

High Throughput Kinetic Assay for Screening Potential Inhibitors of Sick Hemoglobin Polymerization

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Abstract

The current manuscript describes a high throughput assay designed to identify organic compounds with potential inhibitory effects on sickle hemoglobin polymerization. The assay is fast, economic and reproducible. In just 20-minutes, a test compound can be screened for anti-polymerization activity at five different concentrations; each in quadruple; using as little as 10 mg of purified sickle hemoglobin. The assay was conducted in high phosphate buffer concentration (1.5M), a concentration that allows sickle hemoglobin to polymerize at a very low concentration of 50.0 μ M. The new assay was validated by evaluating the inhibitory effects of the amino acid phenylalanine, a standard control used in all gelation assays, and hydroxyl urea, the only FDA approved drug to treat sickle cell anemia. Phenylalanine showed a reproducible and concentration-dependent delay of sickle hemoglobin polymerization at a concentration range of 25-75mM. Hydroxyurea; although its action is thought to be through promoting fetal hemoglobin formation, was found to have direct inhibitory effects on sickle hemoglobin polymerization but at a high concentration range of 64-500mM. The assay was applied for random screening of several organic compounds and 2-thio-salicylic acid was identified to be a powerful inhibitor for polymerization at much lower concentration range of 0.1-1.6 mM.

Keywords: Sick cell anemia; Sick hemoglobin polymerization; High throughput screening assay; Drug discovery; 2-Mercaptobenzoic acid

Introduction

Sickle cell anemia is a genetic disease resulting from a single point mutation that results in the replacement of the hydrophilic glutamic amino acid at the 6th position of the hemoglobin β -chain with the more lipophilic amino acid valine. The mutant hemoglobin, frequently referred to as HbS, polymerizes inside the red blood cells at low oxygen tension leading to cell distortion into the sickle shape, hence the term sickle cell anemia, that in turn, leads to vascular occlusion and the serious deadly complications.

Although all aspects of the disease have been extensively investigated and reviewed [1-3]; a definite treatment for the disease still not at hand. A new gene therapy approach has been recently reported as potential alternative to drug therapy [4].

Attempts to discover new anti-sickling agents have always been hampered by the unavailability of quick screening method to assess potential *in vitro* anti-polymerization activity. In 1976, Hofrichter et al. developed the most widely used protocol to quantitatively evaluate anti-gelling effects of potential inhibitors [5]. In spite of the history and value of the assay in the identification of anti-sickling activities of several organic compounds, however, the assay requires availability of huge amounts of sickle cell blood to purify and obtain and a very high concentration of sickle hemoglobin of 3.0mM to induce the polymerization. For the application of Hofrichter's assay, see several of our previous related publications [6-13]. To offset that need of large amount of sickle hemoglobin, we developed special cells to reduce the needed amounts of hemoglobin to run the assay [14]. Even with success of that modification [15], the modified assay still requires the availability of whole sickle blood as a source of HbS. With the recent advancement in technology and the widespread use of high throughput technology, several new high throughput assay methods have been recently reported in the literature to screen compounds for anti-polymerization activities [16-18], unfortunately, both use whole blood samples.

Current Assay

The absence of rapid high throughput screening assay for anti-

polymerization activities together with the shortcomings of the recently reported assays, prompted us develop the current assay. The assay is fast and economic screening technique that does not use whole sickle blood, rather commercially available purified sickle hemoglobin provided by Sigma-Aldrich. HbS polymerization was achieved at a very low concentration of 50.0 μ M by conducting the assay in high phosphate buffer (1.5M) medium [19]. Using as little as 250 μ l of 40 mg/ml HBS solution; a given test compound can be evaluated at 5 different concentrations; each in quadruple, in a period of 20 minutes. HbS-polymerization was monitored using plate reader technology and through recording the optical density at 700 nm wave length [20]; every 30 seconds for each well over 20-minutes period. The data points are saved as excel file, and the polymerization curve is generated using Sigma Plot program. The current assay monitors HBS polymerization under deoxygenated condition with time for each compound concentration. It must be indicated that monitoring the delay in the polymerization process is a very valuable approach to decrease the precipitation of potential sickle cell crisis *in vivo*. All what is needed is few seconds of delay in the cellular polymerization to allow the red blood cells to go through the vascular venous bed, where the oxygen tension is low, to become re-oxygenated again in the arteries where it does not polymerize [21].

