

# High-throughput screening of glutaminase inhibitors for the pharmacotherapy of schizophrenia: Implementation of a fluorescence-based assay

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## Abstract

Recent studies suggest that reducing synaptic glutamate release may prove therapeutic in schizophrenia. Phosphate-activated glutaminase (PAG), present in human brain mitochondria, is thought to mediate the major flux of glutamate to the neurotransmitter pool. PAG catalyzes the conversion of L-glutamine (Gln) to L-glutamic acid (Glu), so inhibiting PAG seems a promising means of reducing Glu release. The goal of this study was to establish the parameters for a high-throughput screening to identify potential PAG inhibitors. Glutaminase A from *E. coli* was used as a substitute for PAG. Gln was added as the substrate and its conversion to Glu measured using Amplex Red technology as Resorufin fluorescence. First, the linear range for the relationship between glutamate added and Resorufin fluorescence was determined. We measured the kinetics of PAG action, showing that after one hour there was a complete conversion of glutamine to glutamate. However, the relationship between PAG concentration and Resorufin fluorescence was non-linear, consistent with cooperativity in PAG function. We then assayed 6-diazo-5-oxo-L-norleucine (DON), the best known glutaminase inhibitor, and determined an inhibition constant of 73 nM. DON was more effective against bacterial glutaminase than the known millimolar-level inhibition in mammalian neurons, indicating that the bacterial glutaminase would not be suitable for screening for inhibitors of human PAG, and thus for identifying PAG inhibitors with therapeutic potential for schizophrenia. In conclusion, the parameters for the assay of PAG activity and its inhibition were established; and synthesis of human glutaminase should allow us to move forward to high-throughput screening.

Key words: glutaminase inhibition

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## Introduction

### Schizophrenia

Schizophrenia is a debilitating mental condition for individuals and a large economic burden for society. It ranks among the top ten causes of disability in developed countries and affects approximately 1% of the population worldwide. Schizophrenia has both a genetic and environmental basis. It is a complex disorder, in which over seventy polymorphisms have been found to be strongly associated with its biological and behavioral aspects. Schizophrenia manifests through positive symptoms hallucinations, delusions, negative symptoms avolition, apathy and blunted affect and cognitive impairments. There is no known cure for schizophrenia, but appropriate treatment has proven successful in reducing the occurrence of psychotic episodes (positive symptoms). More research is needed for the development of medication effective also for the negative symptoms and cognitive deficits and with reduced side effects.

For a long time, the dopamine hypothesis of schizophrenia was prevalent in schizophrenia research, based on the fact that all known antipsychotic drugs were dopamine D2 receptor antagonists. However, these drugs were only

effective at relieving individuals of the positive symptoms, while being of little benefit for negative symptoms. Recent studies suggest that abnormal glutamate transmission is involved in schizophrenia. Single cell recordings from spinal interneurons in the cat showed that phencyclidine (PCP) and ketamine act as NMDA receptor antagonists (Lodge and Anis, 1982). Consequences of blocking NMDA receptors are excessive release of glutamate (Moghaddam and Adams, 1998) and overstimulation of postsynaptic neurons and these consequences have been associated with the cognitive and behavioral disturbances present in schizophrenia (Olney et al, 1999).

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Consistent with this theory, at the basis of our research is a recent clinical imaging study that reported that an increase in baseline hippocampal activity, especially in CA1 and subiculum, was associated with schizophrenia (Schobel et al, 2007). Another study showed that mutant mice heterozygous for GLS1 with a deficit in glutamatergic transmission were fully viable and represented an imaging phenotype inverse to the one seen in the clinical study performed by Schobel: hippocampal hypoactivity, mainly in CA1 and subiculum (Gaisler - Salomon I, 2007). Additionally, it is known from behavioral and neurochemical measurements that the phenotype of these mice is similar to the one presented by mice treated with antipsychotic drugs. Given these results, there is a high chance that the human increase in hippocampal activity in patients with schizophrenia is directly correlated with increased level of the neurotransmitter glutamate.

The development of the glutamate hypothesis of schizophrenia led to the exploration of new therapeutic targets. A great promise is held by a new compound, LY404039, which reduces glutamate release via metabotropic glutamate receptor 2/3- (mGluR 2/3) mediated presynaptic inhibition (Patil et al, 2007). This drug seems to ameliorate both positive and negative symptoms without the weight gain and potential risk of developing diabetes associated with other anti-schizophrenia drugs. However, undesirable secondary effects still remain (insomnia, nausea, headache, somnolence), emphasizing the need for alternative ways of reducing glutamate release.

