

Duquesne University

Annual Progress Report: 2012 Formula Grant

Reporting Period

July 1, 2014 – June 30, 2015

Formula Grant Overview

Duquesne University received \$100,224 in formula funds for the grant award period January 1, 2013 through December 31, 2015. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

Regulators of Biofilm Formation in the Pathogenic Fungus, Candida. albicans – The fungal pathogen, *Candida albicans*, is one of the most common hospital acquired infections worldwide. Critically ill patients, including those with various forms of cancer, are particularly susceptible to this infection. The pathogenicity of *C. albicans* is enhanced by its ability to grow on the surface of medical devices in the form of biofilms. Biofilms are complex networks of ovoid and filamentous cells that communicate with one another through secreted molecules. The purpose is to identify extracellular signaling molecules produced by *C. albicans* that regulate biofilm formation and thereby contribute to the pathogenicity of the organism.

Anticipated Duration of Project

1/1/2013 – 12/31/2015

Project Overview

Biofilms are complex networks of ovoid and filamentous cells that communicate with one another through secreted molecules, also known as quorum sensing molecules, to relay information about cell density and environmental conditions. To date, only a handful of those molecules have been identified, but it is widely believed that many others exist. Notably, this area of research is understudied, and no published examination of the extracellular metabolome of *C. albicans* biofilms has been reported. Our central hypothesis is that *C. albicans* produces as yet unidentified extracellular signaling molecules that regulate biofilm formation and thereby contribute to the pathogenicity of the organism.

We propose the following two specific aims to investigate our central hypothesis:

AIM 1 - Analyze the extracellular metabolites produced by *C. albicans* biofilms to identify potential regulatory molecules. We will employ a combination of mass spectrometry approaches

to first profile the extracellular metabolites and then to absolutely identify molecules of interest. We will compare the extracellular metabolites produced by planktonic cultures to those produced by biofilm cultures.

AIM 2 - Test candidate regulatory molecules for their role in biofilm formation. Metabolites identified under Specific Aim 1 will be tested for their ability to stimulate or inhibit biofilm formation using various methods, including confocal and scanning electron microscopy. In addition, reporter strains that allow for the determination of the relative amount of yeast versus hyphal cells will be employed as a way of identifying subtle changes in biofilm morphology.

Principal Investigator

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Other Participating Researchers

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Nina Zyvith- employed by Duquesne University- graduate student

Expected Research Outcomes and Benefits

The proposed project is a collaborative effort that utilizes the expertise of two investigators at Duquesne University to attack an important health-related problem. *C. albicans* biofilms are a major source of hospital acquired infection in the US, and patients undergoing cancer chemotherapy, as well as other immunosuppressive treatments, are especially at risk. We expect to identify and test an initial set of biofilm and/or planktonic culture specific metabolites for their role in inhibiting or promoting biofilm formation in the pathogenic fungus, *C. albicans*. By identifying the metabolites that are critical in the biofilm formation/ inhibition process we can then begin to explore the uses of the molecules from a biomedical perspective to stop biofilm formation on catheters, where infections caused by *C. albicans* are critical and potentially deadly for immunosuppressed patients. For example, inhibitory molecules identified here may be infused into new polymer blends used for catheters, thereby reducing infections rates.

Summary of Research Completed

Biofilms are the sources of many implant-related infections. *Candida albicans* are responsible for fungal biofilm infections in catheters and other indwelling devices. They communicate via quorum sensing molecules relaying information about cell density and environmental conditions. No published examination of the extracellular metabolome of *C. albicans* biofilms has been reported. The hypothesis is that *C. albicans* produces unidentified extracellular signaling

molecules that regulate biofilm formation and thereby contribute to the pathogenicity of the organism. We aim to identify regulatory molecules using mass spectrometry in the aims below. We have been working on this project since April 2013. The project has had several significant setbacks due to instrumentation that was down for approximately 1 year followed by training time on the new instrumentation. The data presented below was obtained on the new Agilent Quadrupole-Time of Flight Mass spectrometer (Q-ToF) with a Leman Instruments air generator. This mass spectrometer allows for identification and quantification of the molecules present and the air generator provides reference masses to correct for mass errors.

Aim 1

Analyze the extracellular metabolites produced by *C. albicans* biofilms to identify potential regulatory molecules. We will employ a combination of mass spectrometry approaches to first profile the extracellular metabolites and then to absolutely identify molecules of interest. We will compare the extracellular metabolites produced by planktonic cultures to those produced by biofilm cultures.

C. albicans growth

In order to identify the metabolites in planktonic and biofilm cultures, the *C. albicans* (strain WT-DAY185) were grown for 24 hour and 96 hours. The growth media for both types of bacteria consisted of 2% glucose, 0.5% ammonium sulfate, 75 μ M inositol, 80 μ g/mL uridine, yeast nitrogen base mix, trace salt mixture, vitamin mixture and an amino acid mixture.

