

## **Project #1: Small Molecule and Ion Inhibitors of Human Hexokinase Type II (HKII)**

*S. Braun-Sand, A. Schoffstall, R. Henry, Chemistry; T. Wolkow, K. Newell, Biology*

Hypothesis: Cationic metals and small molecules based on the glucose scaffold will inhibit HKII

Hexokinase enzymes catalyze phosphorylation of glucose to glucose-6-phosphate (G6P), the first step in the anaerobic glycolysis pathway. This enzyme is an attractive drug target because some highly malignant, hypoxic tumors upregulate expression of many glycolytic genes, including HKII [6]. HKII inhibitors could reduce glycolytic activity of the malignant cells and possibly induce apoptosis. G6P and sodium ions are known inhibitors of HKII, but are not appropriate as therapeutics. We plan to investigate the mechanism and efficacy of inhibition of small molecules based on the glucose scaffold and other metal cations.

We have successfully expressed and isolated human HKII at UCCS and are examining its kinetic and thermodynamic behavior with glucose and G6P. Little work has been done to characterize the regulatory sites of HKII; we plan to do so by several methods. 1) Performing concentration studies to determine how many  $\text{Na}^+$  are needed to inactivate it. 2) Determine inhibition effects of variable charges and sizes using a series of metal ions, such as  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Fe}^{2+/3+}$ . 3) Examining inhibition effects using metals with specific coordination number and geometry to give insight into the size and orientation of sidechains in the regulatory sites. 4) Metal complexes will be synthesized that contain ligands with variable numbers of  $\text{OH}^-$  and/or  $\text{PO}_3^{2-}$  functional groups to mimic the effects of glucose/G6P inhibition on HKII.

As an alternative approach to characterizing the regulatory sites, we propose a structure-based method to identify inhibitors using fission yeast, a powerful molecular genetic model system. The fission yeast genome contains a gene (*hvk2<sup>+</sup>*) encoding hexokinase II. To our

knowledge, *hxx2<sup>+</sup>* has not been characterized. We propose to knock-out *hxx2<sup>+</sup>* and characterize the *hxx2Δ* mutant phenotype, which will likely exhibit defects with glucose metabolism. Next, we will test if human HKII functions in fission yeast by cloning the cDNA of human HKII into a fission yeast expression vector and testing if it complements the defects of the *hxx2Δ* yeast mutant. If complementation is successful, we can conclude that human HKII functions in fission yeast. We will use a mutagenic PCR strategy to create a library of randomly mutagenized human *HKII* alleles to identify if G6P has multiple binding sites. Alleles that encode proteins without G6P inhibition will be sequenced to find candidate G6P binding sites. Finally, *in vitro* binding assays will be employed to test if the mutant proteins are specifically defective for G6P binding. These analyses will identify the HKII residues that mediate inhibition by G6P, and will be used to model the 3-D structure of the allosteric site and design small molecules that bind it.

An additional project is currently underway to correlate the structures of glucose/hexokinase complexes, and G6P/hexokinase complexes with their binding affinities through 1) computational calculations and 2) experimental determination of the binding free energy using microcalorimetry. Preliminary computational studies have calculated the binding free energy of G6P and glucose to human hexokinase type I (HKI), which shares significant sequence identity with HKII [7]. Future work will calculate this quantity for other hexokinase isozymes with G6P and proposed inhibitors. Experimentally, microcalorimetry studies are being conducted to measure the thermodynamics of G6P binding to hexokinase isozymes, and future work will measure the binding free energy for proposed inhibitors. Hopefully the calorimetry will confirm that the computations accurately predict binding thermodynamics.

After *in vitro* inhibitors are identified, inhibition of HKII in a cell assay will be studied. With *in vitro* studies, the problem of inhibitor uptake by the cell is avoided, but lack of uptake can make a potent inhibitor virtually ineffective [8]. This makes the cell studies extremely valuable in identifying a potent and effective inhibitor. The cell studies will be done using mouse cell lines L1210 and mouse melanoma cell line B16.F1. An advantage of these cell lines is that they are very well characterized for sensitivity (or lack thereof, in the case of melanoma) to standard drug treatment regimes. These lines are particularly amenable for work by undergraduate researchers, and Dr. Newell's lab has established tissue culture and assay techniques to measure changes in metabolism and susceptibility to cell death. The effects of the newly developed hexokinase inhibitor(s) on these parameters will be assessed using flow cytometry, fluorescent microscopy, and metabolic assays detecting rates of glycolysis and respiration.

### **Project #2: Determination of Neurotransmitters for Studies of Cerebral Blood Flow**

*Associate Prof. David J. Weiss, Chemistry; Assistant Prof. Andrew Subudhi, Biology*

Hypothesis: Microchip electrophoresis with electrochemical detection and on-column sample stacking will allow for rapid, quantitative determinations of neurotransmitters in physiological solutions for studies of cerebral blood flow.

Regulation of cerebral blood flow is necessary to maintain adequate oxygen delivery to the brain. Under normal physiological conditions, regulation of cerebral blood flow is governed, at least in part, by autonomic responses of the central nervous system [9]. Impairment of the neurologic, autoregulatory response is a common factor in the etiology of several cerebrovascular disorders, such as stroke and cerebral edema [9]. Unfortunately, the physiological mechanisms governing cerebral autoregulation are poorly understood. To gain a

clearer understanding of the neurological mechanisms regulating cerebral blood flow, studies are needed which monitor specific neurotransmitter concentrations in arterial and venous blood samples. Research in this area is limited by the lack of simple quantitative methodology for determination of neurotransmitters. Currently only specialized laboratories can obtain a neurotransmitter profile via liquid chromatography (LC) with mass spectrometry (MS) [10]. LC-MS is expensive and slow, making research in this area very limited. A simple, rapid and inexpensive method of analysis for neurotransmitters in physiological solutions is needed.

A complementary method to LC-MS is capillary electrophoresis (CE). CE allows for rapid separation of charged compounds with much higher efficiency than LC. Recently, capillary electrophoretic separations have been transformed into the microchip format. Microchip electrophoresis separations are at the forefront of analytical chemistry due to their high throughput, fast analysis, low cost, and improved disposability and portability [11]. However, since the concentration of major catecholamines in human serum is below the lower detection limits (micromolar range) of this methodology, no quantification of these compounds has been possible in real samples. We propose to develop a method for analysis of the catecholamines serotonin, epinephrine, norepinephrine and dopamine in human serum using microchip electrophoresis with electrochemical detection. Using a Pd decoupler, an electrically decoupled system will be used to improve the detection limits by isolating the system from the high voltage power supply [12]. Second, to address problematic issues with high ionic strength samples, sample stacking by addition of acetonitrile will be used [13]. This method results in both improved peak efficiency and detection limits (up to 50-fold sensitivity enhancement in physiological solutions).