 (B) in 100mM NaCl BPES buffer.

rium between the two structures is shifted predominantly to one. Because hairpin structures are favored at lower pH values, it is likely that the hairpin structure dominates at lower pH, even in the presence of salt.

Invasion assays were performed to evaluate the ability of each ODN to induce invasion in MDA-MB-231 breast cancer cells. The resulting fold increase in invasion over a buffer vehicle for each sequence is shown in **Figure 4**. The 16mer induced invasion at a level comparable to that of the parent ODN M362. It has been demonstrated that the 16mer exists in equilibrium between a duplex and hairpin form. However, the 17mer (Truncated + T) and 18mer (Truncated + TT) are more likely to adopt hairpin structures. These sequences also induced invasion comparable to the parent 25mer. Therefore, the 16mer and the 25mer may also adopt hairpin structures in order to produce the invasive response.

In order to perform the invasion assays, the ODNs were modified to a phosphorothioate (PS) backbone, which con-

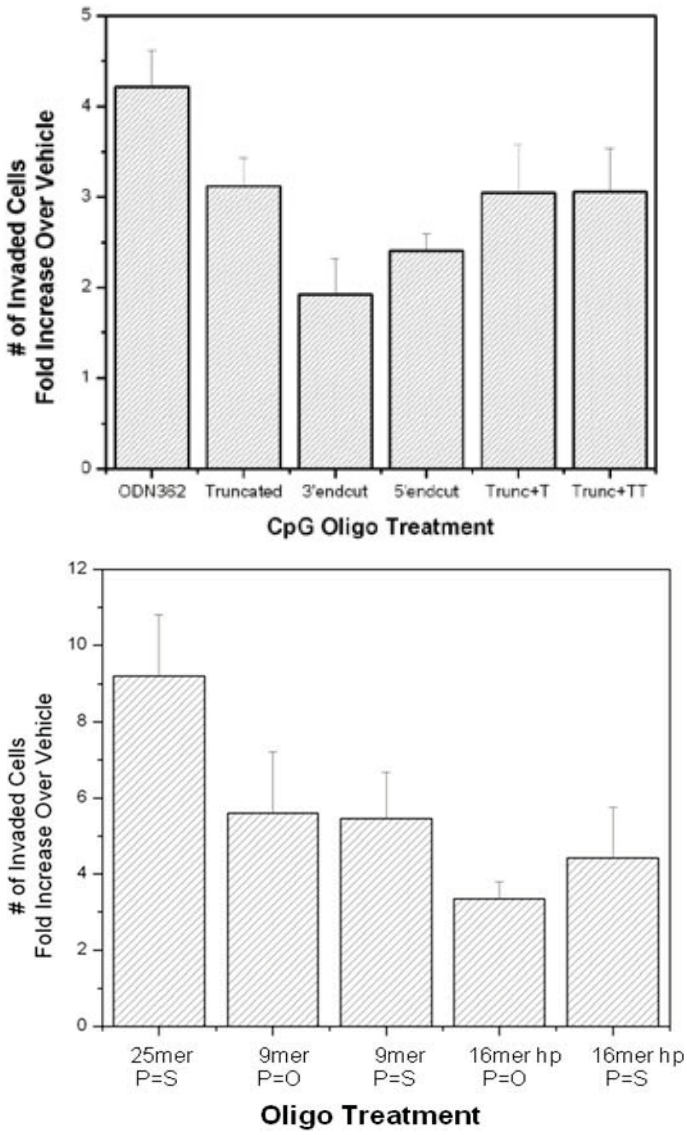

Figure 4. The effects of various oligonucleotides (5μM) on invasion were studied in invasion assays in vitro using MDA-MB-231 cells. The results are expressed as the normalized fold increase in invasion over vehicle. Columns: mean (n = 8) ± SD.

tains phosphate-sulfur double bonds in place of the native phosphate-oxygen double bonds in phosphodiester (PD) backbone. This modification was implemented to make the ODNs resistant to nuclease digestion. However, the apoptotic DNA that is suspected to induce invasion by the same TLR9 mechanism as these ODNs do not contain the modified PS backbone. It has been reported that hairpin structures may offer resistance to nuclease digestion (Yoshizawa, 1994). The effects of a very stable 9mer hairpin with the sequence 5'-d(CGCGAAGCG)-3' on invasion with PD and PS backbone were compared. In addition, the truncated 16mer sequence was modified to contain only purine bases in the loop, in order to provide more favorable stacking interactions to make the hairpin more stable. The effects of this sequence (5'-d(CGTCGTGAAAACGACG)-3', termed "16mer hp") with PD and PS backbone were also studied in invasion as-

says. The results are summarized in **Figure 4**. The increases in invasion due to the PD and PS 9mer hairpin were surprisingly similar. The PD and PS 16mer with purines in the loop also induced invasion at similar levels. This demonstrates that a stable hairpin structure offers resistance to digestion, which allows the ODNs to interact with TLR9 and induce the invasive response.

The hairpin resistance to nuclease digestion was confirmed via CD time course experiments. The CD signal at 250 nm was monitored for a single strand 11mer (PD) that cannot adopt a hairpin structure and for the 9mer (PD) after the addition of S1 nuclease (**Figure 5**). There was a significant change in CD signal for the single strand ODN, while the signal remained constant over thirty minutes for the hairpin ODN.

Conclusions

The work presented here suggests that the biologically active structure is the hairpin. The sequences that contain additional T's in the stem loop of the hairpin structure are more likely to exist as hairpins than in a duplex form. These sequences induce invasion comparable to the truncated sequence. This suggests that the truncated sequence also adopts the hairpin structure. Because the invasive ability of the truncated sequence is not significantly different than that of ODN M362, the biologically relevant species of this sequence may also be the hairpin. These studies also suggest that the predominant and most stable structure is the hairpin.

