Identification of Inhibitors of Phosphate-activated Glutaminase for the Pharmacotherapy of Schizophrenia

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Abstract

In the brain, phosphate-activated glutaminase catalyzes the recycling of glutamine back to glutamate for excitatory neurotransmission. In mice, genetic knockdown of phosphate-activated glutaminase has been shown to confer resilience to schizophrenia-like symptoms, suggesting that inhibition of glutaminase may have therapeutic potential for the pharmacotherapy of schizophrenia. As there are no known neuroactive inhibitors of glutaminase, i.e. inhibitors that get into the brain, high-throughput screening of a library of 58,000 neuroative compounds was conducted using a fluorescence-based assay; this screen identified 320 potential glutaminase inhibitors. A secondary screen was carried out to assess specificity; this yielded 10 hits. Using a kinetic analysis, two leads with low micromolar activity were found.

Introduction

Schizophrenia is a complex and diverse neuropsychiatric disorder that affects approximately one percent of the world population. Symptoms typically present in schizophrenia are placed into three categories: positive, such as hallucinations, negative, such as paucity of speech and social withdrawal, and cognitive, such as conceptual disorganization and deficits in working memory.

The treatment of schizophrenia has focused on targeting dopamine hyperactivity with anti-psychotic drugs. The dopamine hypothesis, which attributes the symptoms of schizophrenia to hyperactive dopaminergic signal transduction in the striatum and, more broadly, the mesolimbic pathway, rose to prominence with the discovery that many common antipsychotic drugs, such as chlorpromazine and clozapine, work by binding to postsynaptic dopamine receptors, notably D2 receptors, and interfering with dopamine signal transduction (Creese, 1976; Seeman, 1975; Van Rossum, 1966; Matthyse, 1973). However, the dopamine hypothesis does not fully explain schizophrenia. One problem is that these dopamine receptor antagonists are largely ineffective in treating the negative and cognitive symptoms of schizophrenia, which suggests that dopaminergic hyperactivity may be unrelated to this class of symptoms (Davis, 1991; Javitt, 2010; Kellendonk, 2006). Moreover, a significant number of patients with schizophrenia do not respond to these drugs, which also may be due to the involvement of other neurochemical pathways (Davis 1991).

More recently, abnormal glutamate transmission has been implicated in the pathophysiology of schizophrenia. Glutamate is the major excitatory neurotransmitter in the central nervous system. Under normal conditions it is produced and maintained at a low extracellular concentration through the glutamate-glutamine cycle (Sanacora, 2008). The importance of glutamate transmission in schizophrenia and psychosis was realized with the discovery that drugs like phencyclidine (PCP) cause symptoms that are nearly indistinguishable from those of schizophrenia by acting as an antagonist to N-methyl D-aspartate (NMDA) receptors to which glutamate is an agonist. Since then, many studies have shown that processes that modulate normal glutamatergic signaling may have beneficial effects on positive, negative, and cognitive symptoms of schizophrenia (Javitt 2010; Vinson and Conn 2011; Gaisler-Solomon et al., 2009).

In particular, mice haploinsufficient for phosphate-activated glutaminase (PAG) show a reduction in glutamatergic synaptic transmission, are behaviorally quite normal, and are resilient to the effects of pro-psychotic drugs. PAG is an enzyme that catalyzes the presynaptic conversion of glutamine to glutamate in neuronal mitochondria, These findings suggest that the reduction of glutamate release through the genetic reduction of PAG confers resilience to a schizophrenia-like phenotype in mice, and that the inhibition of PAG may have therapeutic potential in the pharmacotherapy of schizophrenia (Gaisler-Solomon et al., 2009). PAG inhibition also appears to have therapeutic potential in several diseases beyond schizophrenia, such as in the treatment of cancer, and HIV-associated neurocognitive disorders and the prevention of stroke (Chiaradonna, 2012; Huang et al., 2011; Dhawan et al., 2011).

