

Abstract

Fungal infections occur when fungus invades the tissue, which can grow and affect the whole body if left untreated. These infections are difficult to treat as there are few drugs on the market that can target fungi and they have serious and harmful side effects towards the patient. In an effort to stop fungal infections, we are developing a new method of inhibition. An enzyme critical for life, methionine synthase, has a key difference between fungi and humans that can be exploited. An inhibitory molecule can be made to selectively target fungal methionine synthase based on this difference. Utilizing Autodock, a modelling software, molecular modelling was done to develop theoretical molecules that target the fungal enzyme. Based on the theoretical modelling, a library of potential inhibitors was synthesized. The potential inhibitors were tested in an assay measuring the activity of the fungal enzyme in the presence of our inhibitors. The molecules are further tested in a fungal growth assay which show zones of inhibition that would prove our molecules are active.

Background

Fungal infections are a common public health concern especially in regards to immunocompromised patients who are at a greater risk of death in such cases.¹ As there is an increase of drug resistant fungi, there is a need for new types of antifungal drugs. Methionine Synthase (MetSyn), an enzyme which converts homocysteine (Hcy) to methionine using a substituted folate molecule (Fig. 1), could provide a potential pathway for safe antifungal treatment.

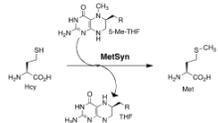


Figure 1: Net reaction of MetSyn

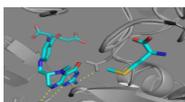


Figure 2: In fungal MetSyn, folate and Hcy bind in close proximity.

Therefore, compounds designed to inhibit fungal MetSyn could serve as antifungal drugs which should not affect the biochemistry of the patient.

The structure of MetSyn used by fungi is different than that used by mammals.² In the fungal enzyme, folate and homocysteine bind to two pockets that are close together (Fig. 2). In humans, the folate and Hcy binding pockets are very far apart. This makes it possible to design synthetic compounds which could bind the folate and homocysteine pockets at the same time in the fungal enzyme, while being unable to competitively bind the mammalian MetSyn form (Fig. 3).



Figure 3: Cartoon model for a molecule binding fungal MetSyn, but will not properly fit inside human MetSyn.

General Design of Molecules

A molecule specific for fungal MetSyn would need a folate mimic attached to a Hcy mimic by some short linker (Fig. 4). This should properly fit in the fungal enzyme, because the two binding pockets are so close together.

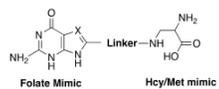


Figure 4: Generalized Inhibitor Molecule

A molecule outlined from Figure 4 would work as a competitive inhibitor. The folate mimic would bind and block natural folate, and the homocysteine mimic would prevent the natural homocysteine from binding.

A molecule such as this would not be expected to interfere with the mammalian MetSyn enzyme, as it would lack the ability to span both binding pockets, resulting in non-specific binding.

Molecular Modeling of Potential Inhibitors

Using the modeling program AutoDock, we can virtually screen potential molecules to confirm they can reach both binding sites (Fig. 5).³

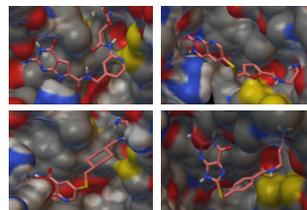


Figure 5: The four molecules above give a representation of four good inhibitors, binding in the folate and Hcy pockets

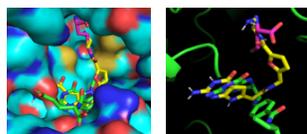


Figure 6: Inhibitor H is a prime example of a good inhibitor as it binds in open conformation (left) and fits in the closed conformation (right) of MetSyn

When modeling the potential inhibitors, we look to see that our folate mimic is in the folate binding pocket and that our amino acid tail is in the Hcy binding pocket. These results confirm that a single molecule can simultaneously bind both pockets within the fungal enzyme.

MetSyn can exist in two conformations: open and close. It conforms to the closed upon substrates binding. A good inhibitor should bind to the open enzyme while still being able to fit in the closed conformation, such as that shown in Figure 6.

Representative Inhibitor Syntheses

Figure 7 represents the synthesis of one of our compounds (inhibitor U). Many of our inhibitors follow similar synthetic steps, putting together a folate linker, and amino acid tail.

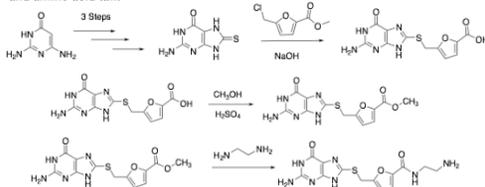


Figure 7: Representative synthesis of inhibitor U, working towards the same general design of a folate mimic, linker, and amino acid tail.