Two compounds were selected to validate the assay: the amino acid phenyl alanine and the drug hydroxyurea. The choice of phenylalanine for evaluation is based on its frequent use as a positive control in the traditional anti-polymerization assay developed by Hofrichter et al.

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and hydroxyurea was chosen for evaluation because it is the only FDA approved drug for treating sickle cell anemia. After confirming the assay validity, several organic compounds were screened for activity; and 2-mercapto benzoic acid (thiosalicylic acid) was identified to be a very potent inhibitor for Hbs polymerization in a concentration-dependent manner. Figure 1 depicts the chemical structures for the evaluated compounds.

Experimental

Material and methods

Potassium dihydrogen Phosphate, Potassium meta-bisulfite, Phenylalanine, 2-mercapto-salicylic acid, hydroxyurea: Sigma Aldrich, Purified Sickle hemoglobin powder provided by Sigma - Aldrich provided as 50% product stabilized with 50% buffer, Synergy Plate reader, 96-Half well plates.

Kinetic gelation assay

A solution of 200.0 μM sickle hemoglobin was prepared by dissolving 10 mg of powdered Hbs supplied by Sigma-Aldrich (containing 50% stabilizing buffer), in 250 μL of 1.5M, pH 7.4 potassium phosphate buffer. Each of the half-well plate cells was charged with 10 μL of this Hbs solution. To each well, 20 μL of the test compound (prepared in 1.5 M phosphate buffer), plain buffer as control, were added. The polymerization process was triggered by adding 10 μL of 1.0 M potassium meta-bisulfite solution to each well and the plate was introduced into the plate reader, preheated to 37°C, and programmed to record the optical density at 700 nm, for each well every 30 second over a total period of 20-minute (total of 40 recorded points for each well). The generated excel file was then loaded into Sigma plot program to generate the kinetic plots showing the delay in polymerization as a function of time in seconds (Figures 2-4). A total number of the