Furthermore, the hairpin structure provides resistance to nuclease digestion, as evidenced by the ability of hairpin ODNs to induce invasion in breast cancer cells, as well as by the CD data demonstrating resistance to S1 nuclease. This allows for connections to be made from the modified PS ODNs used in invasion assays with PD apoptotic DNA. Plans for future work include the correlation of hairpin ODN stability, as determined by DSC and computational methods, with their ability to induce invasion via TLR9. We intend to undertake a direct investigation of the binding of hairpin ODNs with TLR9 using isothermal titration calorimetry (ITC) studies. Finally, we hope to correlate the ODN sequences that induce invasion with sequences contained in apoptotic DNA.

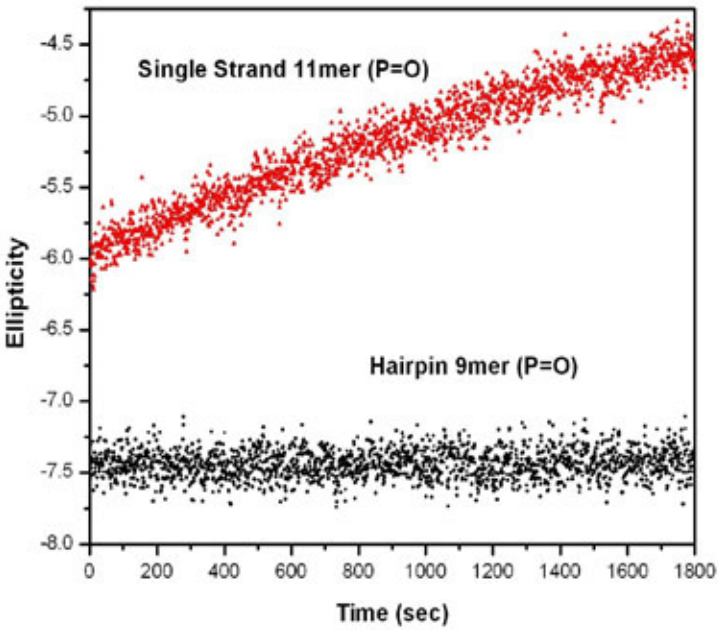

Figure 5. CD signal at 250 nm over 30 minutes after addition of S1 nuclease to a single strand 11mer and the hairpin 9mer.

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faculty interview: physics

Dr. Perry Gerakines: Astrophysicist at UAB Investigates the Origins of Life

Physics, Danuel Laan

As a child, Perry Gerakines was an avid reader of science fiction. Today, he performs the very research that could change science fiction into real technology. He began his career as a high school senior when he was selected to take part in a course at Harvard University. The Nobel Prize winning physicist, Roy Glauber, was his teacher. His first research endeavor was as a sophomore in a Research Experience for Undergraduates (REU) program over the summer of 1991 at the Rensselaer Polytechnic Institute (RPI) in Troy, New York. There he developed an interest in infrared astronomy, a field of astrophysics that analyzes celestial bodies through infrared light to determine structural and functional properties. He carried over his interest in astrophysics to graduate school where he obtained a two-year research degree (M.S.) in Astronomy at Leiden University in the Netherlands. He believes his experience in the Netherlands was invaluable to him as it broadened his perspective and gave him experience in the global science community. He returned to RPI to obtain a Ph.D. in Physics. Before he came to the University of Alabama at Birmingham (UAB), he was a Postdoctoral Research Associate for the National Research Council and the National Academy of Sciences at NASA's Goddard Space Flight Center in Greenbelt, Maryland. UAB's preexisting astrophysics lab was one of the incentives for Dr. Gerakines to join the staff.

Dr. Gerakines' research mainly involves materials found in space either in interstellar clouds, cold dark clouds found between stars, or materials found on the icy surfaces of some planets and planetary moons in our solar system. Some of these moons experience freezing temperatures as low as 10 degrees Kelvin, close to absolute zero, the coldest temperature known. On the surface of these celestial bodies, there are ice-chemical matrices that are of particular interest to him. Dr. Gerakines studies the spectroscopy of these materials in order to see absorption patterns with infrared energy changes associated with physical properties such as cold temperatures, crystalline state, chemicals within the ice, or radiation acting on the ice. The goal of his research is to interpret observations made from space by instruments such as the Spitzer Space Telescope, which records infrared spectroscopies of icy celestial bodies or interstellar clouds and sends these absorptions spectrums back for analysis.

We want to understand what the Saturn system is made of because we want to understand the history of our solar system. The chemistry that has gone on around Saturn is a lot different than what has gone on around earth. Understanding the origins of the materials on these moons tells us about the origin of Saturn and the origin of the universe.

In recent months Dr. Gerakines was awarded a three-year, $408,000 grant for the project, "Vacuum Ultraviolet Spectroscopy of Icy Mixtures Relevant to the Outer Solar System." Gerakines will work with Amanda Hendrix, a Cassini UVIS instrument scientist from the Jet Propulsion Laboratory, to measure the vacuum ultraviolet spectra of thin ice films. This research is part of a larger NASA Outer Planets Research Program and studies the composition of Titan and Enceladus, two of the 52 moons orbiting Saturn. The Cassini mission is a very large space probe that NASA sent to Saturn to analyze

