

## Prototype Summer Research Fellowship

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### **A. Objectives:**

The discovery that Histatin 5 (Hst 5) induces ATP release from *C. albicans* via a non-lytic, channel-like mechanism provides new insight into the potential mechanism of yeast killing and further suggests that extracellular ATP may activate membrane receptors to cause cell death. As a first initial step to identifying the genetic homologs in *C. albicans*, we propose experiments to clone, sequence, and characterize the P2X<sub>7</sub> receptor of *Saccharomyces cerevisiae*.

### **Hypothesis:**

Recent observations from this laboratory have shown that Hst 5 induces a non-lytic release of ATP from *C. albicans* via a channel-like mechanism that is related to killing [1]. Based on evidence that transmembrane ATP efflux can occur in the absence of cytolysis through a channel-like pathway and that released ATP can act as a cytotoxic mediator by binding to purinergic receptors, we evaluated whether extracellular ATP released by Hst 5 may have a further functional role in cell killing. Consistent with this hypothesis, treatment of *C. albicans* with purinergic agonists ATP or analogues BzATP and ATP- $\gamma$ -S resulted in loss of cell viability, while purinergic antagonists prevented Hst 5 induced cell killing. Currently, we do not know whether ATP efflux from *C. albicans* in response to Hst 5 is associated with presence of ABC proteins. However, ABC transporters which carry out transport of a wide range of substrates or function as efflux pumps have been identified in *S. cerevisiae* and resistance to azole antifungal agents in *C. albicans* is mediated by the ABC transporters CDR1

and CDR2 [2]. Although P2 purinergic receptors have not been described in yeast, a regulatory function of released nucleotides may not be unprecedented. cAMP is exported by yeast to regulate intracellular cAMP concentrations, and cell surface cAMP-receptors have been identified [3].

Our findings provide support for the concept that extracellular ATP plays a role in cell killing and raise the possibility of a novel mechanism for Hst 5 cytotoxic action that include (i) killing of *C. albicans* is initiated with release of cellular ATP; (ii) ATP release is via non-lytic, perhaps, conductive pathway; and, (iii) extracellular ATP, released in response to Hst 5, may initiate cellular necrosis or activate purinergic-like receptors on *C. albicans* to ultimately induce cell death.

Characterization of many other important aspects of Hst 5 cytotoxic action will require further study. It is currently unknown whether Hst 5 induced efflux of cellular ATP alone is sufficient to trigger cell death. Further, it is yet unanswered how ATP is released from the cells and whether ATP binding cassette proteins are involved in conductive transport of ATP. The mechanism described here for Hst 5-induced yeast killing has not been evaluated for other antifungal agents and antimicrobial proteins. Consequently, it will be important to determine whether it represents a common antifungal mechanism or it is unique to Hst 5.

**Specific Aims:**

This work we will focus on cloning the P2X<sub>7</sub> homolog in *Saccharomyces*. While it would be ideal to work with *Candida albicans* itself, we feel that *Saccharomyces cerevisiae* offers advantages over working with *Candida* in that (i) it possesses a well defined genetic system; and, (ii) the *S. cerevisiae* genome has been sequenced in full and is easily accessible

through the internet. This latter point will be invaluable in allowing us to conduct database searches with DNA sequences from clones isolated.

## **B. Background and Significance:**

*Candida albicans* is one of the most common human fungal pathogens with the potential to cause severe mucosal and systemic infections in the immunocompromised patient [4, 5]. Incidence of oropharyngeal candidiasis has increased dramatically in the past 20 years due to increased antibiotic and pharmaceutical drug use and longer survival of people with compromised immune systems. While a number of antifungal agents (amphotericin B and azole-based drugs) are available to treat infections caused by this pathogen, the high toxicity associated with anti-fungal drugs coupled with the emergence of antifungal resistant strains has left the clinician with fewer treatment options. A naturally occurring peptide in saliva, Histatin (Hst) has shown to be cytotoxic to *Candida albicans*. These 3-4 kDa salivary peptides are structurally related to the histidine-rich basic salivary proteins and are unique to primate species [6]. Hst 3 (32 amino acids) and Hst 5 (the N-terminal amino acids of Hst 3) are the most potent candidicidal forms in vitro, killing candida species at concentrations found in saliva (15-30  $\mu$ M) [7]. Salivary histatins have promising potential as therapeutic agents for the treatment of oral candidiasis given their potent candidicidal nature and inherent non-toxicity in humans [1]. Understanding mechanisms by which salivary Hsts exert candidicidal activity is the primary focus of this laboratory.