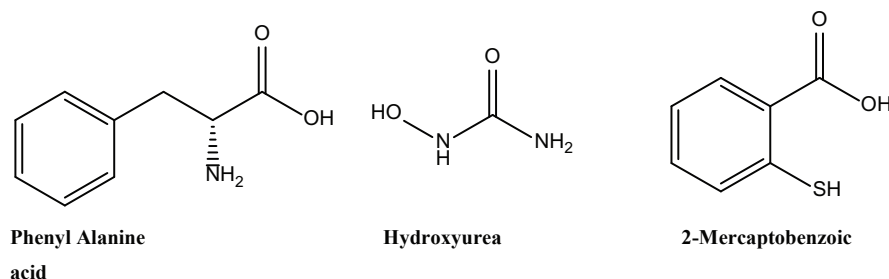
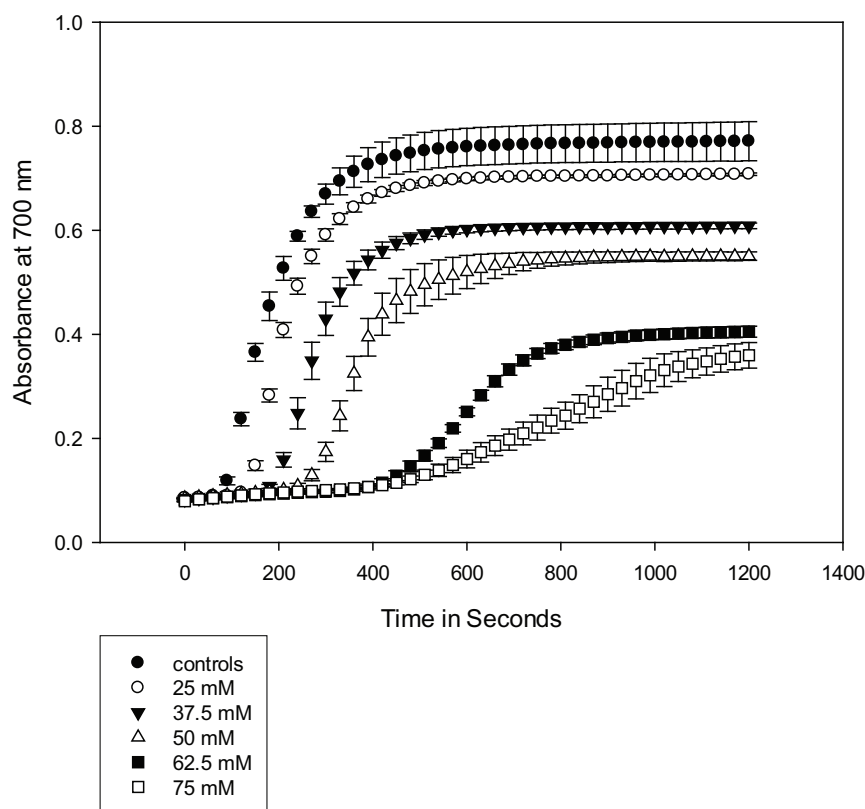
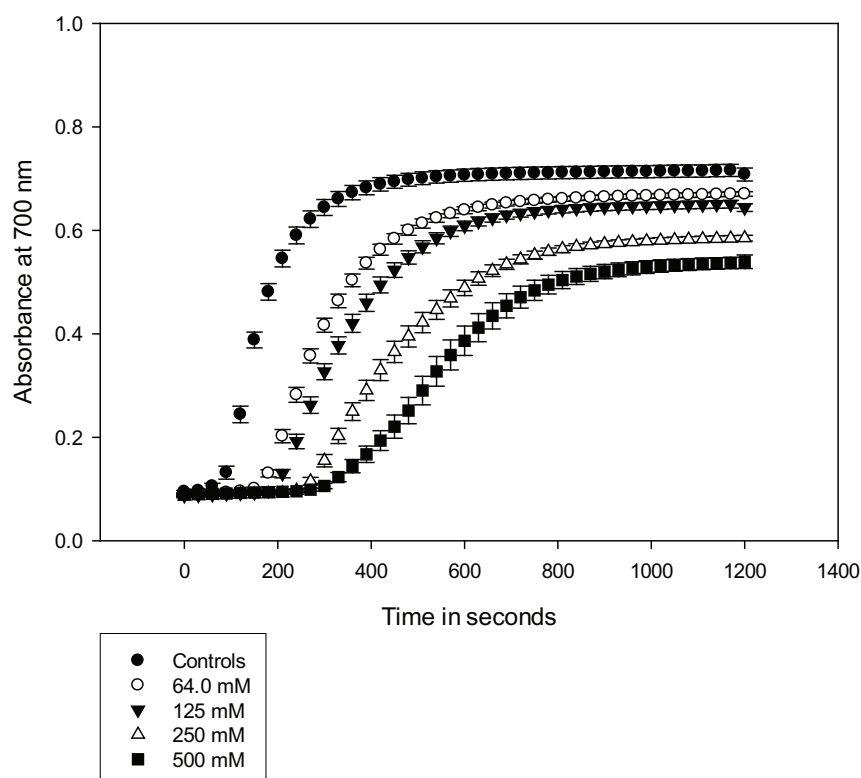


Figure 1: Chemical structures for tested compounds.



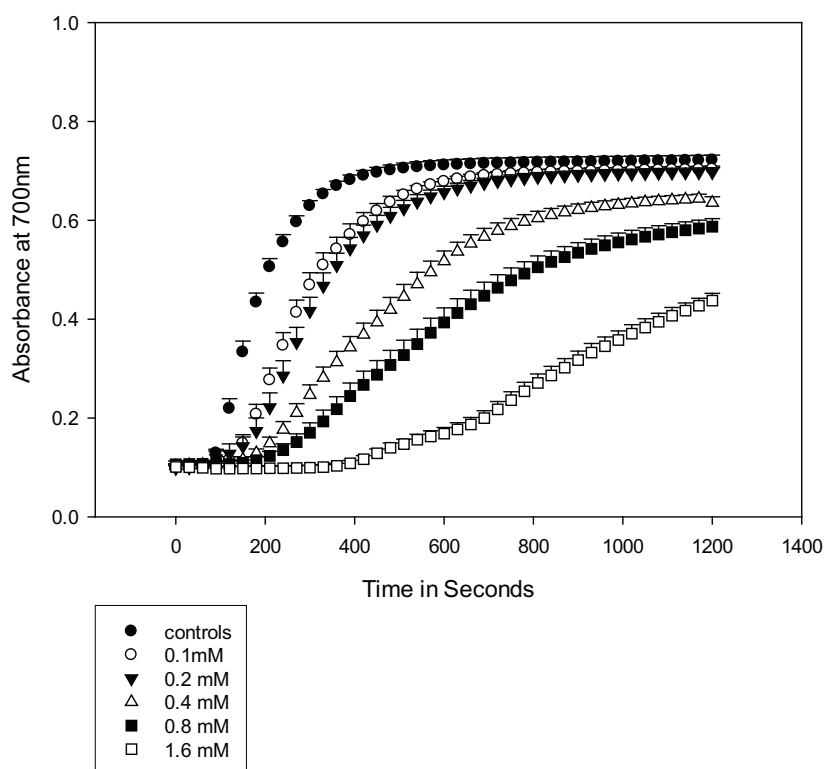
*All data points are average of 5 different runs, in quadruple application for each concentration.