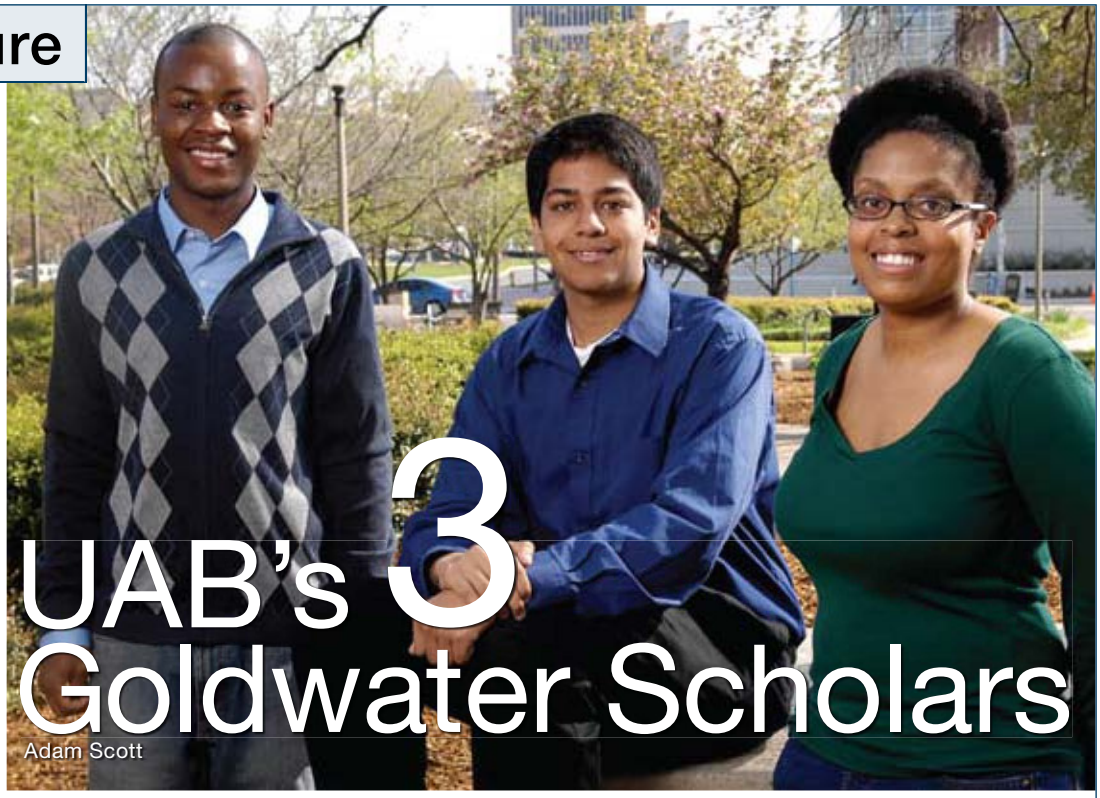
the icy moons that surround Saturn through the ultraviolet spectrum of light. Gerakines will be doing laboratory measurements of what is expected on the surfaces of these planets and then comparing these measurements with the real data as it comes in. Dr. Gerakines says, " We want to understand what the Saturn system is made of because we want to understand the history of our solar system. The chemistry that has gone on around Saturn is a lot different than what has gone on around earth. Understanding the origins of the materials on these moons tells us about the origin of Saturn and the origin of the universe."

Undergraduates that don't have lab or research experience have this idea of what research is or what real measurements entail, but until one does it, one can't actually see that. Research made me feel that I was doing something meaningful; to get real results as opposed to just learning about something that other people had already figured out… It gave me a sense of doing something productive.

In this endeavor, Dr. Gerakines hopes to derive a basic understanding of materials under these extreme conditions. Even though some compounds such as water or carbon dioxide might seem well studied or understood under terrestrial conditions, very little is known about their behavior under extreme conditions such as in the vacuum of space or temperature as low as ten degrees above absolute zero. His research, then, could very well be crucial in the origins of the organic materials present in comets and meteorites or the molecular origins of life itself.

Three graduate students collaborate with Dr. Gerakines in his current research. In addition, every summer undergraduate students assist him in his lab. He is very receptive to students and advises all undergraduates to find a researcher in their particular field of interest and shadow them. He believes that this experience is imperative and clarifies a student's understanding of their potential field while allowing them to truly see if the field is right for them. He says, "Undergraduates that don't have lab or research experience have this idea of what research is or what real measurements entail, but until one does it, one can't actually see that. Research made me feel that I was doing something meaningful; to get real results as opposed to just learning about something that other people had already figured out… It gave me a sense of doing something productive."

He realizes that undergraduates need experience in a specialty to see if it is the best fit for their talents. He also encourages students to explore multiple disciplines and find something that truly excites their interest.



L-R: Taoreed "Larry" Lawal, Pratik Talati and Cierra Spencer

The University of Alabama at Birmingham (UAB) holds a proud reputation of promoting academic success and instilling a passion for scientific research in its students. In the 2008-2009 academic year, UAB upheld this tradition when three of its Honors Program students, Taoreed "Larry" Lawal, Pratik Talati and Cierra Spencer, were selected as Barry M. Goldwater Scholars.

Established in 1986, to honor Senator Barry M. Goldwater and his service to our country, the Goldwater Scholarship is awarded to outstanding undergraduate students who plan to pursue careers in mathematics, the natural sciences, or engineering. Of the 1,035 students nominated nationwide, 321 students were selected, seven from Alabama. The three are among eight UAB students who have received the award in the university's history.

UAB is proud to have a record number of three Goldwater Scholars from Alabama in 2008.

Taoreed "Larry" Lawal is a senior majoring in biology. Lawal's accomplishments are many, and include receiving the NASA Alabama Space Grant Consortium Scholarship in 2007, first place winner in the 2007 UAB Summer Research Intern poster session, and receiving the Excellence in Organic Chemistry Award in 2007. Lawal attributes much of his success to working with Larry DeLucas, O.D., Ph.D., a top UAB researcher in the Center for Biophysical Sciences and Engineering and to his involvement in the University Honors Program. Following graduation, Lawal plans to travel abroad and attend medical school in Fall 2009.

