**Final Report – Chandra Tucker**
**Use of differential scanning fluorometry to identify ligands that stabilize AGT**

Introduction:

A number of patients with Primary Hyperoxaluria Type I contain mutations of alanine: glyoxylate aminotransferase (AGT) that destabilize, but do not completely inactivate, the enzyme. For these patients, pharmacological chaperones, small molecules that bind and stabilize AGT variants, would be of great benefit, as they may correct misfolding and mistrafficking of mutant forms of the protein.

Differential scanning fluorometry (DSF) is a method that can be used to monitor protein stability in a solution. The technique involves involves monitoring the sensitivity of a protein to heat denaturation via the use of an environmentally sensitive fluorescent dye. The technique requires minimal amounts of protein, and can be carried out in parallel in a 96-well qPCR machine. As a protein is exposed to increasing temperature, it undergoes a transition from a folded to unfolded conformation. By monitoring this change using a fluorescent dye, a melting temperature (Tm) for the protein can be observed.

Goals of Project:

The goals of this work were to develop optimal methodologies for using differential scanning fluorometry (also known as DSF or thermal shift assays) to monitor stability of AGT.

Results:

We optimized conditions for using differential scanning fluorometry with wild-type and mutant forms of AGT, using known ligands PLP and AOA. Conditions were developed where we could generate clear melting curves with major allele (AGTma) and minor allele (AGTmi) polymorphic AGT variants using SYPRO orange dye (Fig 1A). We could observe clear differences in the stabilities of AGTma and AGTmi from these studies. We also used DSF to study the stability of wild-type and mutant forms of AGT in the presence of ligands PLP and AOA (Fig 1B,C). PLP alone causes a significant shift in the melting temperature of AGT, and PLP with AOA shifts the melting temperature even further, indicating significant stabilization that is seen with both AGTma and AGTmi variants. Results of these studies are published in Hopper et al. (2008) JBC 283, 30493.

We used DSF to examine stability of a variety of different AGT mutants, including AGTmi-G170R, AGTmi-I244T, AGTmi-F152I, and AGTmi-R233C. These proteins had different stabilities compared with that of AGTmi, and were stabilized by addition of PLP and PLP/AOA.

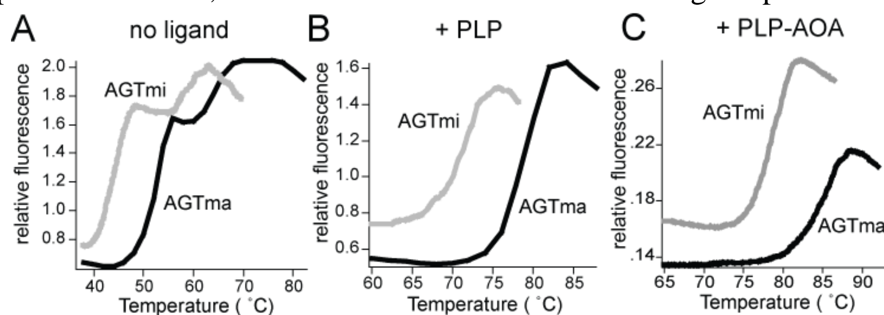


**Figure 1. Differential scanning fluorimetry of AGTma and AGTmi.** Fluorescence changes of 5 μM solutions of AGTma or AGTmi in the presence of SYPRO orange were analyzed A) in the absence of added ligand, (B) in the presence of 70 μM PLP, and (C) in the presence of 70 μM PLP and 200 μM AOA.

To test use of DSF in high-throughput screening for compounds that bind and stabilize AGT, we carried out experiments to test the multiplex capacity of this approach. Using PLP as a test compound, we pooled PLP with 9 other compounds, and screened for stability changes of AGTmi by DSF. Multiplexed pools containing PLP could easily be identified from control pools (Fig. 2A). Subsequently, we carried out a high-throughput screen of 6600 compounds from a Chembridge

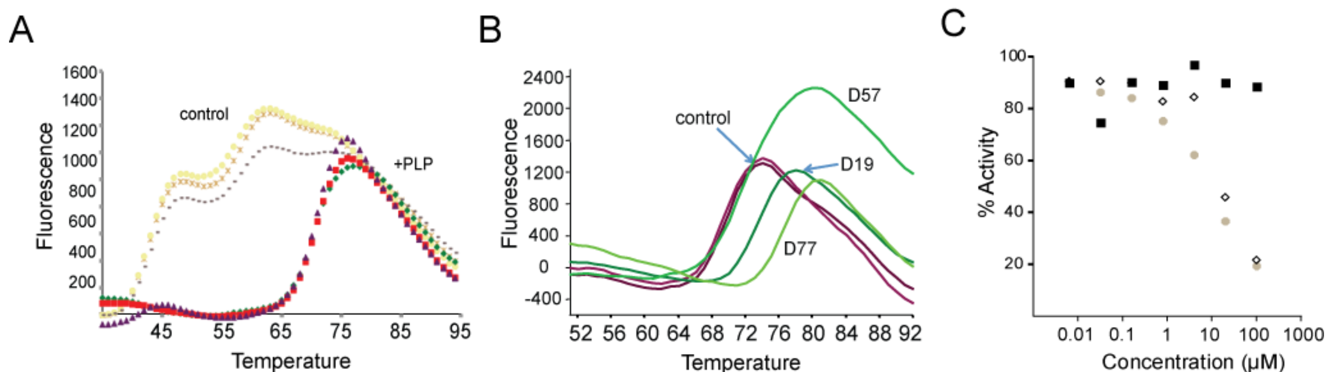


**Figure 2. High-throughput DSF screening for compounds that stabilize minor allele AGT.** (A) Test of multiplex screening parameters. Nine compounds plus a control or PLP were tested for ability to stabilize AGTmi by DSF using a qPCR machine. Samples containing PLP were easily identified by the stability shift obtained. (B) High-throughput, multiplex library screen of AGTmi in the presence of PLP. Shown are stability shifts resulting from three hits from the initial screen, after deconvoluting each pooled hit. (C) Compounds D19 (circles) and D77 (open diamonds) inhibit AGTmi activity with a Ki ~ 10 µM, while D57 (squares) has no inhibitory effect.

DiverSet chemical library, which we multiplexed in groups of 10 chemicals per pool. We obtained three hits from this initial screen (Fig. 2B), which were subjected to activity and toxicity assays. Two of these hits, D19 and D77, inhibited activity of AGT with a Ki of ~10 µM (Fig 2C). One of the compounds is toxic in yeast and mammalian cells, while the other does not appear to be toxic at the levels tested. We tested both compounds in a novel aggregation screen with AGTmi-G170R, a disease variant that is prone to aggregation in vivo and in vitro. Addition of micromolar amounts of both D19 and D77 significantly reduced aggregation of AGTmi-G170R in this cell-based aggregation assay, as did addition of a PLP control. While both compounds also inhibit AGT activity, they represent novel starting points for identifying chemical variants that might stabilize unstable AGT variants.