Colonies of *C. albicans* were grown on yeast peptone dextrose agar. A colony was isolated and inoculated in growth media and allowed to grow overnight in an orbital shaker at 30°C. On Day 1, 4 mL of 100% bovine serum was added into each well of a 6 well plate and incubated for 24 hours at room temperature. Adding bovine serum deposits proteins onto the plastic surface required to initiate biofilm growth. On Day 2, the serum was removed from each well and each well was inoculated with 4 mL of the WT-DAY185 cells (optical density = 0.5 at 600 nm). The cells remained in the wells for 90 minutes at 37°C with orbital shaking at 35 rpm. After 90 minutes, the culture was removed without disturbing the adhered cells. The wells were washed once with phosphate buffered saline to remove any of the non-adhered cells. Another 4 mL of fresh media was added to each well and the well plate was incubated at 37°C with orbital shaking at 35 rpm. After 24 hours, the extracellular fractions were harvested by using a cell scraper to disrupt the biofilm and the contents of each well was placed into a centrifuge tube. Each tube was centrifuged at 3500 rpm for 5 minutes to obtain a pellet with the extracellular material in the supernatant. The supernatant was removed, filtered and stored at -80°C prior to analysis. For 96 hour biofilm growth, 6 mL of growth media was added after washing non-adherent cells to avoid culture drying.

Planktonic cells were grown in culture flasks under vigorous agitation. Each flask contained WT-DAY185 *C. albicans* cells diluted to an optical density of 0.5 (600 nm). The flasks were placed on an incubator/shaker at 37°C and 150 rpm and grown either 24 or 96 hours. The higher shaking speeds prevent cell attachment to the plastic surface. As with the biofilm cultures, the contents of the flask were removed and centrifuged at 3500 rpm for 5 minutes. The supernatant was collected and stored at -80°C until analysis.

Mass Spectrometric Analysis

Using the QToF with the direct infusion method, the samples were analyzed in positive mode using the following settings: Gas Temp: 365°C; Drying gas: 4 L/min; Capillary Voltage: 1900 V; Fragmentor: 175 V; Skimmer: 65 V; OCT 1: 750 V over a two minute sample collection. Samples from the *C. albicans* media at 24 and 96 hour of both planktonic and biofilm cultures were analyzed. The samples were prepared in filtered water with 0.1% Formic Acid with a 1:1000 dilution of media and filter sterilized with a 0.2 um filter. All spectra presented have a blank (filtered water, 0.1% formic acid) and growth media spectra subtracted.

The 24-hour biofilm culture and 24-hour planktonic culture are nearly identical except for the predominate m/z at 195.1007 in the 24-hr biofilm sample (Figure 1). Using MassBank, a high quality mass spectral database, the peak generated a molecular formula of C₉H₁₂N₃O₂. Interestingly, both samples contain the peak at 153.0826 m/z, which is present in both media samples despite the subtraction of the baseline media peaks. This indicates that this compound is more prevalent in both the planktonic and biofilm media. Currently, there are no positive matches for that peak in the MassBank database.

In sharp contrast, the 96-hour cultures differ greatly with only one predominate m/z peak being the same (321.018 m/z) (Figure 2). There are only four significant m/z peaks in the planktonic media (109.0285, 127.0390, 321.0189, 336.9920) while there are numerous peaks in the 96 hour sample with the largest four at m/z 158.9129, 175.0592, 321.0185, and 611.1964. The peak at 109.0285 m/z in the 96 hour planktonic media was identified to have a molecular formula of C₆H₄O₂, with the highest match i.e. largest number of references potentially being p-benzoquinone. The peak at 127.0390 m/z is from a compound with a molecular formula of C₆H₆O₃. Potential matches for this formula are hydroxymethyl furfural, hydroxyquinol, isomaltol, maltol, phloroglucinol, pyrogallol, triacetic acid lactone. These results are interesting as some of the already identified *C. albicans* growth controlling quorum sensing molecules are alcohols and lactones. Lastly, the peak present in both the 96 and 24-hour biofilm and 24-hour planktonic samples at 175.0591 m/z generated a molecular formula of C₅H₈N₃O₄, potentially N-amidino-L-aspartic acid. The true identity of these peaks would be determined by further mass spectral analysis (LC-MS) and comparison with the purported molecule. Additionally, Duquesne now has a license for Mass Profiler Professional which can be used to do PCA analysis on the media samples to compare the components across these samples.

The biofilm media appears to have more components than planktonic cultures, in general (Figure 3). Additionally, the 24-hour and 96-hour biofilms have some overlapping contents within the media (labeled in black; Figure 3 Bottom). Importantly, the 96-hour planktonic cultures appear to have the least amount of components, showing potential nutrient loss.

C. albicans Growth Inhibition

C. albicans cultures were grown in the collected planktonic and biofilm supernatants to test for biofilm growth inhibition. It was previously observed that using the supernatant alone resulted in complete growth inhibition, likely due to the lack of nutrients within the media. *C. albicans* were inoculated into fresh growth media and allowed to grow overnight at 30°C. The culture was then diluted to an optical density of 0.5 using the collected media in a 3:1 ratio of the supernatant of

interest and fresh growth media. The *C. albicans* biofilms were then grown as described in the above section. Planktonic cultures were grown in the well plates alongside the biofilm cultures, but no bovine serum was added to the wells to prevent cell attachment to the plastic.