### Glutamate

Glutamate is the principal excitatory neurotransmitter in the central nervous system, which most likely is a product of the action of phosphate-activated glutaminase, the enzyme that converts glutamine to glutamate and ammonia (Erecinska and Silver, 1990). Three types of postsynaptic ionotropic glutamate receptors have been identified on the basis of their binding affinities for prototypical ligands, i.e., kainate, AMPA and NMDA receptors (Daikhin and Yudkoff, 2000). Glutamatergic neurons mediate many vital processes, including the encoding of information, the formation and retrieval of memories, spatial recognition and the maintenance of consciousness (McEntee and Crook, 1993). A simplified model of the brain glutamate metabolism is provided by the glutamate-glutamine cycle. According to this formulation, glutamate is released from presynaptic terminals and is taken up primarily by astrocytes, where it is converted to glutamine via glutamine synthetase. Glutamine is released back to neurons, in where glutamate is regenerated via phosphate-activated glutaminase, a mitochondrial enzyme (Daikhin and Yudkoff, 2000). The cycling of glutamate to glutamine is essential to maintain a sufficiently low ( $\mu\text{M}$ ) concentration of extracel-

lular glutamate, to avoid the excessive accumulation of glutamate which leads to neuronal cell death (Robinson et al, 2007). Beyond this model, additional degradation of glutamate is achieved through the TCA cycle, where glutamate is converted to malate or oxaloacetate (generating additional energy), which exit the cycle to form one molecule pyruvate (Hertz et al, 2002).

### Glutaminase

In the classic view of glutaminase expression in mammals, presented by Krebs in 1935, there are two main isoenzymes: a liver type and a brain/kidney type which are the products of different but related genes. Recent experimental evidence has shown that the liver type isoenzyme is expressed not only in adult liver, but also in the brain, pancreas and breast cancer cells. The brain/kidney type isoform (PAG) seems to be ubiquitous and is found in all other tissues with glutaminase activity. Simultaneous expression of both isoenzymes appears to be frequent in human tissues and cancer cells (Marquez et al, 2006). The hallmark of differentiation between these two main isoenzymes is that PAG is activated by phosphate (or other polyvalent anions that stabilize it in the active tetrameric form) and inhibited by glutamate, while the liver type does not require phosphate for activation and is not inhibited by glutamate (Curthoys and Watford, 1995).

Given the success obtained in early clinical trials by the compound LY404039, we propose an alternative way of reducing glutamate release by inhibiting the human phosphate-activated glutaminase. Because there is a high degree of similarity among genes coding for the same protein, we attempted to use the commercially available Glutaminase A from *E. coli* in our studies that is a model for PAG.

There are a few known glutaminase inhibitors, but none has therapeutic potential for schizophrenia and none is active at nanomolar concentrations, which are pharmaceutically desirable. The inhibitor DON is a glutamine analogue; it is not selective for glutaminase as it also inhibits other glutamine-utilizing enzymes such as amidotransferases (Ahluwalia et al, 1990). The newly discovered BPTES, with an inhibition constant  $K_i=3 \mu\text{M}$ , is a potent and specific inhibitor that stabilizes glutaminase in an inactive tetrameric form (Robinson et al, 2007), but lacks therapeutic potential because it is too polar to cross the blood-brain barrier and so it would not access brain glutaminase.

Since reducing Glu release in the brain by means of glutaminase inhibitors appears to be a promising therapeutic approach in schizophrenia, the goal of this study was to set up the parameters of an *E. coli* glutaminase inhibition assay by implementing a commercially available kit that provides a fluorescent readout of glutamate production.

## Materials and Methods

### Materials

The similarity between the different variants of glutaminase was determined. A search in the nucleotide Basic Local Alignment Search Tool (BLASTN) provided by the National Center for Biotechnology Information (NCBI) assessed the overlap of the gene coding for the brain/kidney type glutaminase with the gene coding for the liver type glutaminase. The nucleotide sequence for glutaminase from *E. coli* is not fully elucidated, so we could not assess the alignment overlap between these two genes. However, the amino acid sequence is known also for glutaminase from *E. coli*, thus we used BLASTP, to compare the amino acid sequences of the desired enzymes.

L-Glutamine Ultra ( $\geq 99.5\%$ ), Glutaminase from *E. Coli*, grade II and grade V, and DON were obtained from Sigma-Aldrich. Both of the glutaminase varieties were almost entirely Glutaminase A. Glutaminase grade II contained 5-25 units/mg protein and glutaminase grade V contained 50-200 units/mg protein and potassium succinate and ethylenediaminetetraacetic acid (EDTA) as stabilizers. The Amplex Red Glutamic Acid/Glutamate Oxidase assay kit was obtained from Molecular Probes (Invitrogen). BPTES was a gift from Professor Ronald Zielke (University of Maryland).