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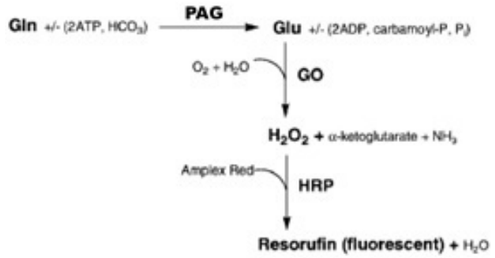


Figure 1 *The steps of the fluorescence-based assay for studying the activity of phosphate-activated glutaminase (PAG). Resorufin fluorescence is a measure of glutamate concentration and, therefore, PAG activity (Modified from McElroy et al. (2000)).*

Currently, there are only two known glutaminase inhibitors: bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl-sulfide (BPTES) and 6-diazo-5-oxo-L-norleucine (DON) (Hartwick, 2011). However, neither compound crosses the blood-brain barrier nor is neuroactive; thus, neither is suitable for pharmacotherapy of schizophrenia. The purpose of this study was to screen for PAG inhibitors in a library of 58,000 neuroactive compounds.

Robotic processing with a fluorescence-based assay was used to assess inhibition of PAG activity in vitro. A secondary assay was used to confirm PAG-specificity of apparent inhibitors, and subsequent kinetic analysis of the top tier of these hits was used to select two leads for further chemical optimization.

Methods

Primary Screen

A library of 58,000 neuroactive compounds was purchased from ChemBridge. Compounds were supplied at a concentration of 10^{-3} M in DMSO and were stored in 384-well plates at -80°C.

Human PAG was synthesized and purified by our lab using a plasmid provided by Professor Norman Curthoys (Colorado State University) as previously described in Kenny et al. (2003).

The fluorescence assay used for the screen was optimized from a commercially available kit (Amplex Red, Invitrogen) and was used to measure PAG activity in the presence of each library compound. Glutamate, the product of PAG catalysis, was converted to a fluorescent compound called resorufin by the addition of a series of kit enzymes, including glutamate oxidase (GO), horseradish peroxidase (HRP), and the fluorogenic substrate Amplex Red (Figure 1). Because the fluorescent molecule resorufin is dependent on glutamate production and glutamate production is the product of PAG catalysis, the rate of change in fluorescence is a measurement of PAG activity. Potent inhibitors of PAG would significantly decrease the fluorescence compared to controls without inhibitor.

The assay was carried out in a 384-well plate, with 80 µL of total liquid volume per well. 320 wells were carried out under experimental conditions, where 20 µL of Amplex Red solution (itself containing 8 mL of Tris-HCl reaction buffer, 60 µL of GO, 25 µL of HRP, and 80 µL of Amplex Red reagent), 40 µL of PAG solution (itself 2 µL of synthesized PAG in 32 mL of Tris-phosphate buffer), 0.8 µL of one library compound (originally 10^{-3} M, but diluted to 10^{-4} M in well), and 20 µL of 40 µM glutamine solution were pipetted into each well. While kit enzyme concentrations were the same as those used in the Amplex Red kit protocol (so that all kit enzymes were in excess), optimum glutamine and PAG concentrations were determined according to Michaelis-Menten kinetics. The concentration of glutamine was selected based on the previously determined value of the K_m of PAG-catalyzed glutamine deamination. K_m is defined as the concentration of substrate (glutamine) required to reach one half of the maximal reaction velocity (vmax) for a particular enzyme-catalyzed reaction (Copeland, 2000).

Control conditions were included in the remaining 64 wells of each plate. Positive control wells, representing 100% PAG activity (0% PAG inhibition), contained PAG, glutamine, and Amplex Red solution. Negative control wells, representing 0% PAG activity (100% inhibition), contained glutamine and Amplex Red solution. 75%, 50%, and 25% inhibition controls were also used, obtained by creating a dilution series of the positive control. Additionally, Amplex Red solution only controls were used as an additional negative control. In control wells, where necessary, Tris-HCl reaction buffer was added to bring wells to the standard volume. Outlined in Figure 2 below is a typical assay plate setup.

Fluorescence measurements were taken at baseline (immediately after glutamine was added to each well), and after a 60-minute incubation at 37 °C, by an Envision fluorescence reader (Perkin Elmer). Pipetting was done by a Mini-Trak Robotic Liquid Handling System (Perkin Elmer) for speed and efficiency.

To calculate percent inhibition of PAG, GO, and HRP, the average negative control fluorescence was subtracted from the experimental group fluorescence and divided by the average positive control fluorescence. Subtracting percent activity from 100% gave percent inhibition.

