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Small-Molecule Activators of Glucose-6-phosephate Dehydrogenase (G6PD) Bridging the Dimer Interface

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Small-Molecule Activators of Glucose-6-phosephate Dehydrogenase (G6PD) Bridging the Dimer Interface

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Abstract: We have recently identified AG1, a small-molecule activator that functions by promoting oligomerization of glucose-6-phosphate dehydrogenase (G6PD) to the catalytically competent forms. Biochemical experiments indicate activation of G6PD by the original hit molecule (AG1) is noncovalent and that one C_2-symmetric region of the G6PD homodimer is important for ligand function. Consequently, the disulfide in AG1 is not required for activation of G6PD and a number of analogs were prepared without this reactive moiety. Our Study supports a mechanism of action whereby AG1 bridges the dimer interface at the structural nicotinamide adenine dinucleotide phosphate ($NADP^+$)-binding sites of two interacting G6PD monomers. Small molecules that promote G6PD oligomerization have the potential to provide a first-in-class treatment for G6PD deficiency. This general strategy could be applied to other enzyme deficiencies where control of oligomerization can enhance enzymatic activity and/or stability.

Protein-protein interactions (PPIs) underpin the vast signaling networks essential for cellular functions. The interactome, estimated at 650,000,[1] is an area ripe for modulating disease pathogenesis.[2] Beyond signaling events, the interactome includes quaternary structural interactions between proteins, which regulate enzyme stability, activity, and function.[3] However, targeting PPIs with small molecules has long been considered a challenging area for drug discovery largely outside the realm of the druggable genome.[4] Yet, a number of methods such as fragment-based drug discovery (FBDD) and rational peptide design have produced leads with translational potential.[5,6] Efforts focused on modulating PPIs have almost entirely centered around the inhibition of these interactions,[7] but a number of natural products and designed ligands derive clinical utility from promoting the interaction of two or more proteins.[8]

G6PD, an ancient metabolic enzyme present in eukaryotes and prokaryotes, catalyzes the first step of the pentose phosphate pathway (PPP).[9] Products of the PPP are important for the biosynthesis of nucleotides and fatty-acids. NADPH is essential for generating reduced glutathione, the cell's primary defense against electrophiles and oxidants. Despite the critical role G6PD plays in the cell, G6PD deficiency is the second most common human enzymopathy; more than 160 single-nucleotide substitutions that alter enzymatic activity and/or stability have been identified in humans.[10] Missense mutations result in a variety of clinical phenotypes depending on the nature and location of the mutation.[11] For example, mutations affecting the homodimer interface of G6PD are particularly pathogenic, as only the homodimeric and homotetrameric oligomers of G6PD are stable and active *in vivo*.[12] G6PD is the only enzyme known to have evolved a second $NADP^+$-binding site, close to the dimer interface; this second, so called structural site, is essential for maintaining activity, stability and oligomeric state of the enzyme.[13] Mutations that negatively impact binding of $NADP^+$ to the structural site also result in pathogenesis.

Despite the prevalence of G6PD deficiency, commonly resulting in mild to severe and chronic hemolytic anemia, no therapies currently exist. Previously, we described a small-molecule activator (Alda-1) of aldehyde dehydrogenase 2 (ALDH2),[14] which activates the most common single-point mutation in humans, ALDH2*2 by robustly correcting its structural defect.[15] Enzyme activation, like PPI stabilization, is a nascent pharmacological modality in drug discovery and identifying novel mechanisms to enhance enzyme function is of significant clinical interest.[16,17] Encouraged by the discovery of Alda-1, we set out to identify small molecules that enhance the activity of pathogenic mutants of G6PD. A biochemical screen was conducted using a common, pathogenic mutant (Canton G6PD, R459L) endemic to South East Asia.[18] A small-molecule activator of several pathogenic forms of G6PD (AG1 (**2**), Scheme 1) was identified from the screen, and a detailed mechanistic understanding of AG1 was required to further our translational objectives.[19]