Glutaminase and DON were dissolved in deionized water and stored as aliquots of 100  $\mu$ L at  $-20^{\circ}$  C. Just prior to the experiment, we prepared solutions of L-glutamine of different concentrations. Until use, the L-glutamine solutions were kept at room temperature, protected from light. The stock solutions of Amplex Red, Tris-HCl buffer, horseradish peroxidase (HRP), hydrogen peroxide, L-glutamate oxidase, L-glutamate pyruvate transaminase, L-glutamic acid and L-alanine were prepared according to the instructions provided in the kit. A series of dilutions of L-glutamic acid were prepared. Just prior to use, we prepared the Amplex Red working solution by adding 50  $\mu$ L Amplex Red stock solution, 12.5  $\mu$ L HRP, 80  $\mu$ L L-glutamate oxidase, 25  $\mu$ L L-glutamate pyruvate transaminase, 5  $\mu$ L L-alanine and 4.83 ml Tris-HCl buffer of pH 7.5 in the proportions written in the kit protocol. The fluorescence of the final product, Resorufin, was measured by using the 96-well plate Continuous Fluorescence Detector Opticon 2 purchased from Bio-Rad Laboratories.

### Methods

#### *The Amplex Red kit*

To obtain the logarithmic correlation of the fluorescence of Resorufin as a function of the glutamic acid concentration, solutions were prepared with final concentrations of Resorufin in the range  $4 \times 10^{-8}$  M –  $4 \times 10^{-6}$  M (Zhou et al, 1997). The 96-well plate was prepared by adding reagents up to a final volume of 100  $\mu$ L. First the negative control

was added, consisting of 50  $\mu$ L solution of reaction buffer and then 50  $\mu$ L of the Amplex Red working solution, then the samples, consisting of 50  $\mu$ L L-glutamic acid of increasing concentrations and then 50  $\mu$ L of the Amplex Red working solution and also a positive control, by adding 50  $\mu$ L freshly prepared  $H_2O_2$  and 50  $\mu$ L of the Amplex Red working solution. The mixtures were incubated for various times ranging from 10 to 130 minutes at  $37^{\circ}$  C, as indicated, with the lid temperature at  $40^{\circ}$  C and the lid shut-off temperature at  $30^{\circ}$  C. The detection of fluorescence was done using the pre-calibrated dye tetramethylrhodamine methyl ester (TMR), with an emission spectrum similar to the one of Resorufin.

Establishing that we can determine the amount of L-glutamic acid present in the reaction system, L-glutamine was introduced in the reaction system as the precursor of L-glutamic acid. Firstly, we made sure that L-glutamine does not convert to L-glutamic acid in the absence of glutaminase. As expected, the fluorescence values when no glutaminase was added to the system were equal to the ones of the negative controls, showing that the conversion of glutamine to glutamate does not happen in the absence of glutaminase.

We compared the fluorescence obtained when we started from glutamine and glutaminase to the fluorescence obtained when we started from L-glutamic acid. In the first type of reaction mixture, we added 5  $\mu$ L glutaminase, 5  $\mu$ L  $K_3PO_4$ , 25  $\mu$ L Amplex Red working solution and 25  $\mu$ L glutamine of various concentrations, for a total volume of 60  $\mu$ L. In the wells containing glutamic acid, we added 30  $\mu$ L glutamic acid of various concentrations and then 30  $\mu$ L Amplex Red working solution. The concentrations of glutamic acid and glutamine were calculated so that they would yield the same concentrations of Resorufin in the final reaction volume.

In further experiments, the well composition was modified in order to include different concentrations of glutaminase and have a pseudo-inhibitor assay. Since the activity of glutaminase is defined as number of moles of glutamine deaminated/min, sooner or later the existent glutamine will be completely converted to glutamate. Triplicates were used in wells containing 10  $\mu$ L glutaminase or glutaminase diluted 2-fold or glutaminase diluted 5-fold, 10  $\mu$ L Tris-HCl buffer and 40  $\mu$ L Amplex Red working solution. The substrate, consisting of 40  $\mu$ L L-glutamine of various concentrations, was added last to a final reaction volume of 100  $\mu$ L. Fluorescence values were read after 20 minutes from the start of the reaction.

#### *Known glutaminase inhibitors*

For the inhibitor BPTES, the same amounts and the same order of reagents were used, but only 3 concentrations of BPTES were tested:  $10^{-5}$  M,  $5 \times 10^{-4}$  M and  $10^{-4}$  M.

Additional tests were performed to see if BPTES is fluorescent itself. 50  $\mu$ L of BPTES were added to 50  $\mu$ L buffer,

then to 50  $\mu\text{L}$  Amplex Red working solution and these results were compared with the negative and positive controls.

In order to test the action of the known inhibitor DON, 10  $\mu\text{L}$  of inhibitor of concentrations ranging from  $10^{-2}$  M to  $10^{-7}$  M were added to 10  $\mu\text{L}$  glutaminase, followed by the addition of 40  $\mu\text{L}$  Amplex Red working solution, and lastly the substrate, 40  $\mu\text{L}$  glutamine of various concentrations. Using the same amounts of reagents, the concentrations of DON were varied again, this time in the range  $10^{-4}$  M to  $10^{-10}$  M.