	1	2	3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20	21	22
A	positive control: PAG + glutamine + Amplex Red		320 library compounds	positive control: PAG + glutamine + Amplex Red	
B					
C					
D					
E	negative control: glutamine + Amplex Red			negative control: glutamine + Amplex Red	
F					
G					
H					
I	75% inhibition control			75% inhibition control	
J					
K	50% inhibition control			50% inhibition control	
L					
M	25% inhibition control			25% inhibition control	
N					
O	Amplex Red			Amplex red	
P					

Figure 2 *A typical plate from the primary screen.*

Secondary Screen

To select true inhibitors of PAG, it was necessary to eliminate the hits that were inhibitors of the kit enzymes GO and HRP. To eliminate GO/HRP inhibitors, another resorufin assay was designed for this purpose. This assay was similar to the original glutamine-glutamate Amplex Red assay except that glutamine was replaced by the GO/HRP substrate glutamic acid, and PAG was not included. Because the conversion of glutamate to resorufin is much faster than PAG-catalyzed glutamine deamination, it was necessary to determine optimal substrate and enzyme concentrations that would produce a linear increase in product over a given time frame. Based on a preliminary kinetics assay, it was determined that replacing the 40 μM glutamine with 10 μM glutamic acid and maintaining previous kit enzyme concentrations produced a reaction where the concentration of product (resorufin) increased linearly with time (and did not saturate).

Once the glutamic acid assay parameters were determined, the primary screen assay and glutamic acid assay were carried out in parallel on the primary screen hits. Percent inhibition of PAG, GO, and HRP (primary screen) and of GO and HRP (glutamaic acid assay) were determined for each hit, and percent inhibition of PAG only was calculated by subtracting GO/HRP inhibition from PAG/GO/HRP inhibition.

'Hit' Kinetics Analysis

Of the compounds that were picked up in the secondary screen, the ten with the highest percent PAG inhibition were selected for kinetic analysis and purchased from ChemBridge. 0.1 M aliquots of each compound were prepared with DMSO and stored at -80°C. From these stock solutions, 10-fold dilution series ranging from 10^{-3} M to 10^{-8} M were prepared for each compound. The same assay that was used in the primary screen was used for the kinetics analysis experiment, except that the inhibitors were used in

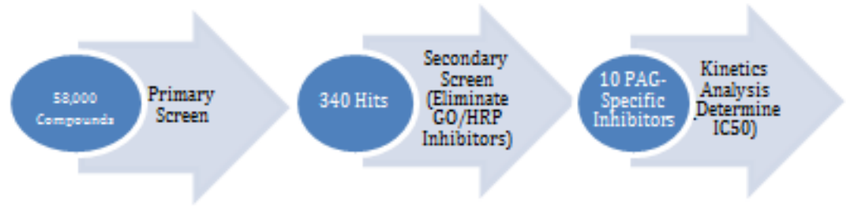


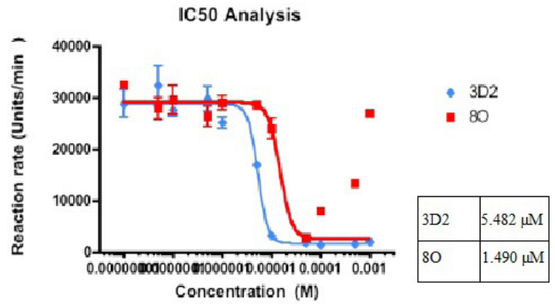
Figure 3 *A schematic diagram outlining the screening process for the selection of PAG-specific inhibitors*

varying concentrations. Additionally, fluorescence readings were taken every 10 minutes so that reaction rates could be calculated based on the slope of the graph of fluorescence versus time. GraphPad Prism 4 was used to transform the fluorescence data to fit a characteristic Michaelis-Menten curve and to calculate the IC_{50} value for the inhibitors. IC_{50} is a measure of inhibitory power; it is the concentration of inhibitor at which the reaction rate catalyzed by a particular enzyme is halved.