C. albicans biofilms grown in 24 hour planktonic supernatants resulted in hyphal formation. The presence of hyphae indicate that *C. albicans* growth is not being inhibited and the culture is growing as a biofilm. Cultures grown without bovine serum on the well plate resulted in a yeast cell morphology, indicating growth inhibition. *C. albicans* biofilms grown in 96 hour planktonic supernatant resulted in small yeast cells, indicating growth inhibition. The same was observed for cultures grown without bovine serum. *C. albicans* biofilms grown in the 24 hour biofilm supernatants resulted in yeast cell formation with some pseudohyphae. These pseudohyphae are precursors to hyphal cell development. In the absence of bovine serum, cells grew as uninhibited yeast cells. Results for biofilms grown using 96 hour biofilm supernatants were the same as biofilms grown in 24 hour supernatants. Among the supernatants tested, 96 hour planktonic supernatants resulted in the least amount of biofilm growth where *C. albicans* cells remained in their initial yeast cell state (Data not shown). Examining the supernatant from 96 hour planktonic cultures using mass spectrometry therefore may give information regarding molecules involved *C. albicans* growth inhibition.

Aim 2

Test candidate regulatory molecules for their role in biofilm formation. Metabolites identified under Specific Aim 1 will be tested for their ability to stimulate or inhibit biofilm formation using various methods, including confocal and scanning electron microscopy. In addition, reporter strains that allow for the determination of the relative amount of yeast versus hyphal cells will be employed as a way of identifying subtle changes in biofilm morphology.

We have not made any progress on this aim since we have not identified any new molecules.

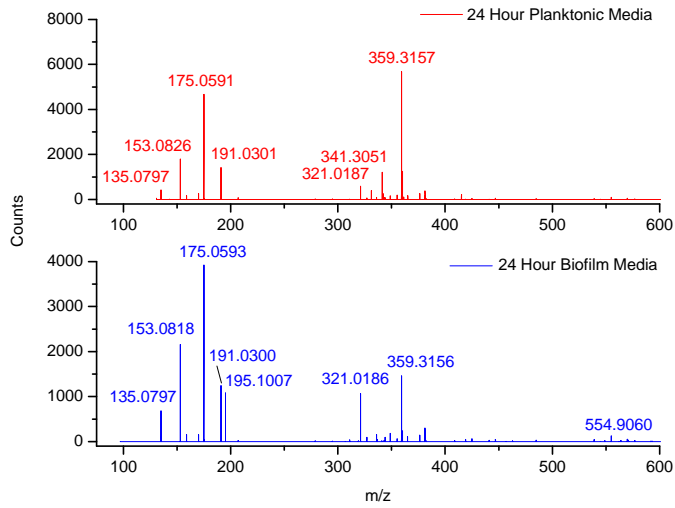


Figure 1. Mass spectrum using QToF direct infusion Top: 24 hour planktonic media. Bottom: Media from 24 hour biofilm media. Spectrum of water, formic acid and control media were subtracted.

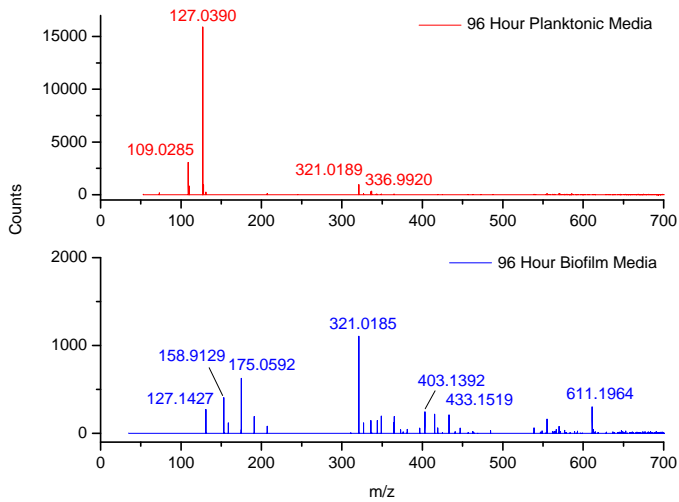


Figure 2. Mass spectrum using QToF direct infusion Top: 96 hour planktonic media. Bottom: Media from 96 hour biofilm media. Spectrum of water, formic acid and control media were subtracted.