Pratik Talati, a junior majoring in mathematics and chemistry, maintains a high level of academic excellence. Talati is a student in the Chemistry Scholar and Math Fast Track programs. This past summer, Talati represented UAB while traveling to the Max Planck Institute of Psychiatry in Munich, Germany, through the DAAD-RISE program. Talati says this experience was his "most personally satisfying and significant accomplishment at UAB," affording him the opportunity to work with top researchers while exploring Europe.

In his spare time, Talati enjoys working with Habitat for Humanity and reading to patients through Healing Words. After graduating in May 2010, Talati plans to pursue an MSTP program for a joint M.D./Ph.D.

Cierra Spencer is a senior chemistry major specializing in biochemistry. Spencer's numerous academic accolades include the Excellence in Freshman Chemistry Award, the Excellence in Organic Chemistry Award, and a Chemistry Scholar fellowship from the UAB Department of Chemistry. Her most recent accomplishment was receiving the 2008 UNCF Merck Undergraduate Science Research Scholarship Award. As part of this award, Spencer spent last summer at the Merck Research Lab in Rahway, NJ, in the Process Research division. While there, she gained invaluable research experience and submitted a paper for publication in October to Tetrahedron Letters.

Spencer will be graduating in May 2009. She plans on attending graduate school to study pharmacology, with a focus on drug metabolism and resistance.

UAB is proud to have a record number of three Goldwater Scholars from Alabama in 2008. All three students emphasize gaining a diversity of training in research as an undergraduate, and promote getting involved and being proactive early in your academic career. For information on nominations for the Goldwater Scholarship, students should visit the UAB website of the fellowships and scholarships office: www.uab.edu/fellowships.

Wear Testing of Zirconia Coatings for Applications in Total Hip Replacement

Carrie Stewart, Alan Eberhardt

Abstract:

Polyethylene wear is recognized as one of the leading contributors in the long-term failure of total hip replacements (THR). Improving the tribological properties of the articulating surfaces in THR reduces wear and extends implant life. Zirconia is a biocompatible material that has been used in femoral heads because of its high hardness and smooth finish. Presently, twelve different metallic-coating combinations were tested, including anodized and non-anodized commercially pure titanium (CP-Ti) and titanium alloy (Ti6Al4V), each with one, two or no coats of zirconia. Zirconia-coated CP-Ti and Ti6Al4V were wear-tested against ultra high molecular weight polyethylene (UHMWPE) as a preliminary screening of the coatings as candidates for use in THR. CP-Ti coated with one zirconia layer was found to cause less UHMWPE mass loss than the uncoated alloys and twice coated alloys. The remaining coated samples, however, showed more mass loss than the uncoated alloys. Meta-analysis suggested the thicknesses for the twice coated specimens may have exceeded the critical thickness values for zirconia coatings, leading to the poor performance in the present comparisons.

Introduction

Ultra high molecular weight polyethylene (UHMWPE), the most common articulating surface used in the acetabular component of total hip replacements (THR), exhibits low friction and wear behavior; however, there remain concerns regarding its longterm performance. In the 1990s, UHMWPE wear debris became recognized as one of the central causes for initiating osteolysis, a major factor that limits joint prosthesis life. [1] Particulate wear debris circulates in body-fluids around the total joint prosthesis and initiate a foreign body reaction in local bone. Studies suggest that over 10^5 polyethylene particles are formed with each step. [2] Cells responsible for destroying foreign matter are activated by the polyethylene particles, and this process simultaneously destroys peri-implant tissues, including bone, through osteolysis. [2]

Titanium alloys are suitable materials for total hip replacement because they are very resistant to corrosion, which is a good indicator of biocompatibility. An oxide film, TiO_2, passively forms and protects both commercially pure titanium (CP-Ti) and its alloy (Ti6Al4V). [5] A drawback to titanium and its alloy is the relative softness as compared to other metals. Titanium alloys are not commonly used for femoral heads in total hip replacement because they perform poorly in comparison to the cobalt-chromium (CoCr) bearing surfaces currently popular in THR.

Modern THRs are typically modular and allow different material combinations of metals, ceramics and polyethylene components, which affect tribological behaviors. The CoCr and UHMWPE combination has been the standard for THR since the 1970s. Metal-on-metal implants were one of the earliest successful combinations, and newer, alternative, biocompatible bearing surfaces have the potential to increase the lifespan of a THR. [4] Increased implant life would be especially beneficial to younger patients and patients with high activity levels. Material choices in THR are limited by manufacturing restrictions and precise implantation techniques. [5]

Many bearing surfaces have been coated with different materials to manipulate tribological behavior. Alumina, zirconia, nitriding, and nitrogen ion implantation of Ti alloys [7], and amorphous diamond have all been used to try to improve tribological properties in THR. Alumina, with high hardness, fracture toughness, and biocompatibility, has been clinically used as a bearing surface in alumina-UHMWPE configurations. [8,17] Wear rates of zirconia ceramics, yttria-stabilized tetragonal zirconia polycrystal (Y-TZP), have shown lower wear rates when articulated against UHMWPE as compared to alumina. [6]

The purpose of this study was to screen zirconia surface coatings on Ti6Al4V and CP-Ti for potential bearing surfaces of femoral heads against UHMWPE in THR. It was hypothesized that of the materials tested, zirconia coated Ti6Al4V and CP-Ti would outperform the uncoated titanium surfaces. In other words, the hard, smooth coating of zirconia on the metallic was expected to cause less UHMWPE wear.

Materials and Methods

Materials

CP-Ti and Ti6Al4V alloy samples were provided by an external university source. Coatings were applied to the samples by the source using proprietary sol-gel spin coating methods. There were three or four samples of each specific metallic and coating combination. Table 1 illustrates the testing matrix for the different alloys.