Previous studies using radiolabeled Hst 5 detected a class of functional binding sites on *C. albicans* cells and the presence of a 67 kDa yeast Hst binding protein (HstBP), which may be the basis for the selectivity of Hst 5-yeast killing and lack of toxicity to human host cells. Although, the exact role of HstBP in Hst 5-induced yeast killing is unknown, its location

suggests that it may function as a surface receptor or mediate the entry of Hst 5 into the cell. Hst 5 binding, while required for killing, is only the initial interaction of a multistep mechanism, consistent with the recent finding that internalization of Hst 5 correlated with killing [8]. Work by Koshlukova et al. [1] attempted to identify a specific intracellular target for Hst 5 by testing pharmacological agents for effects on Hst 5 candidacidal activity and found that the uncouplers carbonyl cyanide m-chlorophenyl-hydrazone (CCCP), dinitrophenol (DNP), and azide inhibited Hst 5-induced killing of *C. albicans*. In examining potential alterations in the intracellular ATP levels as a result of the uncoupler treatment, it was found that DNP, CCCP and azide had little or no effect on cellular ATP production. In contrast, *C. albicans* exposed for 10 min to Hst 5 had a drastic reduction of intracellular ATP content which was a result of efflux of cellular ATP. CCCP, DNP, and azide at concentrations that inhibited Hst 5 killing of *C. albicans* also inhibited Hst 5 induced ATP efflux, thus establishing a correlation between ATP release and killing. Cell lysis is one scenario to account for this ATP release from cells. However, *C. albicans* cells were actively respiring and had polarized membranes at the time of ATP release and 80 min following treatment, implying a non-lytic export of ATP from the cells in response to Hst 5.

Intracellular ATP plays a fundamental role in energy metabolism, nucleic acid synthesis, protein modification and enzyme regulation. Concentration of ATP in the cells is an indicator of viability, and gradual depletion of cellular ATP can induce structural and functional alterations leading to inhibition of macromolecule synthesis and cell death via necrosis or apoptosis [9]. Therefore, Hst 5-induced massive loss of intracellular ATP may in turn cause a series of biochemical alterations that result cells unable to replicate or initiation of processes leading to later loss of cellular structure. Alternatively, extracellular ATP released from *C.*

*albicans* in response to Hst 5 may have a further physiological role in cell death. In higher eukaryotic cells, ATP released non-lytically through specific membrane channels can function by activating membrane purinergic receptors to cause increased  $K^+$  and  $Cl^-$  permeability and even cell lysis [10, 11].

Although the intracellular role of ATP has been recognized for many years, under appropriate conditions purine nucleotides and nucleosides can be selectively released from cells where they interact with specific surface receptors (purinoreceptors; P1 receptors recognize adenosine; P2 are activated by ATP) [12]. Release of cytoplasmic ATP as a physiological regulatory mechanism is only recently beginning to be understood. A range of biological effects include platelet aggregation, neurotransmission, vascular tone and cardiac function or even cytotoxicity [13].

Many cell types are killed by sustained exposure to high concentrations of extracellular ATP and the plasma membrane ATP receptors (P2X) involved have been intensively studied during the past decade. P2X<sub>1-7</sub> receptors are ATP gated channels and share structural motifs with other ion channels such as degenerins (known to be involved in cell death of *C. elegans*) and amiloride-sensitive Na-channels. Depending on the ATP dose, length of stimulation and receptor subtype, P2X receptor stimulation may cause necrosis or apoptosis. The cytotoxic P2Z/P2X<sub>7</sub> receptor has dual functions as an ion channel and ATP-gated pore. Stimulation of cells expressing these receptors with ATP or ATP analogues leads to pore opening and eventually lysis [14]. The physiological meaning of P2X mediated cytotoxicity is not understood, but involvement in death of aging and damaged cells or immune-mediated reactions is postulated [15].

**Significance:** Histatins have great potential as therapeutic agents against oral candidiasis, being potent antifungal molecules with no associated toxicity in humans. Their efficacy against azole resistant *Candida* species makes them potentially significant agents in treatment of patients with disease caused by resistant strains. An understanding of the mechanism of Hst-induced killing and the nature of its specificity for yeast cells over human cells is critical in development of Hsts as fungicidal drugs.