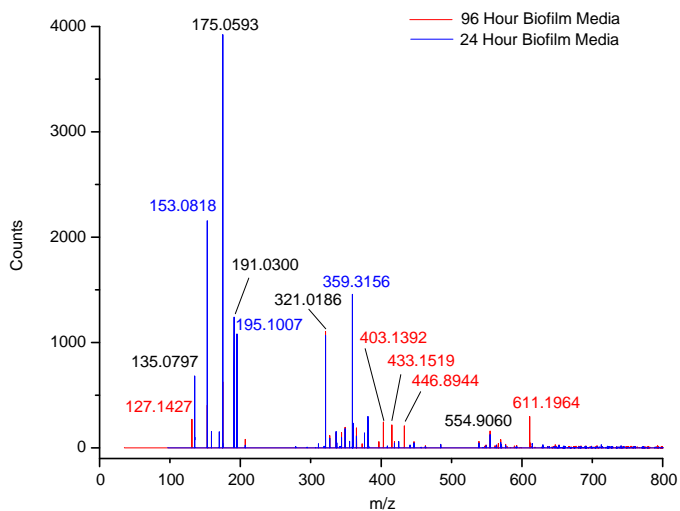
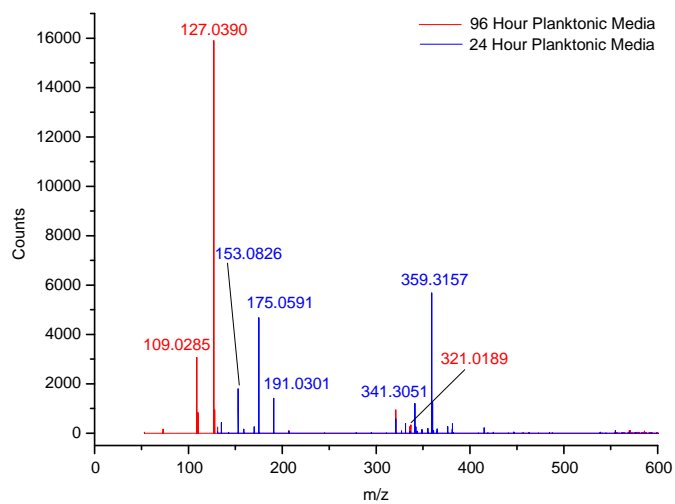


Figure 3. Mass spectrum using QToF direct infusion Top: 24 and 96 hour planktonic media overlay. There are no common peaks and the 96 hour spectrum has only three major peaks Bottom: Media from 24 hour planktonic and biofilm media overlay. The four overlapping peaks are indicated in black. Spectrum of water, formic acid and control media were subtracted.

Research Project 2: Project Title and Purpose

Impact of N-Acetyl Cysteine on Heat Shock Protein 70 – The goal of our study is to understand the mechanism of action of N-acetyl cysteine (NAC). NAC has long been used to raise the critical antioxidant glutathione. However, our recent data suggest that it also increases heat shock protein 70, a chaperone that helps reduce the cellular burden of misfolded proteins. This may better explain why NAC has benefited patients with Alzheimer’s disease in clinical trials, because Alzheimer’s disease is primarily a disorder of protein misfolding. If we can show that NAC protects cells by raising heat shock protein 70, this would yield novel insight into its efficacy. Such a finding would usher in a new field of research on the impact of NAC on the heat shock family of proteins, a set of protective chaperone proteins.

Duration of Project

1/1/2013 – 12/31/2014

Project Overview

The overarching goal is to understand why N-acetyl cysteine (NAC) protects against animal models of neurodegeneration and improves cognitive function in Alzheimer’s patients. Although NAC is well known to be a glutathione precursor, recent data collected in the Leak laboratory show that NAC also raises the folding chaperone heat shock protein 70 (Hsp70). Furthermore, pilot data suggest that inhibition of Hsp70 activity prevents the protective action of NAC. These novel findings reveal an unanticipated level of complexity to this inexpensive over-the-counter dietary supplement and warrant deeper mechanistic investigations. We propose two Specific Aims to test the main hypothesis that *NAC protects cells through a rise in Hsp70*. Aim 1: We will test the hypotheses that an adenosine triphosphatase (ATPase) inhibitor of Hsp70 (MAL3-101) and knockdown of Hsp70 messenger Ribonucleic Acid (mRNA) levels with Ribonucleic Acid (RNA) interference will both result in loss of the protective effect of NAC against MG132 toxicity in neuronal N2a cells and primary astrocytes. In order to elicit degeneration, we apply the toxic proteasome inhibitor MG132 to neurons and glia, the two basic cell types in the brain. MG132 raises the levels of misfolded proteins by inhibiting misfolded protein degradation. If our findings generalize across two cell types, this will support the robustness of the effect. Furthermore, using two different means of inhibiting Hsp70 will reduce the likelihood that the results are from non-specific inhibition of other proteins and will help solidify the role of this chaperone. Other heat shock proteins (Hsp90, Hsp40, and Hsp25) will also be measured to test the hypothesis that NAC raises multiple members of this protein family. Aim 2: We will test the hypothesis that NAC raises Hsp70 levels in the brain of animals *in vivo* and that it protects neuronal and glial cells from infusions of MG132 into the rat hippocampus. The hippocampus is involved in learning and memory and is severely affected in Alzheimer’s disease. Aim 2 will accomplish 4 goals. First, it examines the impact of NAC in live animals and corroborates our *in vitro* Aim 1. Second, the impact of NAC on Hsp70 can be simultaneously examined in multiple cell types (neurons and glia) and various tissues (brain, liver, and blood) to reveal how well the findings can be generalized. Third, other heat shock proteins will also be assessed to examine the impact of NAC on the heat shock protein family *in vivo*. Fourth, a model of hippocampal loss in

Alzheimer’s will be developed for general use by the Leak laboratory by infusing MG132 into this vulnerable brain region.