UHMWPE (Arcom®, Biomet, Inc., Warsaw, IN) was machined into cylindrical wear pins 9.5 mm in diameter with a step down to 4.76 +/- 0.03 mm diameter on the contacting surface, giving a contact area of approximately 17.8 mm^2 . The pins were soaked in bovine serum (Hyclone, Logan, UT) for a minimum of two weeks prior to wear testing.

1. *Frictional Wear Testing*
Before tests began, pins and disks were sonicated in a 10% LiquiNox® (Alconox Inc., White Plains, NY) solution, rinsed with deionized water, and submerged in ethyl alcohol. Then the pins and disks were dried in a vacuum for thirty minutes. To obtain a change in mass, the initial and final pin weights were recorded. The pins were each weighed five times on a Mettler Toledo AG245 microbalance (Columbus, OH) with a resolution to .00001 grams and averaged, before and after wear testing.

During each test, the pins and disk samples were submerged in a lubricant that contained 53% bovine serum water, anhydrous ethylenediaminetetraacetic acid (EDTA) and sodium azide. EDTA (Sigma-Aldrich, St. Louis, MO), was added to bind the calcium in the solution and prevent precipitation of abrasive components onto the bearing surfaces. Sodium azide was also added to prevent bacterial depredation. The mixture was filtered with a .22 micron filter, and warmed to 37° C. The lubricant temperature was maintained at body temperature throughout testing.

Wear testing was performed in an OrthoPOD® six station pin-on-disk machine (Advanced Mechanical Testing, Inc., Watertown, MA). Nine tests were performed. The CP-Ti samples were tested separately from the Ti6Al4V samples. Each test, of the same metallic type, had a coated or uncoated sample at five of the six stations. A mixture of coating types was used during the test. Six pins and five disks were used during each test. The sixth pin was used for as an unworn soak control.

The polyethylene pins were installed into the OrthoPOD® and articulated against the flat, coated test faces of the metal disks. Figure 1 shows the configuration of the pin and disk. The wear path followed a figure eight pattern at a frequency of 1.5 Hz and a sliding speed of .4 m/s at constant load of 10.7 MPa. Testing conditions were chosen in accordance with ASTM standards G99 and F732. The OrthoPOD® software calculated coefficient of friction based on three triaxial load cells at intervals of 25,000 cycles. The horizontal force measurements had an accuracy of +/- 2N, and the vertical forces were accurate +/- 8N. Each test length was 500,000 cycles.

After 500,000 cycles, the pins and disks were removed from the machine, soaked for two days, mass was measured again, and mass loss was calculated. The soak control accounted for any change in mass due to fluid absorption.

Statistical Analysis
Kruskal-Wallis one-way analysis of variance by ranks was applied to test if there were overall differences in mass loss among the different test groups. This form of non-parametric statistics substitutes the ranking of the wear value for the actual value and the sum of ranks is used to test for differences among the groups. Additional t-tests were performed to compare different treatments, with a level of statistical significance of $\alpha = 0.05$. StatView software was used to perform both the non-parametric and parametric tests. The treatments compared were anodized and non-anodized samples as well as coated and uncoated samples. Samples were randomly selected for visual observation of wear paths using light microscopy (KEYENCE, Osaka, Japan).

Results
The four best performing surfaces were CP-Ti once coated with ZrO_2, uncoated anodized CP-Ti, uncoated Ti6Al4V, and uncoated anodized Ti6Al4 with average mass loss for the surfaces ranging from 660 μg to 933 μg. The four worst performing surfaces were all twice coated with ZrO_2, with average mass loss for the surfaces ranging from 51.5 mg to 7.5 mg. Uncoated CP-Ti, anodized CP-Ti once coated with ZrO_2, Ti6Al4V once coated with ZrO_2, and anodized Ti6Al4V once coated with ZrO_2 coated were ranked among the middle of the pack. Figure 2 illustrates the mass loss associated with each coating.

The Kruskal-Wallis analysis indicated that there were statistically different wear rates among the groups ($p < 0.001$), and provided a ranking of best to worst. The mean mass losses associated with the coated specimens was 57% higher than the uncoated specimens; however the differences were not significant (p=.0634). Among the anodized and non-anodized samples, mean mass losses were 54% higher among the anodized; however, again these results were not significant (p=.0687).

Evidence of material transfer was apparent along the wear path on the coated specimens following wear testing. Figure 3 shows a magnified image confirming material transfer onto the surface of the disk. The transfer film was likely UHMWPE, since UHMWPE has been known to transfer to counter-surfaces in wear testing.[16] Due to the proprietary nature of the coating processes, all specimens were returned to the manufacturer after wear testing was completed, and no further analyses were performed.

Discussion
The original hypothesis, that the ZrO_2 coatings on the titanium surfaces would create less wear than the uncoated titanium surfaces was supported in the case of the once coated CP-Ti. The next best performing surfaces were all uncoated. The remaining coated surfaces exhibited poorer wear behavior than the once coated CP-Ti. CP-Ti may have outperformed Ti6Al4V because of the latter material's surface instability. Ti6Al4V surface breakdown has been reported as one of the main causes of poor polyethylene wear performance. Delamination of the titanium alloy's surface layer has been reported

to increase polyethylene wear. Polymer binding increases surface roughness and has been associated with the poor wear performance exhibited by Ti6Al4V. [5]

The history of zirconia in femoral heads reveals mixed results with regard to polyethylene wear due to the complex nature of the material. Each zirconia implant has unique properties specific to the manufacturer, and therefore not all zirconia coatings have the same tribological properties. Y-TZP has high fracture toughness, as compared to other ceramics like alumina, which has been equated to better reliability. Also, a Y-TZP has a lower Young's modulus and ability to be polished to a superior surface finish as compared to other ceramics [11,3] Y-TZP undergoes transformation toughening on machined and polished bearing surfaces, whereby three to five percent transforms from tetragonal phase to the monoclinic phase. The monoclinic phase change results in small volume increases and there is a volumetric expansion of the ceramic grains that cause a compressive surface layer that helps to resist crack initiation and propagation. [10]