Because of the acknowledged lack of specificity of DON, we tried to see if DON does actually inhibit glutaminase or it actually acts on another enzyme from the Amplex Red kit (Glutamate oxidase, HRP or L-glutamate pyruvate transaminase). To accomplish this, we started from L-glutamic acid and we evaluated the fluorescence results in the absence and presence of different concentrations of DON. 10  $\mu\text{L}$  DON of various concentrations were added to 10  $\mu\text{L}$  Tris-HCl buffer and then 40  $\mu\text{L}$  L-glutamic acid and 40  $\mu\text{L}$  Amplex Red working solution were added. Furthermore, it was tested if DON does not generate fluorescence by itself by adding 50  $\mu\text{L}$  DON and then 50  $\mu\text{L}$  Amplex red working solution. The results were evaluated after 40 minutes from the beginning of the reaction and they are the averages of 3 determinations.

## Analysis

The reaction rates for the uninhibited and inhibited reactions presented in figure 5 were calculated with the formula

$$v = \frac{\Delta c}{\Delta t} = \frac{\Delta \text{Fluorescence}}{\Delta t}$$

The values were evaluated as the mean values after 30 minutes from the beginning of the reaction. The functional strength of the inhibitor ( $\text{IC}_{50}$ ) was calculated with the GraphPad Prism 4.0 software, and the  $k_i$  value was calculated by using the Cheng - Prusoff equation:

$$k_i = \frac{\text{IC}_{50} [S]}{1 + k_m}$$

## Results

### Choosing glutaminase A from *E. coli* as a model for our studies

The similarity between the different variants of glutaminase was determined. BLASTN determined that the similarity of the nucleotide sequences of the gene coding for the brain/kidney type glutaminase and the gene coding for the liver type glutaminase was 78%. Using BLASTP to compare the amino acid sequences of the desired enzymes, we

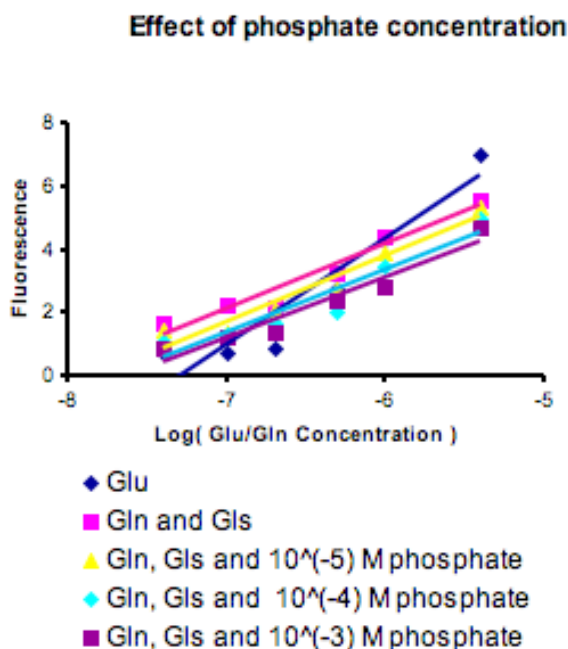
found that Glutaminase A from *E. coli* presents 38% identities, 56% positives and 2% gaps compared to the human brain/kidney type glutaminase. We have chosen to study the glutaminase from *E. Coli* because it was easily available, keeping in mind that the results may not be fully applicable to the brain/kidney enzyme.

### Effect of glutamate concentration on the conversion from glutamine to glutamate

As the next step, it was determined that the Amplex Red kit was reliable for assessing the amount of glutamate present in the reaction system. We confirmed the established range of linear dependence on concentration for Resorufin:  $4 \times 10^{-8}$  M –  $4 \times 10^{-6}$  M. The linear correlation of fluorescence as a function of concentration for 14 different concentrations of L-glutamic acid was established.

### Phosphate dependence of Glutaminase A from *E. coli*

The brain/kidney type glutaminase requires phosphate or other polyvalent anions for activation (Curthoys and Watford, 1995). Our results showed that glutaminase A from *E. coli* does not require phosphate for activation. The attempt to ensure physiological concentrations of phosphate (20-30 mM) failed since it destabilized the pH of the buffer and the emission spectrum of Resorufin that is pH-dependent. Lower concentrations of phosphate in the interval  $10^{-3}$  M to  $10^{-5}$  M did not affect the fluorescence values. ANOVA analysis showed no statistically significant difference between the values



**Figure 1**  
 Effect of phosphate concentration on the conversion from glutamine to glutamate

of fluorescence obtained from L-glutamine with the mentioned concentrations of phosphate and without phosphate ( $p$ -values  $>0.05$ ).

It was established that glutaminase from *E. coli* does not need phosphate for activation and the next experiments were performed without phosphate.

### The effect of time on the conversion from glutamine to glutamate

The effect of time on the progress of the reaction from glutamine to Resorufin was compared to the one from glutamic acid to Resorufin. The results are presented in Figure 2. When we added glutaminase, the fluorescence values from glutamine were lower than the ones from L-glutamic acid in the beginning of the reaction. At 30 minutes, the difference between the advancement of the glutamine and L-glutamic acid reaction becomes significant. ANOVA analysis revealed that after 60 minutes from the beginning of the reaction, the difference between the values of fluorescence when starting from L-glutamine and respectively from L-glutamic were not statistically significant ( $p$ -value  $>0.05$ ). At 130 minutes, the fluorescence values obtained when starting from glutamine and respectively from L-glutamic acid are visibly overlapping. The correlation coefficients were higher than 0.9 in all cases, confirming once again the linear dependence of the fluorescence on the logarithm of the concentration of the substrate.