The steps in the screening of the ChemBridge CNS-Set library are detailed in Figure 3. A visual basic loop program was used to rank in descending order and in terms of percent inhibition the 320 library compounds on each of the 180 plates. Because of variability in fluorescence values of the controls in different plates, results of the primary screen were sorted based on their dynamic range. The DR of fluorescence for a particular plate was defined using the control fluorescence values:

$$DR = \frac{100\% * (\text{positive control} - \text{negative control})}{(\text{positive control})}$$

Plates were then placed into three categories: good



3D2	5.482 μM
8O	1.490 μM

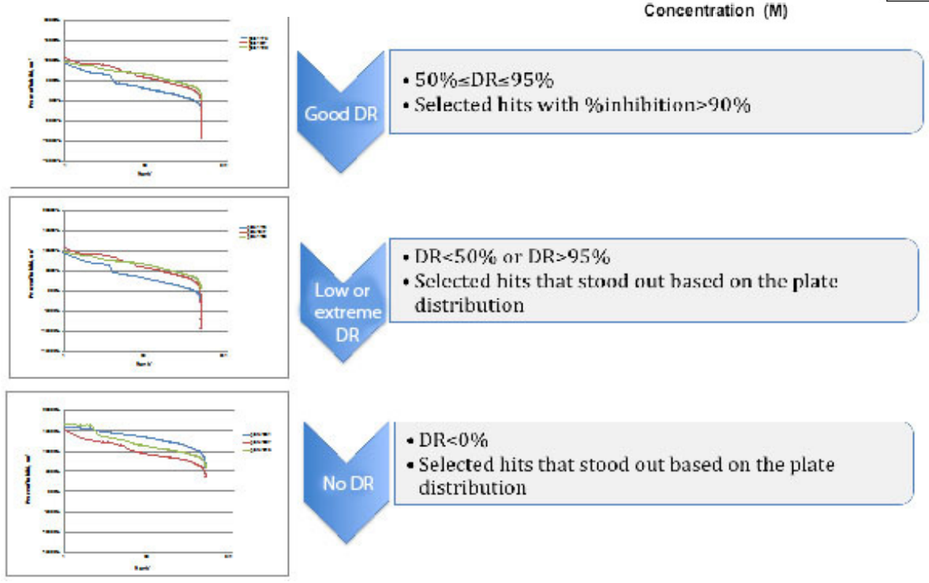


Figure 4 *Reaction rate vs. concentration plotted using Prism software. IC_{50} values are displayed in the table to the right*

Trial	1	2	3	4	Average ± SE
IC50 (uM)	5.482	3.647	4.655	3.303	4.272 ± 0.572
R^2	0.951	0.982	0.944	0.956	

Figure 5 *IC_{50} values determined for 3D2 with corresponding R^2 values. *8O was not tested on 10/17/2011*

(DR=95-50%), low or extreme (DR<50% or DR>95%), and poor dynamic range (DR≈0%). For plates with a good dynamic range, compounds that exhibited at least 90% inhibition were selected. For plates with a low or extreme dynamic range or non-existent dynamic range, compounds were selected if they stood out in the plate distribution. Representative distributions of compound inhibition data for three plates in each of the three categories are shown in Figure 4. For plates with a good dynamic range, there were typically very few or no compounds with percent inhibition above 90%; however, for plates with a low or extreme dynamic range or a poor dynamic range, there were significantly more compounds whose percent inhibition was greater than 90%, and in fact, there were a significant number of compounds with percent inhibition greater than 100%.

The hits picked up by this process were tested in the secondary screen to eliminate inhibitors of GO and HRP. The compounds that had the least impact on GO and HRP were ranked based on their selective inhibition of PAG. From this list of compounds, the top 10 were selected for kinetic analysis.

For 2 of these 10 compounds, the inhibition curve fit Michaelis-Menten kinetics. Plots for these 2 compounds, 3D2 and 8O, of reaction velocity versus inhibitor concentration are shown below, and Figure 5 indicates the IC_{50} values 3D2 in four independent experiments.

The average IC_{50} value for 3D2 was 4.272 ± 0.572 μM. As indicated by the regression coefficients of each experiment, the Michaelis-Menten model of enzyme kinetics was applicable to the activity of 3D2.