While transformation toughening is said to account for the high strength of the material, under certain manufacturing conditions of stress and moisture, the Y-TZP can transform more aggressively to the monoclinic phase than is desired. Increased monoclinic phase in zirconia increases the surface roughness and has shown poor performance and wear results when articulating with polyethylene.[10] Additionally, several studies have reported difficulties in obtaining crack-free coatings with variable thicknesses of zirconia.[12]

Recent studies have shown that ceramics can have properties such as decreased surface roughness, increased hardness and scratch resistance, which are a benefits over metallics. The benefits have shown clinical significance in improving wear behavior of articulating surfaces. [10,3] In the present study, the zirconia coatings tested may have been thicker than the critical thickness for zirconia coatings, which the manufacturer of the samples reported as 200nm.[9] Critical thickness measurements are based on the internal stresses of the zirconia and are related to the sol-gel deposition methods.[15] No additional information was provided by the manufacturer, and no analysis of coating thickness was conducted in the present study.

One potential benefit of using ceramics over metallics in implants is the possibility of obtaining superior surface finishes. However, in this experiment, only the single coated CP-Ti outperformed the uncoated samples. The majority of surface treatments of the metallics in this study did not display advantages in wear performance against polyethylene as compared to single coated CP-Ti. The titanium alloys coated twice with zirconia performed poorly, among the worst of the samples, suggesting that the added coating thickness was not advantageous to the tribological properties of the titanium and ceramic combination. The manufacturer provided no information regarding the polishing or final surface roughness values.

Future studies dealing with tribological properties of titanium metallics coated with zirconia ceramics should concentrate on applying crack-free coatings by obtaining a coating on the alloy less than the critical thickness. The thicknesses at which coatings are crack-free vary based upon application processes, materials, and methods used to apply the ceramics to alloys. [12,13] The applied thickness of coatings should be slightly less than the critical thickness to prevent crack formation.[14] Optimizing the coating fabrication process will be important for the future of ceramic coatings on titanium alloys for hip implants.

Conclusions

In summary, a series of coated and uncoated specimens were wear tested against UHMWPE to compare the behaviors of two alloy substrates (CP-Ti and Ti6Al4V), under anodized and non-anodized conditions, and with one or two layers of zirconia coating. It was hypothesized that the zirconia coated specimens would outperform the uncoated Ti-based substrates. The results indicated that the twice coated specimens were associated with mass losses that were an order of magnitude greater than the non-coated or once coated specimens. No significant differences in mass lost were detected when comparing the base substrates, nor between the anodized and non-anodized specimens. Meta-analysis suggested that coating thicknesses exceeding critical thickness may have been linked to the poor wear performance of the twice-coated zirconia specimens.

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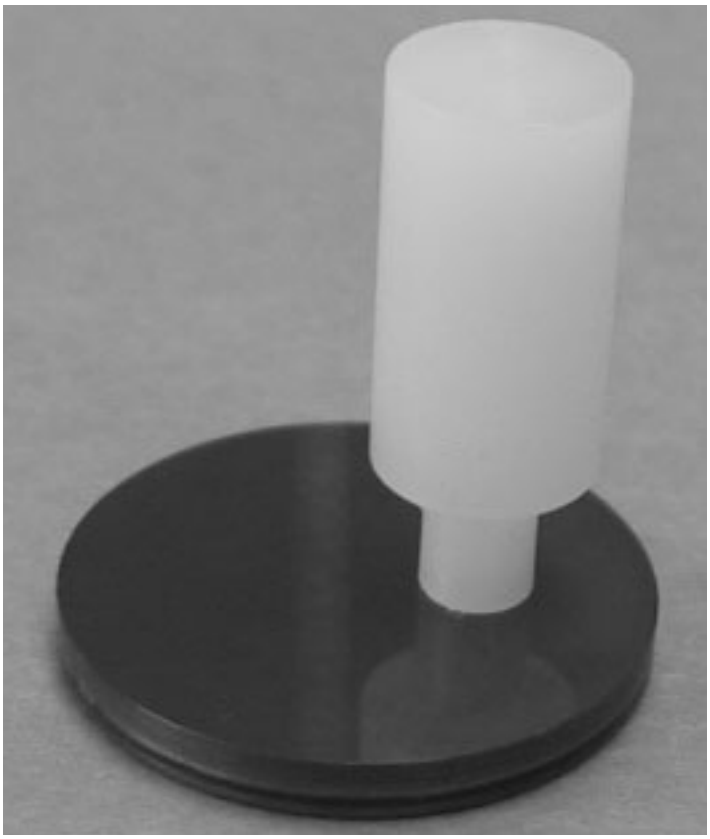
Table 1

Base Material	Treatment
CP-Ti N=4	No coating ZrO_2 Coated at 550° C ZrO_2 Coated at 550° C, 2 x ZrO_2 Coated layers
50V Anodized Cp-Ti N=4	No Coating ZrO_2 Coated – 550° C Anodized, ZrO_2 Coated at 550° C, 2 x ZrO_2 Coated layers
Ti6Al4V N=3	No Coating Ti6Al4V - ZrO_2 Coated at 550° C Ti6Al4V - ZrO_2 Coated at 550° C, 2 x ZrO_2 Coated layers
50V Anodized Ti6Al4V N=3	No Coating ZrO_2 Coated at 550° C ZrO_2 Coated at 550° C, 2 x ZrO_2 Coated layers

Figure 1. The pin and disk configuration for articulation.