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Other Participating Researchers

Paid researchers: Amanda M. Titler, BS; Jessica M. Posimo, BS – employed by Duquesne University

In addition, the following students have contributed to this project:

Last Name, First Name	Position Title
Jiang, Yiran	Pharm.D. student
Unnithan, Ajay	Undergraduate student
Choi, Hailey	PharmD student
Pulugulla, Sree	Master’s student
Pant, Deepti	Undergraduate student
Crum, Tyler	Graduate student
Mason, Daniel	Graduate student
Broeren, Matthew	PharmD student
Heinemann, Scott	PharmD student
Weiland, Nicole	PharmD student
Negin Nouraei	Graduate student
Weilnau, Justin	Graduate student
Jenn Rumble	Undergraduate student

Expected Research Outcomes and Benefits

This research is expected to shed light on the underlying mechanism of action of N-acetyl cysteine (NAC). NAC benefits Alzheimer’s patients’ cognitive function and is currently in clinical trials for Parkinson’s disease. This grant is an example of “bedside-to-bench” medicine, where drugs known to be useful in the clinic are reexamined in experimental models to better understand how they function. This reverse approach helps to 1) shed light into disease etiology, 2) identify novel therapeutic targets, and 3) design even better drugs with fewer side effects. This approach has already been employed successfully in psychiatry for many decades and it is time it is leveraged against the devastating diseases associated with the aging population of America. The present study hopes to identify additional, novel targets of NAC other than its well-known

impact on glutathione. Our pilot data suggest that heat shock protein 70 (Hsp70) is one such promising target. Although heat shock proteins are protective in animal models of disease, whether Hsp70 protects patients against degeneration is not known. Thus, our long-term goal is to help focus clinical research on this protein. For example, blood levels of Hsp70 before and after an oral NAC regimen could be measured to see if Hsp70 levels correlate with improved human cognition. Furthermore, we hope to examine the breadth of NAC's actions on the entire heat shock protein family. Hsp25, Hsp90, and Hsp40 will be measured in the present study to begin this process. All these studies will benefit patients because they help to identify additional therapeutic targets of a compound already known to be protective. Finally, the studies may directly improve health status by encouraging the use of this inexpensive dietary supplement by those at high risk for disease, such as the elderly with a family history of neurodegeneration.

Summary of Research Completed

Aim 1: test the hypotheses that an adenosine triphosphatase (ATPase) inhibitor of Hsp70 (MAL3-101) and knockdown of Hsp70 messenger Ribonucleic Acid (mRNA) levels with Ribonucleic Acid (RNA) interference will both result in loss of the protective effect of NAC against MG132 toxicity in neuronal N2a cells and primary astrocytes.

We have performed additional experiments on NAC in primary cortical astrocytes and primary olfactory bulb and cortical neurons. The experiments support the hypothesis that NAC is protective against proteotoxic stress and that this effect is mediated by heat shock protein 70 (Hsp70) in a glutathione-independent manner. We have verified the effects of NAC using multiple assays of glutathione and multiple inhibitors of Hsp70 to better support our interpretations. Furthermore, in order to rigorously verify that Hsp70 protects against proteotoxic stress, we have used inhibitors and/or activators of this molecule in two recent publications (see below). Note that the primary olfactory bulb and cortical neuron models replace the old neuroblastoma N2a model because the latter cells are genetically transformed, undifferentiated, and not truly neuronal. The olfactory bulb model was developed in the Leak lab because the olfactory bulb is highly vulnerable to both Parkinson's disease and Alzheimer's disease, leading to an early loss of smell in both conditions. The cortical neuron model is a standard neuronal model for research in Parkinson's and Alzheimer's disease because pathology in this brain region is linked with cognitive dysfunction in both disorders.

The *in vitro* experiments described below were performed on at least three occasions. All cell counts were performed by a blinded investigator. The *in vivo* experiments described below were performed on at least 3 mice per group. All statistical analyses were performed by an ANOVA followed by the Bonferroni *post hoc* correction when there were multiple groups and by a two-tailed *t* test when there were only two groups. Data were deemed significant when $p \leq 0.05$.

Our injury model involves proteotoxic stress from the proteasome inhibitor MG132, which increases the burden of misfolded proteins and elicits cell death. Previously, we found that NAC attenuated the MG132-induced increase in ubiquitinated proteins in primary cortical astrocytes, suggesting that NAC permits more effective degradation of misfolded proteins through the proteasome. Thus, we subsequently examined whether NAC increased the levels of proteasome subunits to account for increased degradation of misfolded proteins by this system. We found

that levels of the PA28a subunit were decreased by MG132 in primary cortical astrocytes but NAC did not alter this response ($*p \leq 0.05$ versus 0 μM MG132, Figure 1A). Furthermore, no significant changes were observed in the 42 kDa or the 46 kDa PA700 subunit (Figure 1B, C). These results show that NAC does not modulate proteasomal subunits in primary astrocytes.

Previously, we examined several heat shock proteins other than Hsp70, in order to test the hypothesis that NAC increases multiple heat shock proteins in stressed cells. We continued those studies by examining the impact of NAC and MG132 on the phosphorylated form of Hsp25, a heat shock protein that battles apoptosis. MG132 increased phosphorylation of Hsp25 in primary cortical astrocytes (Figure 1D, E, $*p \leq 0.05$ versus 0 μM MG132), but this effect was reduced in NAC-treated cells ($+p \leq 0.05$ versus 0 mM NAC).