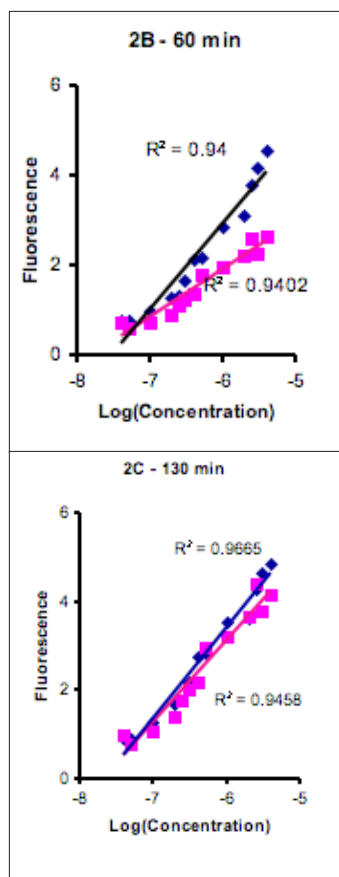
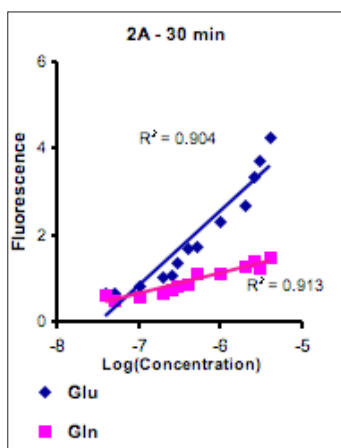


Figure 2

Effect of time on the conversion of glutamine to glutamate

### The effect of glutaminase concentration on the conversion from glutamine to glutamate

Figure 3 presents the results of simulation of inhibition of glutaminase by working with different amounts of glutaminase (glutaminase, glutaminase diluted 2fold and glutaminase diluted 5fold). At previous time points, the fluorescence values for the glutamine solutions were not significantly higher than the negative control. ANOVA analysis performed showed that there was no statistical significant difference between the values of fluorescence measured in the presence of the chosen concentration of glutaminase.

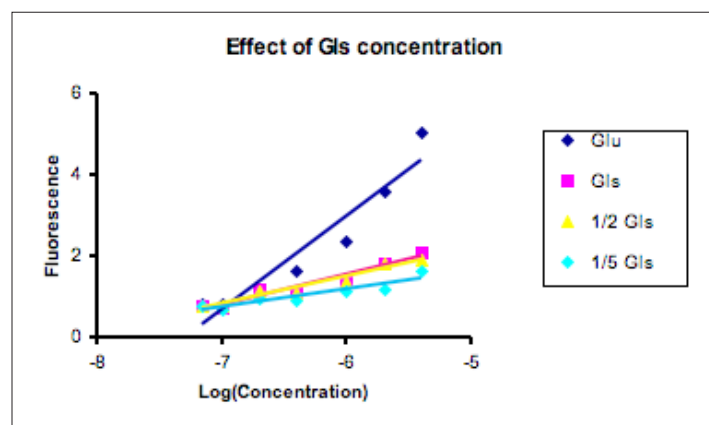


Figure 3

Effect of glutaminase concentration on the conversion of glutamine to glutamate

Working with such low concentrations of glutamine necessary for the detection range of Resorufin, implied that not only glutaminase, but also L-glutamine was a limiting reagent, which complicated the kinetics. The attempt to work with physiological concentrations of L-glutamine, in the micromolar range and very short time periods so that only a low amount of glutamine would be converted to glutamate and Resorufin concentration would still be in the detection range was unsuccessful. No increased fluorescence was observed with the addition of higher concentrations of glutaminase.

### The rate - limiting step

In figures 4A and 4B, we present the dependence of fluorescence of Resorufin on time for various concentrations of L-glutamic acid, and correspondingly, of L-glutamine.

The conversion from L-glutamic acid to Resorufin is almost instantaneous, the fluorescence values start off from very high values, while for L-glutamine the fluorescence values start off from nearly 0. Therefore, the conversion of L-glutamine to L-glutamic acid is the rate-limiting step, making it theoretically possible to perform the kinetical analysis.