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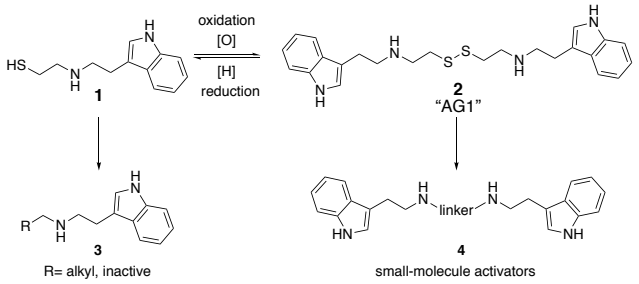


Scheme 1. Analogs (**3**) developed to mimic the primary thiol in **1** are inactive, but compounds (**4**) designed from the pharmacophore of **2** activate G6PD.

First, we found that analogs (e.g. **3**, Scheme 1) prepared using the pharmacophore of the structure provided by the vendor (**1**) were inactive. We reasoned that the original molecule **1** can undergo oxidation to the disulfide under ambient conditions.[20] When the dimeric molecule AG1 was independently synthesized and purified, it recapitulated the activation originally observed in the high-throughput screen; a 20% and 60% increase in the activity of the wild-type and the Canton mutant, respectively. Biochemical experiments indicated that AG1 activates G6PD in a non-covalent fashion (Figure S1).

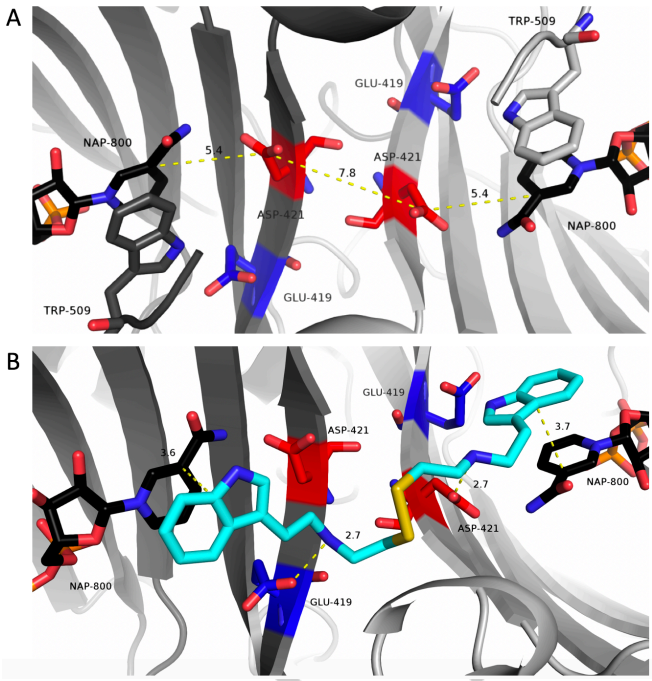


Figure 1. Residues expected to interact favorably with the pharmacophore of AG1. (PDB: 1QKI) A) The dimer interface of Canton G6PD located between the structural NADP+ (black, designated as NAP-800) with monomers shaded in dark and light grey. B) AG1 (teal) docked to the dimer interface using the Glide program in the Schrödinger software suite.

G6PD is active as a dimer or tetramer, but not as a monomer.[21] Considering that the active ligand is a dimer, which promotes oligomerization in a dose-dependent manner, it seemed possible that AG1 spans part of the C_2 symmetric dimer interface directly stabilizing the G6PD homodimer. Examination of the two C_2 symmetric regions at the dimer interface identified one region which can accommodate the pharmacophore of AG1 (Figure 1A). In the G6PD structure, the pyridinium ring of the structural NADP+ forms a π-stack with Trp509 (W509), which is approximately 5 angstroms from Asp421 (D421). This feature is repeated on the other monomer, with the two Asp421 separated by 7 angstroms. AG1 was docked into this region after removing the unstructured C-terminal residues in the enzyme up to Trp509 (Figure 1B). Docking of the ligand between the structural NADP+ sites reveals an asymmetric pose (Figure 1B), which places the two amines in AG1 near Asp421 and Glu419 (E419). A new π-stack of the indole in AG1 is formed with the pyridinium ring of the structural NADP+.