Previously, we showed that NAC does not raise glutathione levels in MG132-treated cortical astrocytes by infrared fluorescent In-Cell Western analyses. In order to verify those data with an independent assay, we performed the luminescent Glutathione-Glo assay (Promega) in primary cortical astrocytes (Figure 1F). The luminescence data agreed with the previous fluorescent assay in that 1) MG132 raised glutathione levels ($***p \leq 0.001$ versus 0 μM MG132), 2) NAC reduced the MG132-induced increase in glutathione ($+p \leq 0.05$, $+++p \leq 0.001$ versus 0 mM NAC), and 3) buthionine sulfoximine (BSO) decreased glutathione levels ($^{\wedge}p \leq 0.01$, $^{\wedge\wedge}p \leq 0.001$ versus 0 μM BSO). The effect of buthionine sulfoximine was greater in the luminescent assay, probably because only reduced glutathione levels are measured. In contrast to the luminescent assay, the In-Cell Western fluorescent assay measures total glutathione levels, which include both reduced and oxidized forms. Taken together, these findings continue to support the hypothesis that NAC can protect astrocytes independent of glutathione because we have shown previously that buthionine sulfoximine does not abolish NAC-mediated protection despite loss of glutathione.

Previously, we showed that two independent Hsp70 inhibitors, VER155008 and PES, both abolish the protective effects of NAC in primary cortical astrocytes. Because all pharmacological inhibitors suffer from non-specific side effects, we used a third inhibitor of Hsp70 (MAL3-101) to further validate the VER155008 and PES findings (Figure 1G). MG132 elicited significant loss of Hoechst-stained astrocyte nuclei ($*p \leq 0.05$ versus 0 μM MG132) and NAC significantly prevented this effect ($+p \leq 0.05$ versus 0 mM NAC). In the presence of MAL3-101, the protective effects of NAC were abolished as expected ($^{\wedge}p \leq 0.05$ versus 0 μM MAL3-101). Thus, data with three Hsp70 inhibitors with independent modes of action strongly support the hypothesis that Hsp70 mediates the protection afforded by NAC.

If NAC protects through the Hsp70 protein, it follows that there should be examples in which inhibitors of Hsp70 are toxic and activators of Hsp70 are protective. In order to verify the role of Hsp70, we examined primary olfactory bulb and primary cortical neurons. The choice of these models is described above. As expected, loss of Hsp70 activity with two independent inhibitors synergistically exacerbated the toxicity of proteasome inhibitors, demonstrating that Hsp70 normally protects primary neurons from proteotoxic stress. In Figure 1H-M and Figure 2A-E, we have used two independent proteasome inhibitors with independent mechanisms of action (MG132 and lactacystin), two independent Hsp70 inhibitors (VER155008 and MAL3-101), and three independent, unbiased methods of measuring viability (DRAQ5-stained nuclei, the MAP2

neuron marker, and ATP levels) in primary olfactory bulb neuron cultures to convincingly show that Hsp70 inhibitors act in concert with proteasome inhibitors to synergistically increase proteotoxic cell death (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ versus 0 μM lactacystin or MG132; + $p \leq 0.05$, ++ $p \leq 0.01$, +++ $p \leq 0.001$ versus 0 μM VER155008 or MAL3-101). In other words, loss of Hsp70 protein refolding activity potentiated the toxicity of the proteasome inhibitors. MG132 was more effective than lactacystin at revealing this phenomenon. Furthermore, we demonstrated that an inhibitor of heme oxygenase 1, tin protoporphyrin (SnPPx), did not have a similar effect on lactacystin or MG132 toxicity (Figure 1H-M), thereby acting as a negative control. [We have shown representative images of the DRAQ5 and MAP2 assays in Figure 2D-E. No images are shown of the ATP assay as it is not image-based]. Heme oxygenase 1 is a heat shock protein but plays no role in protein folding. It serves as part of the cellular antioxidant defense system.

Western blotting data confirmed that proteasome inhibitors elicited an increase in Hsp70 protein levels in this model (not shown here, shown in final progress report). These findings reveal that primary olfactory bulb neurons increase Hsp70 levels as a self-protective measure in response to proteotoxic stress and when this defense is inhibited, toxicity is greatly enhanced. In order to verify that the olfactory bulb results were generalizable to other neuronal systems, we have performed similar experiments with Hsp70 inhibitors in neo- and allocortical primary neurons (Figure 2F-I). MG132-treated primary allocortical neurons were more vulnerable to Hsp70 activity loss with VER155008 and MAL3-101 than primary neocortical neurons (+ $p \leq 0.05$, ++ $p \leq 0.01$, +++ $p \leq 0.001$ versus 0 μM MG132; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ versus neocortex; ~ $p \leq 0.05$, ~~ $p \leq 0.01$, ~~~ $p \leq 0.001$ versus 0 μM VER155008 or MAL3-101). In addition, Hsp70 activation with the compound 115-7c robustly protected neocortical but not allocortical neurons against MG132 (data published and included in final progress report, not shown here). Consistent with these findings, Western blotting analyses revealed that allocortex exhibited higher Hsp70 protein levels when treated with MG132, supporting the view that allocortex needs to rely more on endogenous Hsp70 defenses than neocortex (data in final progress report). We interpret these findings to suggest that allocortical defenses are more easily compromised by proteotoxic stress and loss of Hsp70 protein folding activity, entirely consistent with the greater development of Parkinson's and Alzheimer's pathology in the human allocortex. These studies support the notion that compounds that increase Hsp70 activity safely will protect neurons and astrocytes from protein misfolding stress.