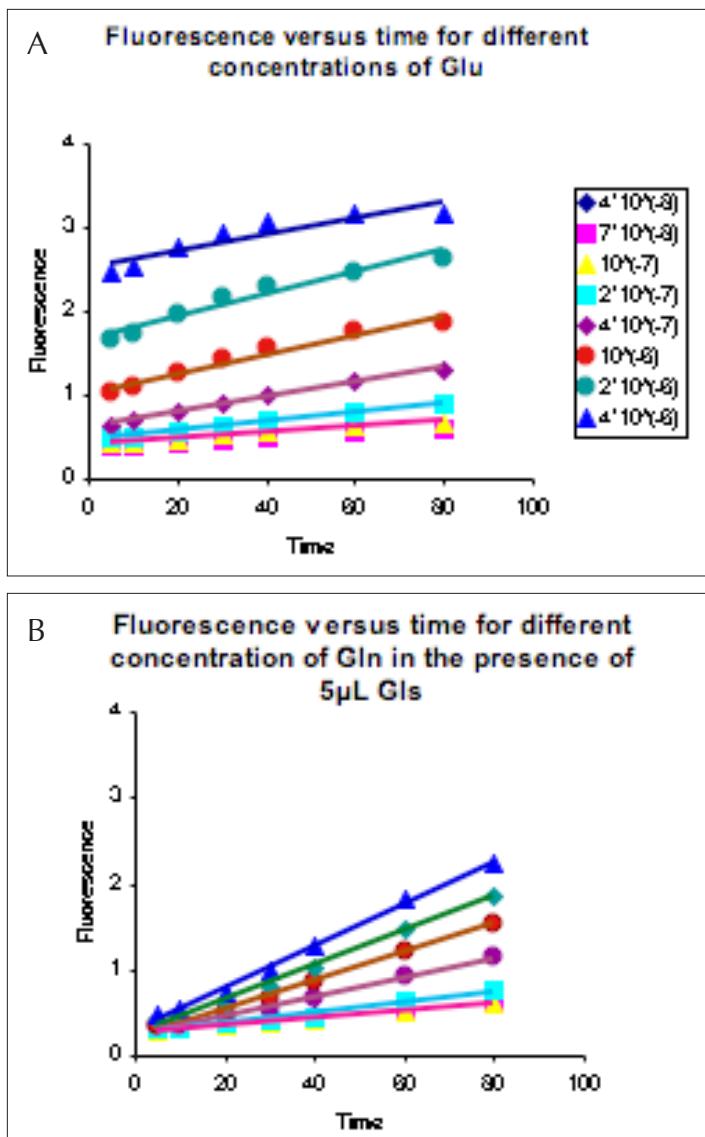


Figure 4

A. Fluorescence as a function of time for different concentrations of L-glutamic acid  
 B. Fluorescence as a function of time for different concentrations of L-glutamine

Not being able to see differences in the fluorescence values when varying the amounts of glutaminase, an analysis of slopes of the lines presented in graphs 4A and 4B was performed, but no significant difference was found, having p-values > 0.05.

**Assays with the known glutaminase inhibitors**

Next, we present the data obtained from the known glutaminase inhibitors. By working with three different concentrations of BPTES, no difference among the fluorescence values from the solutions with different concentrations of inhibitor and without inhibitor (p-value>0.05) was found.

We performed additional tests to see if BPTES interferes

with the Resorufin fluorescence. The results, the means of 3 determinations are presented in table 1:

Reagents Added	50µL buffer + 50µL Amplex red	50µL buffer + 50µL BPTES	50µL BPTES + 50µL Amplex Red	50µL H <sub>2</sub> O <sub>2</sub> + 50µL Amplex red
Fluorescence	1.0819	0.2471	1.5053	3.6665

Table 1

BPTES fluorescence results show increased values in the presence of Amplex Red relative to negative controls

By itself, BPTES decreases the fluorescence values under the value of the negative control (buffer+Amplex Red), while in combination with Amplex Red it increases them. We conclude that the assessment of the inhibition by BPTES is delicate because of its interference with the fluorescence of Resorufin.

The second available inhibitor was DON. The *k<sub>i</sub>* of DON was determined to be in the millimolar range (Shapiro et al, 1978). First of all, inhibition for a wide range of DON concentrations, from 10<sup>-2</sup> M to 10<sup>-7</sup> M, varying 10fold was tested and the fluorescence values were plotted as a function of the logarithm of L-glutamine concentration. The maximum inhibition was observed for DON concentrations of 10<sup>-4</sup> M and 10<sup>-5</sup> M, which is not in concordance with the accepted inhibition constant that is in the millimolar range. The fluorescence decreased with the decrease in concentration until 10<sup>-4</sup> M and 10<sup>-5</sup> M, and then it started increasing. For 10<sup>-2</sup> M DON, it was actually observed an increase in fluorescence. This pattern of variation of percent inhibition with concentration of inhibitor observed is not known.