To determine if Asp421 and Glu419 participate in AG1 binding, a mutational analysis around the two C_2 points of symmetry was initiated in the Canton G6PD mutant enzyme (Figure 2). First, residues which could complement the ligand at the C_2 symmetric site distal from the structural NADP+ were mutated to serve as a control (Figure 2A, bottom). Two anionic residues, Asp228 and Glu347, were mutated to alanine (Ala, A). Additionally, a hydrophobic pocket in this region near Gly222 (G222), which could accommodate an indole ring, was mutated to an alanine to occlude this space. When AG1 was docked to this region of the enzyme, none of the poses generated were favorable when compared to the conformation of AG1 docked to the structural NADP+ sites. Anionic residues at the predicted site between the structural NADP+ (figure 2A, top), Asp421 (red), Glu419 (blue) and the double mutant (purple), were mutated to alanine. To assess the effect of the mutations, the AC_{50} (concentration at half-maximal activation) of AG1 in the relevant mutated enzyme was normalized to the AC_{50} of AG1 in unmutated Canton G6PD (figure 2B); values greater than 1 indicate that the given mutation attenuated the activation by AG1. Analysis of these mutant enzymes indicated that none of the structural changes affected the efficacy of the ligand on the site distal to the structural NADP+ within error. In contrast, mutations at the dimer interface between the structural NADP+, where AG1 was predicted to bind, significantly perturbed the AC_{50} (D421A and E419A/D421A).

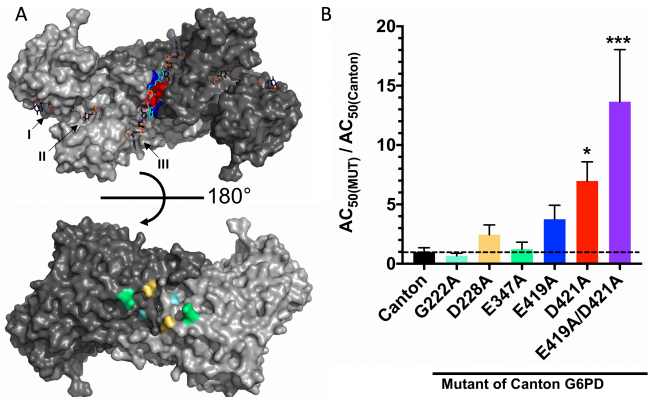


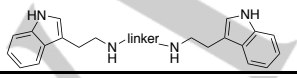
Figure 2. Mutagenesis of residues expected to interact favorably with the pharmacophore of AG1 about both C_2 symmetric dimer interfaces suggests the region between the structural NADP+ is important for ligand binding. (PDB:

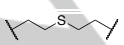
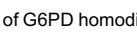
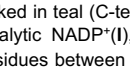
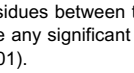
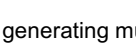
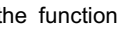
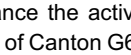
1QKI) A) Surface model of G6PD homodimer colored with the mutated residues (color coded), AG1 docked in teal (C-terminus deleted to residue 501) and the cofactors in black (catalytic $NADP^+$(**I**), G6P (**II**), structural $NADP^+$ (**III**)). B) Mutations of anionic residues between the structural $NADP^+$ attenuate activity of AG1, but do not have any significant effect on the opposite dimer interface. ($^*p = 0.0075$, $^{***}p < 0.0001$).

In addition to generating mutations, which were predicted to negatively impact the function of AG1, we predicted that one mutation may enhance the activation and/or binding of AG1. In the crystal structure of Canton G6PD, Trp509 forms a π-stack with the structural $NADP^+$ (Figure 1A). Previous studies demonstrated that the presence of the structural $NADP^+$ is crucial for maintaining the activity and stability of the enzyme.[22] We therefore reasoned that removing Trp509 and replacing it with alanine would impair G6PD function, but that AG1 should have a greater compensatory effect in this mutation. Additionally, because this Trp appears to compete with AG1 for binding to the structural $NADP^+$ site, we expected that mutating Trp509 should not affect the AC_{50} negatively or possibly lower the AC_{50}. The ligand, which had an A_{max} of 1.2-fold in wild-type G6PD and an AC_{50} = 4.2 μM, activated the Trp509Ala mutant enzyme 1.7-fold with an AC_{50} = 0.8 μM (Figure S2).