**C. Preliminary Studies – None.**

**D. Methods and Materials:**

*Preliminary screening of Saccharomyces cerevisiae cDNA library*

Preliminary screening for the yeast P2X7 homolog will be carried out using an EcoRI 5' STRETCH cDNA isolated from *Saccharomyces cerevisiae* and cloned into  $\lambda$ gt11 genomic library (Clontech Laboratories, Inc., Palo Alto, CA). The host strain *Escherichia coli* Y1090r will be infected with the *S. cerevisiae*  $\lambda$ -phage library, plated onto Luria-Bertani agar, and incubated to produce approximately 1500 bacteriophage plaques per plate. Plaques will be transferred to nitrocellulose membranes (MSI, Westboro, MA) for subsequent antibody screening.

A commercially available rabbit anti-P2X7 polyclonal antibody, raised to a synthetic peptide corresponding to the C-terminal 20 amino acids of the murine P2X<sub>7</sub> receptor (Alomone Labs, Jerusalem, Israel), will be used to screen the *S. cerevisiae* cDNA library. Nitrocellulose plaque blots will be blocked using 1% BSA/tris-buffered saline/0.05% Tween-20 solution and then incubated with the anti- P2X<sub>7</sub> antibody at a concentration of 1:750 in a TBS for 1 hour followed by extensive washing with TBS/0.05% Tween-20. Detection of positive clones will be accomplished by incubation with a secondary antibody directed against the anti-P2X<sub>7</sub>

antibody (goat anti-rabbit horse radish peroxidase) and use of the ECL detection system (Amersham International PLC, Buckinghamshire, England). Positive plaques will be isolated and stored in 0.5-mL SM buffer/20  $\mu$ L chloroform at 4°C until used for PCR analysis.

*PCR amplification of inserts from antibody reactive clones*

Polymerase chain reaction (PCR) will be used to amplify *S. cerevisiae* insert DNA from the anti- P2X<sub>7</sub> reactive lambda plaques. PCR primers corresponding to the 5' and 3' flanking regions of the *Eco*RI cloning sites of the  $\lambda$ gt11 will be used (5' flanking primer, 5'-GACTCCTGGAGCCCG-3'; 3' flanking primer, 5'-GGTAGCGACCGGCGC-3'). The SuperTaq Plus PCR system (Ambion, Austin, TX) will be used following the manufacturers instructions with modifications to the temperature cycling reaction made as needed.

*Cloning and sequencing of amplified DNA*

DNA modifying enzymes will be obtained from MBI Fermentas, Inc. (Amherst, NY). DNA from PCR reactions will be viewed by agarose gel electrophoresis and ethidium bromide staining. Amplified DNA will be used either directly from reactions or agarose gel purified (QIAquick gel extraction kit; QiaGen, Valencia, CA) subsequent to insertion into the pT-Adv PCR cloning vector (Clontech Laboratories, Inc., Palo Alto, CA). This vector carries resistance genes to ampicillin and kanamycin for easy selection; the vector also contains 3' thymidine overhangs withing the multiple cloning site to facilitate rapid and easy cloning of PCR amplified DNAs, which normally have terminal adenine overhang residue added by the Taq polymerase. Ligations will be performed using T4 DNA ligase under standard enzymatic conditions. Ligations will be transformed into the host strain *Escherichia coli* TOP10F' (Clontech Laboratories, Inc., Palo Alto, CA) using a modified heat shock method [16]. Plasmid transformants will be selected on antibiotic containing medium and screened for loss

of  $\beta$ -galactosidase activity, indicating insertion of foreign DNA into the cloning site. Plasmid DNA from selected clones will be isolated using the QIAprep spin mini-prep kit (QiaGen, Valencia, CA). Enzyme restriction digests and agarose gel electrophoresis will be used to verify the size of the cloned PCR DNA inserts. Confirmed inserts will be submitted for DNA sequencing at the CAMBI facility at SUNY/Buffalo (Buffalo, NY).

#### *DNA sequence analysis*

DNA sequences obtained from cDNA library screening will be used to search the Saccharomyces Genome Database (<http://genome-www2.stanford.edu>) and the NCBI database (<http://www.ncbi.nlm.nih.gov/Genbank>). These sites are easily accessed using a web based server and the search queries will use the BLAST search algorithm of Altschul et al. [17].

#### **E. Statement on the Use of Recombinant DNA**

This project entails cloning of non-virulent *Saccharomyces cerevisiae* genes into standard *Escherichia coli* laboratory and, as such, is exempt from specific regulation as spelled out in Appendix C section II and Appendix G of *The NIH Guidelines for Research Involving Recombinant DNA*. Biosafety level 1 facilities will be used with close attention to standard laboratory hygienic practices and aseptic techniques [18].

#### **F. References**

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