Discussion

Based on its average IC_{50} value, inhibitor 3D2 appears worthy of further investigation. Because combinatorial chemistry can be used to optimize inhibitory power by up to three orders of magnitude, it appears that optimization of 3D2 particularly would produce a compound that would inhibit PAG in the nanomolar range (Jorgensen, 2009). At concentrations lower than 10^{-4} M, 8O also exhibits similar dose-dependent behavior, but because of its unusual behavior for concentrations greater than this, where reaction velocity appears to increase with greater concentrations of inhibitor, it is necessary to subject 8O to further kinetic scrutiny. For both compounds, before they are to be optimized, it is necessary to confirm their inhibitory power through alternative assays and to increase the number of hits to be optimized.

The organization of the primary screen experiments into three categories based on the dynamic range of control fluorescence enabled comparison between plates and appropriate selection of hits. For plates with a good dynamic range, there were few or no compounds with greater than 90% inhibition. However, for plates with an extreme (low or high) or non-existent dynamic range, there were many compounds with greater than 90% inhibition and even some well above 100%. This result most likely does not indicate a greater number of hits for these two categories of plates; indeed, the >100% inhibitors suggests that the large number of apparent hits with percent inhibition >90% is an artifact of an abnormal dynamic range. Therefore, the division of the plates into the three categories and the selection of outliers from the two categories of plates with abnormal values for dynamic range were used to correct this artifact so that only outliers were selected from every plate.

The secondary screen was designed to eliminate non-specific inhibitors and false positives from the screen. In testing for inhibition of glutamate oxidase and horseradish peroxidase, the secondary screen was able to exclude compounds that were potential inhibitors of these kit enzymes. In addition to this secondary assay, it is necessary to confirm the inhibitory power of 3D2 using another assay without the use of Amplex Red; one such assay involves the direct determination of glutamate as a product of PAG catalysis.

From the IC_{50} curve of 8O, it appears that for concentrations of inhibitor greater than 10^{-4} M, the reaction rate is actually higher than it is for lower concentrations; otherwise, for concentrations below 10^{-4} M, 8O appears to adhere to the dose-dependent pattern normally seen with good inhibitors. A likely explanation for this unusual pattern is that 8O is itself fluorescent and enhances the fluorescence signal at high concentrations.

One caveat to Michalis-Menten kinetics determinations is that the value of the IC_{50} measurement is in itself limited; IC_{50} depends on the concentration of substrate, enzyme, and inhibitor, as well as other experimental conditions. Ki, the dissociation constant of the inhibitor to the enzyme, is a more valuable measurement because it is an intrinsic thermodynamic quantity independent of substrate concentration, but dependent on enzyme and inhibitor concentrations. For this reason, K_i measurements are more replicable across different experimental methods (Cer, 2009).

There are ways of measuring K_i based on IC_{50} values, one of which is the Cheng-Prusoff equation:

$$K_i = IC_{50}/(1+[S]/K_M)$$

However, this equation is used to calculate K_i for inhibitors known to be competitive (Copeland, 2000).

Another method that can be used to determine K_i is through the use of the Lineweaver-Burk plot, which Graphpad Prism 4 can generate. These plots are obtained by varying both inhibitor and enzyme concentration, and can both elucidate the nature of the inhibitor (competitive, non-competitive, or uncompetitive) and be used to calculate K_i (Copeland, 2000). Knowing the nature of the inhibitor will allow prioritization of the leads for optimization. Non-competitive inhibitors will be favored for optimization over competitive inhibitors. Competitive inhibitors, binding to the enzyme's active site, are less likely to be specific to PAG, as they are also likely to bind to the active sites of other enzymes in the glutamate cycle. Therefore, since PAG-specificity is one of the selection criteria, and competitive inhibitors are unlikely to be PAG-specific, non-competitive inhibitors will be preferentially selected for optimization.

Once these leads are optimized, they will be assessed for drug-like character and their suitability in the pharmacotherapy of schizophrenia. For example, PAG activity measurements in mice administerd the leads, electrophysiological recordings of their brain slices, and behavioral tests analyzing their response to amphetamine-induced hyperactivity can be used to assess the value of these leads in pharmacotherapy. Additionally, ADME-tox (Absorbtion, Distribution, Metabolism, Excretion, and toxicity) analysis will be necessary in assessing suitability for drug development.

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