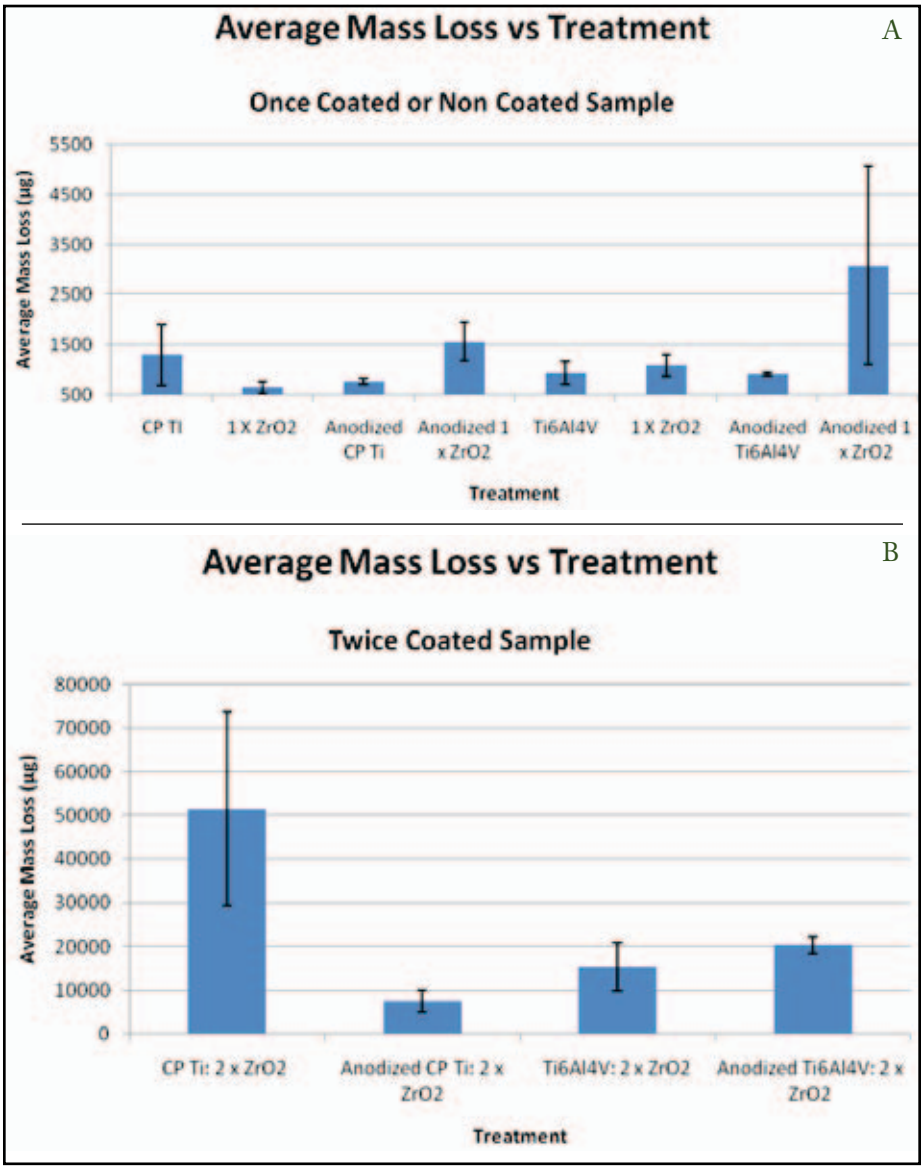


Figure 2. (a) Mean mass loss (bars indicate standard deviation) for each of the 8 metallic and once-coated combinations used as counter faces during wear testing; (b) Mean mass loss for the twice zirconia-coated specimens illustrating values one order of magnitude higher than the non- or once-coated specimens.

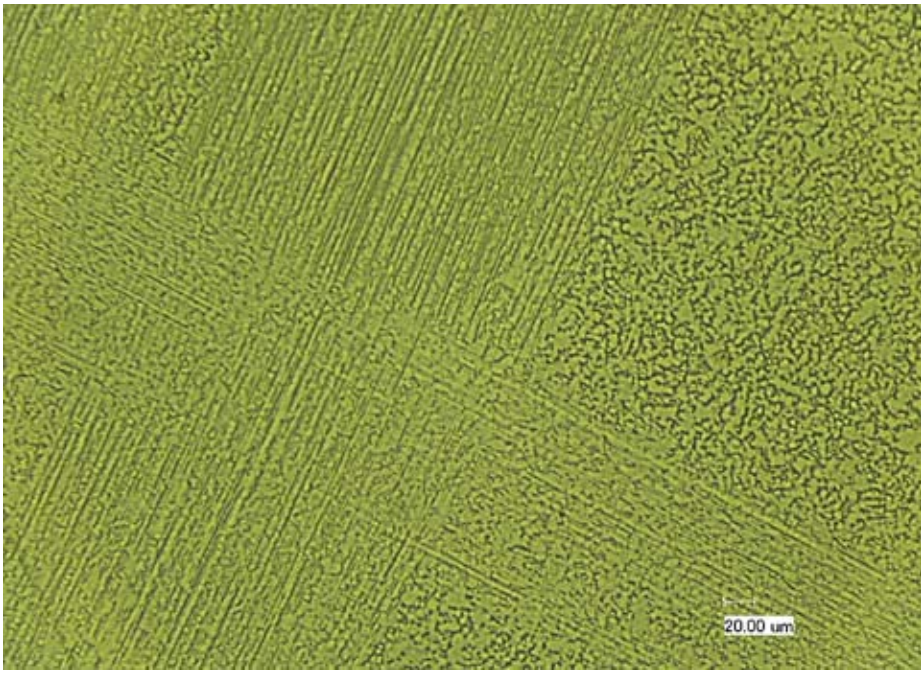


Figure 3. Keyence micrograph reveals evidence of a transfer film at the crossing point of the Figure-8 wear path between the UHMWPE pin and twice-coated disk.

inquiro staff

Larry Lawal
Chief Editor

Larry Lawal is a senior majoring in Biology. He works in the crystallography lab at the Center for Biophysical Sciences and Engineering with Dr. Larry DeLucas. He is a 2008 Goldwater Scholar and has previously conducted research in multiple labs under three National Science Foundation REU awards. Larry is a member of Alpha Epsilon Delta, Phi Kappa Phi, and the University Honors Program, where he served as a 3rd year teaching assistant. His interests include playing golf, tennis, and reading. Upon graduation, he plans to attend medical school in 2009.