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However, the correlation of concentration of substrate and velocity for the uninhibited reaction presented the predicted shape. The results are presented in Figure 5:

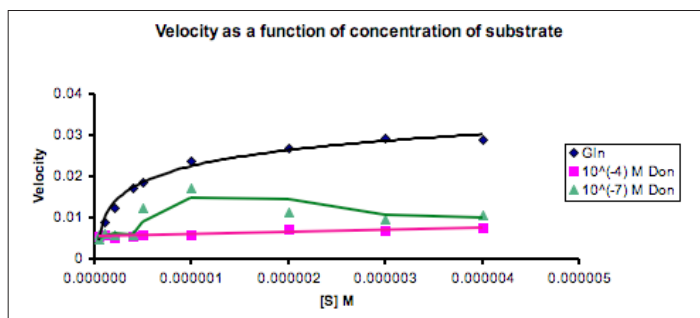


Figure 5

Velocity of the reaction as a function of the molar concentration of the substrate (L-glutamine).

The uninhibited reaction plot has the predicted shape and the plateau, corresponding to the maximum velocity ( $v_{max}$ ) occurs at  $2 \times 10^{-6}$  M L-glutamine. In the presence of  $10^{-4}$  M inhibitor, the inhibition is total since the fluorescence values are at the level of the negative control. In the presence of  $10^{-7}$  M concentration of inhibitor, the plot has the shape expected in the case of non-competitive inhibition.

The results in Table 2 show that DON does not inhibit any of the enzymes from the kit, on the contrary it produces an increase in fluorescence, increase that is slightly more pronounced at higher concentrations of DON. The test of DON fluorescence showed that at high concentrations, greater than  $10^{-3}$  M, DON generates fluorescence by itself.

Concentration of L-glutamic acid	Fluorescence without inhibitor	Fluorescence with $10^{-4}$ DON	Fluorescence with $10^{-7}$ DON
$10^{-7}$ M	0.8400	1.0673	0.9698
$10^{-6}$ M	2.0464	2.4494	2.3672

Table 2

Comparison of the fluorescence results when we started from L-glutamic acid with the fluorescence results obtained when we added different concentrations of DON to L-glutamic acid.

The results of the test of the activity of DON in the range  $10^{-4}$  M -  $10^{-10}$  M are presented in Figure 6.

Maximum inhibition is observed for concentrations of DON higher than  $10^{-6}$  M and the minimum inhibition is observed for concentrations lower than  $10^{-8}$  M DON. The calculated  $k_i$  of DON is  $7.26 \times 10^{-8}$  M.

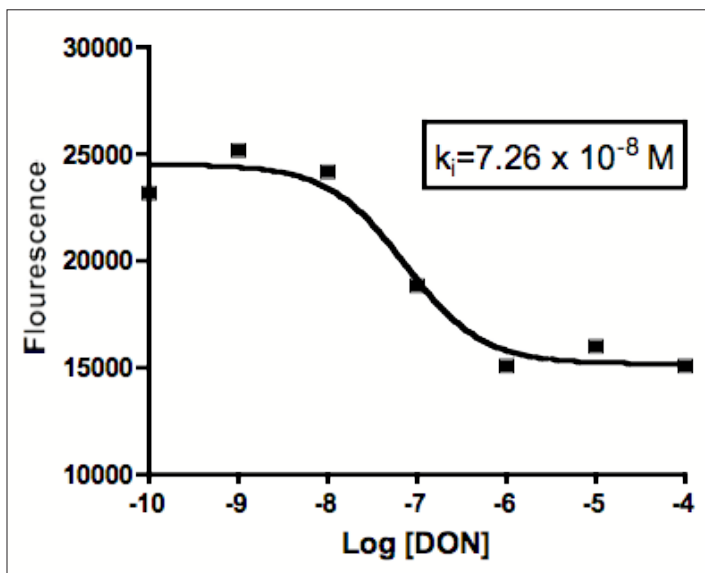


Figure 6

Effect of different concentrations of DON on the formation of Resorufin. The inhibition constant of DON on glutaminase A was determined to be approximately 73 nM

## Discussion

In this study, we set the parameters of a fluorescence-based assay for the identification of glutaminase inhibitors. Working with glutaminase A from *E. coli*, we found that it is not phosphate-dependent, that the complete conversion from glutamine to glutamate under the studied conditions takes 60 minutes and that DON is a much better inhibitor of the bacterial glutaminase compared to PAG.

Because of temporal and financial constraints, we used in our assays the commercially available glutaminase from *E. coli*. The first question that arises is its homology to other forms of the protein, and potential differences in the properties of the various forms. Almost all of the studies previously done on the brain-kidney type glutaminase used glutaminase extracted and purified from animal mitochondria. It is known that proteins with corresponding functions from different animal species have similar sequences yet different physical and chemical properties. The results show that the similarity of the amino acid sequences of the liver and brain/kidney type glutaminase was 78%. Despite this relatively high similarity, the brain-kidney type glutaminase is inhibited by glutamate, while the liver type is not, making questionable the possibility to find an inhibitor for the human brain type glutaminase using glutaminase A from *E. coli*. The ability of glutaminase A to be active independently of phosphate was another contrast to the human brain type glutaminase.

The nucleotide sequence for glutaminase from *E. coli* is not fully elucidated, so we could not assess the overlap between these two genes. However, the amino acid sequence is known for glutaminase from *E. coli*, thus we used BLASTP, to compare the amino acid sequences of the desired enzymes. We found that Glutaminase A from *E. coli* presents 38% identities, 56% positives and 2% gaps compared to the human brain/kidney type glutaminase.