Aim 2: test the hypothesis that NAC raises Hsp70 levels in the brain of animals in vivo and that it protects neuronal and glial cells from infusions of MG132 into the rat hippocampus.

We are still in the process of setting up an *in vivo* model of neurodegeneration. As outlined previously, we experienced issues with MG132 dissolution in saline. Vehicles such as dimethyl sulfoxide are commonly used for MG132 but are toxic to the brain *in vivo*. The proteasome inhibitor lactacystin, however, dissolves readily in phosphate-buffered saline, which is non-toxic. We therefore began studies with this compound, in addition to the 6-hydroxydopamine *in vivo* studies outlined in the prior report. Lactacystin infusions into the CA1 sector of the mouse hippocampus elicited loss of the neuronal marker NeuN in the ipsilateral hemisphere in a dose-responsive manner (Figure 2J, *** $p \leq 0.001$ versus 0 μg lactacystin, +++ $p \leq 0.001$ versus contralateral hemisphere). As high doses of lactacystin lead to severe loss of NeuN signal, as visualized in Figure 2J, we have been working with lower doses of lactacystin. Furthermore, we

will have to count individual NeuN⁺ neurons by higher resolution microscopy in order to verify that the loss of hippocampal NeuN signal reflects true loss of cell numbers. Once we have set up this model, we will be able to test the hypothesis that NAC protects hippocampal neurons against proteotoxic stress from lactacystin *in vivo*.

In our last progress report, we described the impact of NAC on tyrosine hydroxylase levels in the 6-hydroxydopamine (6-OHDA) model of Parkinson's disease. As tyrosine hydroxylase can be modulated by stress, this marker suffers from the caveat that loss of tyrosine hydroxylase does not necessarily reflect loss of dopaminergic neurons. In order to label dopaminergic neurons without this confound, we have been developing fluorescent tract-tracing of the dopaminergic nigrostriatal pathway with the retrograde tracer FluoroGold (see injections in striatum and retrogradely labeled dopamine neurons in Figure 2K; cc=corpus callosum, SNpc=substantia nigra, pars compacta, SNpr=substantia nigra, pars reticulata). FluoroGold is not modulated by stress because it is not an endogenous protein and counts of FluoroGold⁺ neurons should be in proportion to dopaminergic cells whether or not they are stressed. We have injected FluoroGold into animals treated with 6-OHDA and NAC and quantified the data, but are not including those findings here because they were collected after the project end date of December 2014.