Felix Kishinevsky
Chief Editor

Felix is a senior double major in Psychology and Biology. After graduation, he plans to pursue a Masters in Public Health, and then enroll in medical school. He is a member of Phi Kappa Phi, and a chapter officer of Psi Chi. He is currently president of the Jewish Student Association. He serves as a medical translator for members of the Russian community and as a Sunday school teacher. He has been working on a fMRI neuroimaging study of decision making in obese women under Rosalyn Weller, Ph.D. as part of his psychology honors thesis research.

Shweta Patel
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Shweta Patel is a sophomore Biology major with a concentration in Molecular and a Chemistry and Spanish minor. She is a member of the University Honors Program and the pre-health honor society Alpha Epsilon Delta. With a combined MD/PhD degree, her professional career goal is to teach and to conduct research in Pathology at a leading medical research university where scientific projects and clinical practices complement each other. Outside of academics, Shweta plays just about every intramural sport she can join.

Kimberly Trawick
Staff Writer

Kimberly Trawick is part of the 5th Year's Master's Program in Biology, and has worked in Dr. Stephen Watts' lab since 2003. She hopes to gain a Masters Degree and use her research experience to rebuild the environment. She is also a member of Alpha Omicron Pi, and enjoys mountain biking and scuba diving.

Andrew Buie
Staff Writer

Andrew Buie is a sophomore Biochemistry and Spanish double major at UAB. He is a member of the Chemistry Fellowship and the University Honors Program. Andrew is a member of Alpha Lambda Delta and AMSA and is currently the secretary of SAACS. Upon graduation, Andrew plans on attending medical school at UAB in 2011.

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Staff Writer

Matt Morton is a junior Biomedical Engineering and Pre-medicine major. He is a member of the Science and Technology Honors Program and co-chair of its volunteer committee, as well as a member of the Tau Beta Pi engineering honor society. His interests include traveling, genetics, and advances in computer technology. Currently, he is working on his honors thesis research in the Program in Epidemiology of Immunity and Infection with Dr. Richard Kaslow. After completing his degree in 2010, Matt plans to attend medical school.

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Atbin is a freshman Biology and Mathematics double major. He enjoys reading, history, and watching movies. His research interests include movement disorders, medical drug synthesis, and modeling. Atbin is a member of Science and Technology Honors Program and Math Fast-Track program.

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Danuel Laan is a junior at UAB. His major is Molecular Biology with a minor in Chemistry and Spanish. He is also a member of the University Honors Program (UHP). Danuel currently serves as a teaching assistant (TA) in the UHP and in 2007 was a TA in general chemistry. He serves as the Delta Sigma Phi Philanthropy chair and Alpha Epsilon Delta Fundraising chair. His hobbies are reading, playing guitar and volunteering at Camp ASCCA, The Alabama Special Camp for Children and Adults.

Pratik Talati
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Pratik Talati is a junior Chemistry and Mathematics double major at UAB. His interests include reading, volunteering, and learning foreign languages. Pratik currently works as a student assistant in the Department of Psychiatry and Behavioral Neurobiology under Dr. Rita Cowell. After graduating, he plans on joining a Medical Scientist Training Program for a joint MD/PhD.

Adam Scott
Staff Writer

Adam Scott is a senior molecular biology major. He is currently working in the Alzheimer's Disease Research Center within the UAB Department of Neurology. He is actively involved in Alpha Epsilon Delta, Phi Sigma, and Outreach Abroad. He also enjoys volunteering with Big Brothers Big Sisters and Grace House Ministries. Adam is an avid mountain biker and plays basketball frequently. Following graduation, he plans to attend medical school in 2009.

Courtney Sparkman
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Courtney Sparkman is a sophomore majoring in Biology at UAB. Currently, she is a lab assistant in the Department of Psychiatry and Behavioral Neurobiology with Dr. Rita Marie Cowell. She is a member of the University Honors Program, UAB Ambassadors, Alpha Lambda Delta, and Alpha Omicron Pi.

2009 inquiro submission guidelines

Any student participating in scientific research at UAB is invited to submit a research paper for submission in the 2008 issue of Inquiro. Papers will be subject to student and faculty review.

The deadline for submissions is **May 15, 2009**; however, students participating in summer research at UAB or another institution are encouraged to submit by **August 17, 2009.**

Initial submissions should follow these guidelines:

1) 12 point font, double spaced, pages numbered with the author's name appearing in a header on every page (further formatting will be required upon acceptance).

2) Figures, tables, and graphs should be submitted in their original formats as separate files.

3) All research papers should be submitted with the Inquiro Permission to Publish Form.

Staff also invites students to submit research narratives, interviews with faculty members, and science related editorials.

Short Reports: These reports are short papers derived from the text of science posters. Please convert the original poster to a Word document, which includes all text, figures, tables, and images from the poster. As above, images should be submitted additionally as a separate file. The suggested length is 2,500 words.

Research Narratives/Other: If students would like to submit editorial or narrative pieces relating to scientific research, they may certainly do so. The journal staff will review the article and consider it based on relevance and quality. The suggested length is 900 words.

Anyone who whishes to join the inquiro staff should fill out the application on our website.

Pleases send submission or questions to sciencejournal.inquiro@gmail.com. For more information or to view previous publications visit the website at www.uab.edu/undergraduate-research

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