Unfortunately, we know nothing about the location of the enzymes' active sites and if they fall within the identities, positives or gaps. We hope that studies regarding human brain type glutaminase's crystal structure will cast more insight into this problem. Knowing the enzymes' active sites locations is relevant only to the most common types of inhibition, competitive inhibition and non-competitive, where the inhibitor competes with the substrate for the enzyme's active site or binds to a site located near the active site. This still leaves open the possibility of allosteric inhibition to occur through different mechanisms beyond our ability to predict, by not to predict it by knowing the position.

It is known that the activity of glutaminase A from *E. coli* is at its maximum at pH 4.9 and is highly depen-

dent on pH. If our reaction conditions had included a pH of 4.9, the conversion of L-glutamine to L-glutamic acid would have been completed in less than a minute. However, Resorufin required a pH environment of 7.5. This sub-optimal pH caused a significant reduction in the activity of this enzyme. The results presented in figures 2A, 2B and 2C show that in 60 minutes the conversion from L-glutamine to Resorufin can be considered total. From a kinetical perspective, this time is favorable. Theoretically, it ensures that the conversion from L-glutamine to L-glutamate is the rate-limiting step and it allows us to observe differences in glutaminase activity at time points between 5 and 10 minutes, suitable for high throughput screening. However, the human glutaminase will be faster-acting at a pH physiological pH and the reaction might reach completion in less than 5 minutes.

For a while, the inability to simulate glutaminase inhibition by varying the amounts of the enzyme was attributed to an impurity present in the grade II glutaminase, glutamate decarboxylase that converts glutamic acid to gamma - amino butyric acid (GABA) and carbon dioxide (CO<sub>2</sub>). By increasing the amount of glutaminase, we would also increase the amount of glutamate decarboxylase, destroying more of the formed glutamate. Since glutamate decarboxylase and glutamate oxidase have binding constants in the same millimolar range, we thought that a competition between these two enzymes might occur. However, if the amount of glutamate decarboxylase were significant, the fluorescence values obtained from L-glutamine would never reach the values obtained from L-glutamic acid (see in figures 2B and 2C), making this possibility unlikely.

The phenomenon of cooperativity provides a better explanation. Above a critical concentration, the activity of glutaminase likely reaches a plateau. So, we diluted glutaminase below the critical concentration; however this increased the reaction time too much (after 3 hours the fluorescence values were still at the negative control level), making it impractical for high-throughput screening. The non-linearity in the relationship between glutaminase concentration and glutamate production is characteristic of reactions involving cooperativity, and glutaminase is known to function in a tetrameric configuration, which would account for this. The attempt to use glutaminase of grade V, without glutamate decarboxylase was unsuccessful because of its significantly decreased activity: after two hours, the fluorescence values from glutamine were not significantly higher than the one from the negative control. We thought that interference of its stabilizers (phosphate succinate and EDTA) with the reaction system may account for the decreased activ-

ity. As a preliminary step to setting up a reliable library screen methodology, we tested the known glutaminase inhibitors: BPTES and DON. We did not see inhibition with BPTES, possibly because it has an extensively conjugated structure that might have interfered with the fluorescence of Amplex Red. Despite our inability to mimic inhibition by varying the amounts of glutaminase added, inhibition by DON was observed for all concentrations of DON tested, with the exception of 10<sup>-2</sup> M. We tested further if DON does not inhibit other enzymes from the reaction system or if DON does not generate fluorescence by itself. Our results showed that DON generates fluorescence at high concentrations. Hartman presented a plausible explanation for this phenomenon: glutaminase catalyzes hydrolysis of the C5-C6 bond in DON to yield glutamic acid and diazomethane. (Hartman, 1973). Additional amount of glutamic acid formed by the degradation of DON will lead to an increase in the overall fluorescence.

The constant for inhibitor binding  $k_i$  of DON found in our studies, approximately 10<sup>-7</sup> M is not consistent with the one found in literature for the phosphate activated brain/kidney glutaminase, which is in the millimolar range (Shapiro et al, 1978). It is likely that DON is a much better inhibitor of the bacterial glutaminase than for the mammalian glutaminase. If this is the case, performing the high throughput screening assay on the bacterial glutaminase would not be useful, as we would identify a large number. We will identify a large number of good bacterial glutaminase inhibitors that would be significantly less potent as inhibitors of phosphate-activated glutaminase.

Nevertheless, we conclude that the search for glutaminase inhibitors holds great promise as a therapeutic target in schizophrenia and beyond. Recently, we received a low-copy plasmid, pET-15b, which contains the gene for the human phosphate-activated glutaminase (PAG) that will enable the synthesis of large quantities of this protein (Kenny et al, 2003). Obtaining PAG would ensure a more precise test for discovery of specific brain/kidney type glutaminase inhibitors.

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