Given these results, we reasoned that the disulfide is not required for activation and could be replaced with non-reactive linkers (Table 1). The preferred method for synthesis of these analogs was via the Fukuyama amine synthesis,[23] which was superior in all cases examined to the initial route of amide formation and reduction. As expected, from the analysis of the dimer interface and the pharmacophore, shorter linkers – analogs **6** (4-carbon linker) and **7** (5-carbon linker) – did not function as activators but in fact, inhibited enzyme function. Simply replacing one atom in **7** with a sulfur atom resulted in conversion of an inhibitor to a dose-dependent activator (**8**). Analogs **5** (3-carbon linker), **9** (6-carbon linker), **10** (7-carbon linker) and **11** (8-carbon linker) modestly activated the enzyme without prominent sigmoidal dose-response curves. Analogs **8**, **2**, **12** (9-carbon linker), **13** (10-carbon linker) and **15** (12-carbon linker) all exhibited approximately equal activation (1.6-fold) and sigmoidal, dose-dependent activation. Analog **12** was only 2-fold less potent than the original disulfide-containing analog **2**. Similarly to AG1 (**2**), analogs **10**, **12** and **13** increased the oligomerization of the enzyme (Figure S3).

Table 1. Impact of linker on activation of Canton G6PD.



Compound[a]	Linker	AC_{50} [μM][a]	A_{max} [%][a]
5		-[b]	135%[b]
6		-[c]	-[c]
7		-[c]	-[c]
8		33	155%
9		-[b]	119%[b]
2		3.3	160%
10		-[b]	123%[b]
11		-[b]	130%[b]
12		7.7	160%
13		15	159%
14		-[b]	104%[b]
15		12	146%

[a] AC_{50} values were obtained by titration of activators in a biochemical assay of Canton G6PD activity with substrate concentrations close to physiologically relevant conditions (95% confidence intervals of AC_{50} values are available in the supplementary information). [b] Significant dose-response curves are not obtained with these compounds and activation is instead reported at 50 μM. [c] Compounds were found to inhibit the enzyme in a dose-dependent manner (SI).

Pharmacological modulation of PPIs, a promising area of drug discovery, remains elusive. Moreover, ligands capable of stabilizing PPIs are rarer still. As discussed, many pathological G6PD mutations have been identified in humans, and AG1 promotes activation of at least several pathogenic mutants. Our current study sheds light on the mechanism by which such a poly-genotypic activator of G6PD acts. This novel class of molecules was originally discovered due to the correct identification of the active species (AG1, **2**) in samples of the primary thiol **1**. Mutational analyses and biochemical experiments support a mechanism of action whereby AG1 promotes G6PD dimerization by bridging two structural $NADP^+$. Efforts to optimize these G6PD activators and evaluate their translational potential as a first-in-class treatment for G6PD deficiency are ongoing. Given the omnipresence of PPI regulation in both cellular signaling events and enzyme activity/stability, we anticipate that small-molecule stabilization of PPIs will be increasingly valuable in drug discovery.

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Keywords: small-molecule activator • glucose-6-phosphate dehydrogenase • protein-protein interactions • bivalent ligand • enzyme catalysis

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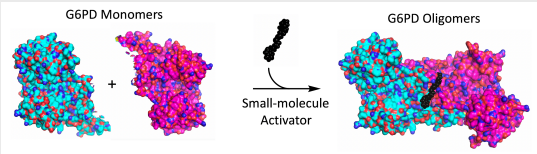

A novel class of small-molecule glucose-6-phosphate dehydrogenase (G6PD) activators is described. Structural studies support a mechanism of action (MOA) whereby the small molecules bind across the dimer interface to promote oligomerization to the more catalytically competent enzyme state(s).

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