We are also exploring alternative strategies for the inhibitor, such as the use of a pyridine linker and glutamic acid as the source of the amino acid tail (Figure 8). This method uses a key DBU-promoted reaction developed in our lab.⁴ We are in the process of improving these reaction conditions to allow for better recovery of this new potential inhibitor.

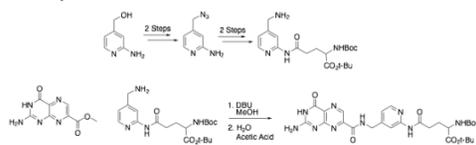


Figure 8: Synthesis of a new inhibitor candidate currently under development

Inhibitor Candidates

By using methods similar to those shown in Figures 7 and 8, a number of similar potential inhibitors have been synthesized (Fig. 9). This library of molecules provides a number of subtle structural differences. The hope is that these small changes will help identify a molecule that has the perfect "fit" in

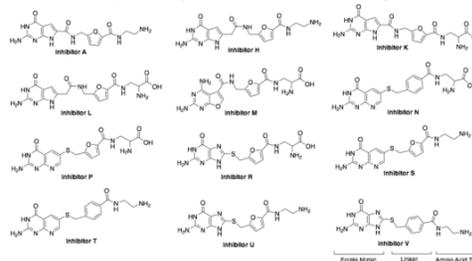


Figure 9: Current library of inhibitors that are being tested, all with slight variation but following same general design.

Testing inhibitors via Enzyme Assay

To test whether our inhibitors can effectively inhibit fungal methionine synthase, a fluorescence assay is used.⁵ A fluorescent reagent is added, and the fluorescence correlates to Hcy concentration. This allows us to track the activity of the enzyme indirectly with the concentration of Hcy (Fig. 10).

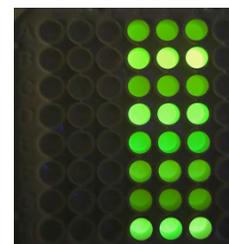


Figure 10: Well plate showing results from a fluorescence assay. High fluorescence represents high Hcy concentration and low enzyme activity.

The first set of triplicates are blanks, followed by a triplicate of the positive control which contains Hcy and fluoresces the most. Third is the enzyme reaction, which consumes Hcy and lowers the fluorescence. The last five triplicates are the various inhibitors tested in that trial, with varying amounts of inhibition. High fluorescence means successful inhibition, as the enzyme can't consume Hcy to make methionine.

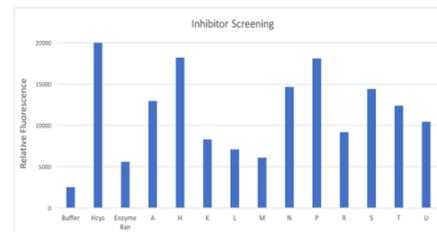


Figure 11: Preliminary inhibitor screen; High Fluorescence correlates to low enzyme activity

This assay identifies which inhibitors show the most promise. From these results, inhibitors H and P appear most active, as the high fluorescence indicates low production of methionine.

Fungal Growth Assay

Another way we tested our inhibitors was through a fungal growth assay, referred to as a Kirby-Bauer test. Discs containing our compounds are exposed to various microorganisms. Successful inhibitors will prevent fungal and bacterial growth, which can be shown by zones of inhibition on the plates.

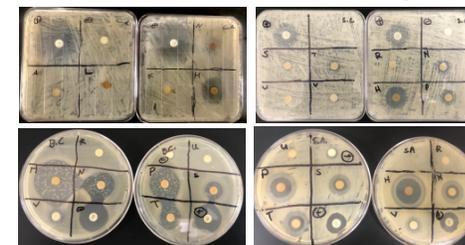


Figure 12: Inhibitor activity against two strains of fungi *Candida albicans* (C.A) and *Saccharomyces cerevisiae* (S.C), as well as two bacterial strains *Bacillus cereus* (B.C) and *Staphylococcus aureus* (S.A).

Inhibitors such as H, P, N, S, and T show promise in the fungal growth assay, which agree with the results of the fluorescence assay. When these results are paired together, these molecules can successfully inhibit the methionine synthase enzyme, and are able to stop the growth of various fungi and bacteria. From this test, we can show that our inhibitors are successful in killing and stopping the growth of various pathogenic organisms.

Conclusions and Future Directions

Using molecular modelling, potential inhibitor candidates were found and then synthesized based on the best structures. The library of synthesized inhibitors were tested in a fluorescence assay to determine activity against the fungal enzyme. These compounds were then tested against various microorganisms in the fungal growth assay. From these, we have developed a library of working inhibitors that stop the growth of various microorganisms. Moving forward, the mechanism for inhibiting the growth of the fungi can be tested in a modified Kirby-Bauer test. More modelling can be done to develop more potential inhibitors, and our library of inhibitors can be expanded on and tested further.

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