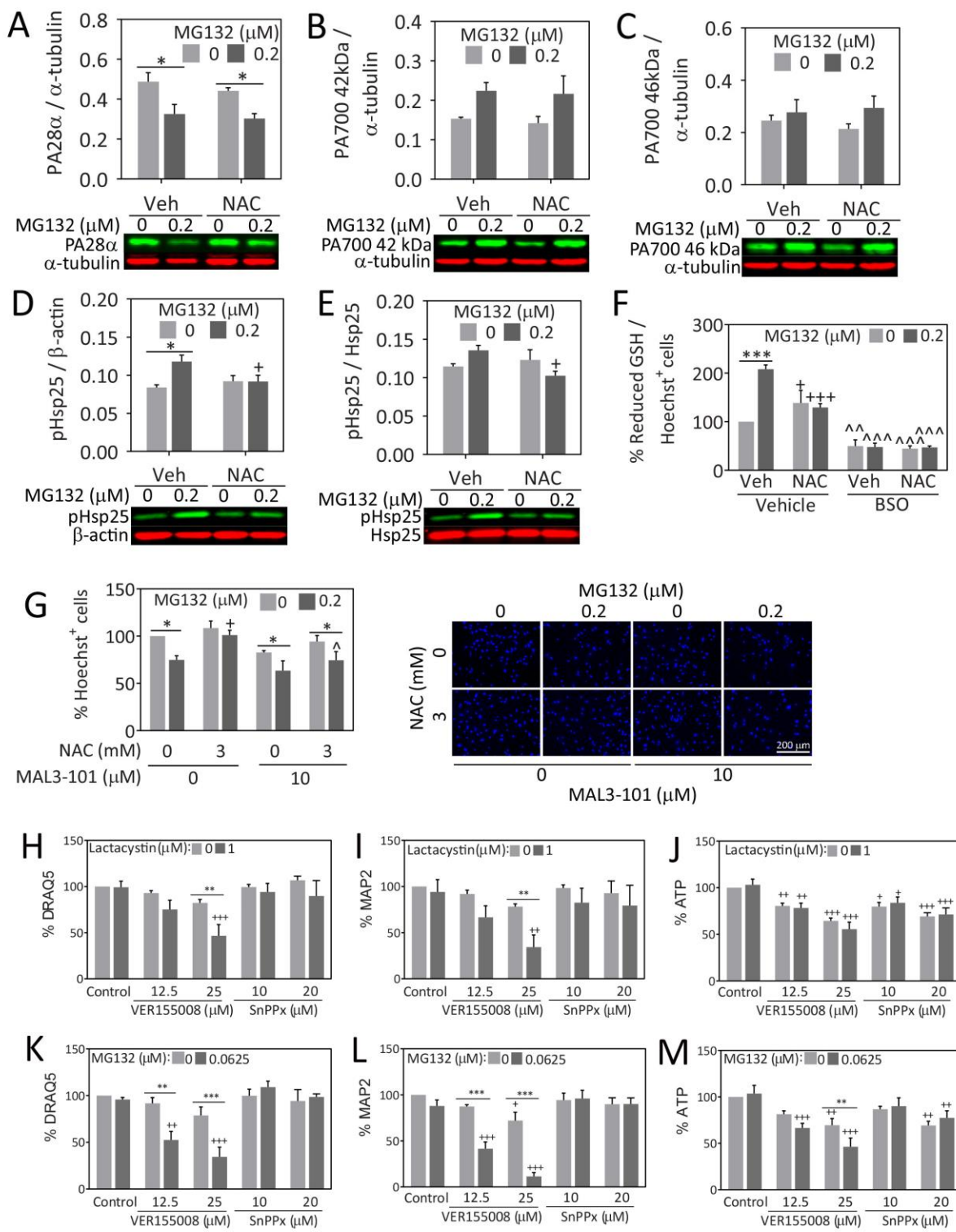


Figure 1

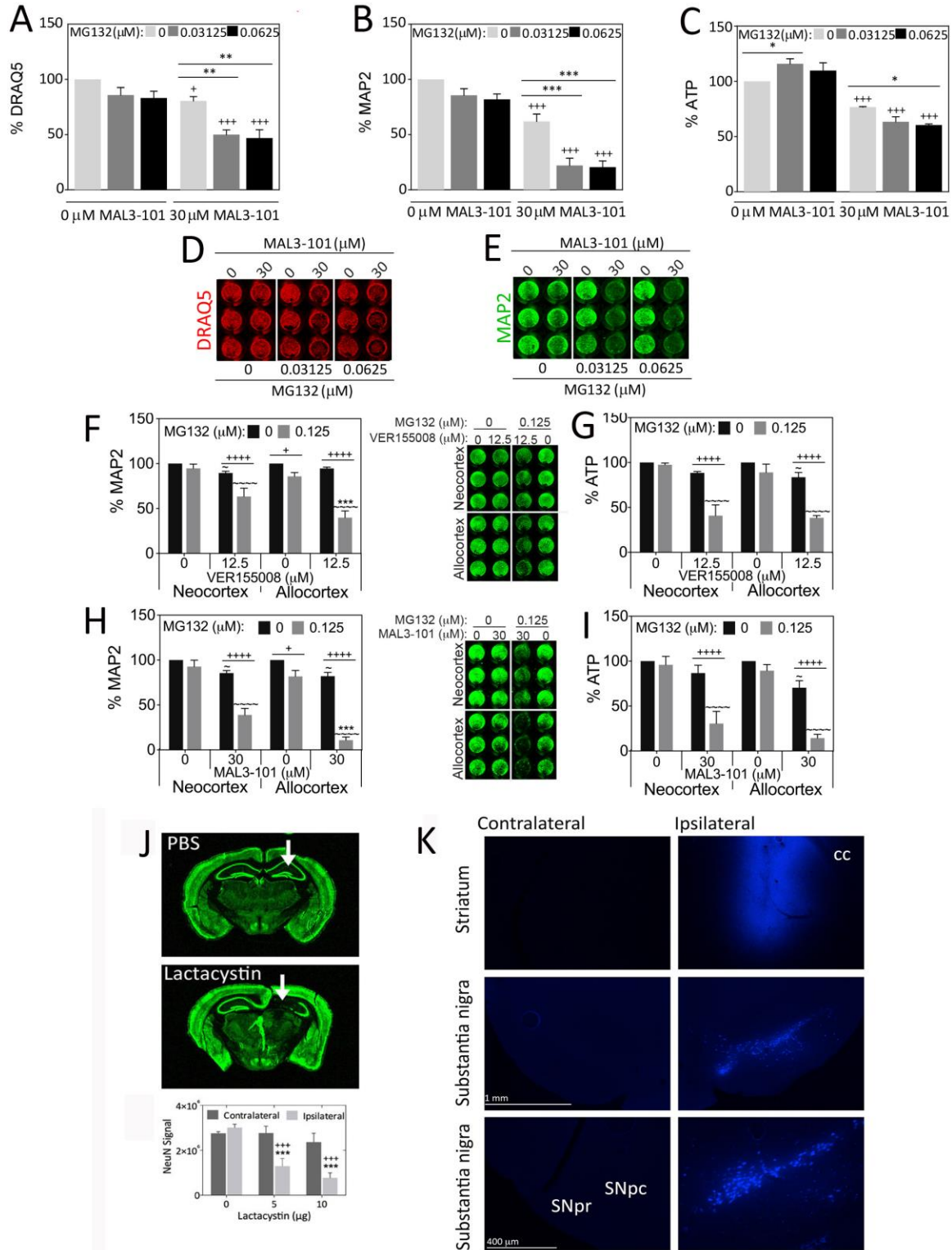


Figure 2