Figure 2: Inhibitory effects of phenylalanine on HBS polymerization*.



*All data points are average of 5 different runs, in quadruple application for each concentration.

Figure 3: Inhibitory effects of hydroxyurea on HBS polymerization*.



*All data points are average of 5 different runs, in quadruple application for each concentration

Figure 4: Inhibitory effects of 2-mercaptobenzoic acid on HBS polymerization*.

plate wells employed were 24: one as blank, three as controls (Hobs, buffer, and sodium meta-bisulfite), and twenty wells for 5 different concentrations of the test compound; each in quadruple. The total assay volume in each well is 40 μ L. The final Hobs concentration after mixing with all reagents is now 10 mg/ml (50.0 μ M).

Results and Discussion

Phenylalanine

Figure 2 depicts the effect of phenylalanine concentration on the kinetics of Hobs polymerization. The amino acid was found to delay the polymerization and shift the polymerization curve to the right, in a concentration-dependent fashion, over a concentration range of 25-75mM.

Hydroxyurea

On the other hand, hydroxyurea was evaluated for direct inhibitory effects on the polymerization process under the current assay conditions. As stated earlier; hydroxyurea was chosen for evaluation because it is currently the only FDA approved drug for treating sickle cell anemia. The widely accepted mechanism of its action is through promoting the biosynthesis of fetal hemoglobin (HbF), a hemoglobin species that, unlike HSB, does not polymerize at low oxygen tension. We were interested to assess whether that is the only beneficial effect of the drug, or it may have direct inhibitory effect on the polymerization process as well. Figure 3, depicts that hydroxyurea has direct inhibitory effects on the polymerization process, though at very high concentration range of 64-500mM. The latter, confirms that the *in vivo* mechanism of action of the drug cannot be through direct inhibition of the polymerization process. However, testing of hydroxyurea in our assay in addition to excluding the direct inhibitory effects on Hobs polymerization, as a possible second mechanism, provides evidence that the assay can assess the anti-polymerization activity in a concentration dependent manner.

2-Mercapto benzoic acid (Thiosalicylic acid)

The reproducibility in collecting data screening data for phenyl alanine and hydroxyurea prompted us to randomly screen few compounds that existed in the lab. To our surprise and delight; thiosalicylic acid was found to have inhibitory activities at much lower concentration range compared to that of phenylalanine and hydroxyurea. As depicted in Figure 4, thiosalicylic acid inhibits Hobs polymerization in a concentration range of 0.1-1.6 mM. It is must be indicated that thiosalicylic acid has been previously approved in the past for use as analgesic and anti-inflammatory drug. The latter pharmacological effects are required for patients with sickle cell disease who always suffer from painful crises.

Conclusion

A new high throughput kinetic assay has been developed to screen organic compound for potential anti-sickling activity. The assay has been proven to be accurate, reproducible and economic regarding the amount of sickle hemoglobin needed to screen compounds. The new assay allows screening of the test compound at 5 different concentrations, each in quadruple in a total time of 20 minutes. Screening of several organic compounds with the current assay revealed that 2-mercaptobenzoic acid is a potent inhibitor for Hbs polymerization at a concentration range of 0.1-1.6 mM.

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