Point of VIEW

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Smaller Is Better for Antibiotic Discovery

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ABSTRACT A recent study demonstrates the use of fragment-based drug discovery and virtual screening to develop inhibitors of biotin carboxylase that exhibit antibacterial activity. The work not only further validates biotin carboxylase as a target for antibiotic research but also provides a framework for using fragment-based drug discovery in antibiotic development.

As the problem of antibiotic-resistant pathogenic bacteria steadily worsens, there remains an urgent need to develop new antibiotics and identify new targets for antibiotics (*1*). Historically, antibiotics have been discovered by natural product screening. However, natural product screening has been replaced by high-throughput screening (HTS), where a target molecule is screened against a library of a million or more potential inhibitors. Unfortunately, for antibiotic discovery HTS has not lived up to expectations. O'Shea and Moser have argued that the physicochemical properties of molecules that can penetrate the membrane(s) of prokaryotes are substantially different from the properties of molecules effective against eukaryotic targets (*2*). Yet, in an article in this issue Mochalkin *et al.* (DOI 10.1021/cb90000102) (*3*) point out that most pharmaceutical HTS libraries were designed for targets within eukaryotic cells. To circumvent this bias, Mochalkin *et al.* use a combination of fragment-based drug discovery (FBDD), virtual screening, structure-based drug design, and combinatorial synthesis to develop a molecule with antibacterial activity. The target for that molecule is biotin carboxylase.

Biotin carboxylase is one component of the multienzyme complex acetyl-CoA carboxylase (ACC), which catalyzes the first committed step in fatty acid biosynthesis (*4*). As with all biotin-dependent enzymes, ACC utilizes a two-step reaction mechanism shown in Scheme 1.

In the first half-reaction, biotin carboxylase catalyzes the ATP-dependent carboxylation of biotin. In the next reaction, catalyzed by the carboxyltransferase subunit, the carboxyl group is transferred to acetyl-CoA to make malonyl-CoA. *In vivo*, biotin is covalently attached to the biotin carboxyl carrier protein (designated as Enzyme-biotin in Scheme 1). In bacteria each of the three components of ACC are separate proteins where biotin carboxylase and carboxyltransferase retain their enzymatic activity after purification and will utilize free biotin as a substrate in place of the carrier protein (*4*). In eukaryotes, all three functions of ACC are combined within a single polypeptide.

Validation of biotin carboxylase as a target for antibiotic development was recently obtained by Pfizer (*5, 6*). Using bacterial cell screening, the Pfizer group found that pyridopyrimidines exhibited antibacterial activity against certain types of Gram-negative bacteria. By making pyridopyrimidine-resistant mutants of *Hemophilus influenzae* and mapping the resistance-conferring gene, biotin carboxylase was identified as the target of the pyridopyrimidines. A particularly potent (K_i = 0.8 nM) member of this class of molecules is shown in Figure 1, panel A. The pyridopyrimidines were bactericidal both *in vitro* and *in vivo* and most importantly did not inhibit human biotin carboxylase. Crystallographic analysis revealed the inhibitor binds in the ATP binding site. Comparison of the binding of ATP (*7*) (Figure 1, panels C and D) and pyridopyrimidines (Figure 1, panels A and B) illustrates how the same active site residues are used to bind ATP and the pyridopyrimidines. These interactions between biotin carboxylase and the pyridopyrimi-

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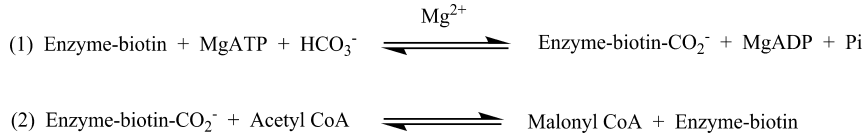
Scheme 1.



dines were used as a guide for developing inhibitors using virtual screening and FBDD.

The basic premise behind FBDD is that molecules of less than 250 Da are screened, which means that a greater proportion of chemical space can be tested for binding to a target when compared to conventional HTS methods (*8, 9*). In other words, libraries containing large, complex molecules are less likely to yield a molecule that efficiently binds to the target (as gauged by the binding affinity relative to the molecular weight), whereas a library of smaller, simpler molecules is more likely to find a ligand efficient "hit". Another advantage of using small, simple molecules to screen a target is that the modest number of compounds comprising these libraries allows them to be assembled with an eye toward ease of synthetic follow-up. This aspect of FBDD is especially important because the major drawback to screening small simple molecules is that the binding affinity is usually low. To identify molecular fragments that bound to biotin carboxylase, Mochalkin *et al.* screened fragment libraries virtually and with a combination of high-concentration biochemical screening and saturation transfer difference NMR (STD-NMR).

Virutal screening was based on three-dimensional shape and electrostatic complementarity. Of the 2.2 million compounds screened, 525 were selected for testing as inhibitors. Interestingly, the number of compounds with an IC_{50} of 10 μM or less was 200-fold higher than was obtained by a conventional HTS screen, which shows that FBDD combined with virtual screening can be more fruitful than a standard HTS approach. The second method to identify fragments that bind to biotin carboxylase was to screen a library of 5200 molecules using high-concentration biochemical screening and STD-NMR; STD-NMR is particularly useful for FBDD because it detects weak binding molecules. The combined biochemical and STD-NMR approach yielded six fragments with IC_{50} values of 95 μM or less.

All of the fragments discovered by the fragment and virtual screening approaches that bound to biotin carboxylase had very similar pharmacophore features. Characterization of the fragments by X-ray crystallography revealed that they all bind in the ATP site of biotin carboxylase just like the pyridopyrimidines. In fact, the pharmacophore features common to all of the fragments are also observed in the pyridopyrimidines. For example, in Figure 1, panel E an amino-oxazole fragment detected by virtual screening has the same complement of hydrogen bond donors and acceptors as the pyridopyrimidines (Figure 1, panels A and E, atoms shown in red). As a result, the amino-oxazole fragment interacts in the same manner and with the same active site residues in biotin carboxylase (Figure 1, panel F) as the pyridopyrimidines (Figure 1, panel B) and ATP (Figure 1, panel D). While the amino-oxazaole fragment in Figure 1, panel E only had an IC_{50} of 21.5 μM the structure was very accessible to further modification to increase the binding affinity.

One of the major advantages of FBDD compared to the large complex molecules found in traditional HTS libraries is that fragment libraries are often selected to facilitate chemical modification in order to increase the affinity and generate lead compounds. There are several strategies for chemical modification such as fragment growing, merging, and morphing. In the work by Mochalkin *et al.*, a very impressive example of fragment growing is the use of the amino-oxazole fragment in Figure 1, panel E to generate a compound with higher affinity for biotin carboxylase that also has antibacterial activity. Amide analogs of the amino-oxazole fragment could be easily synthesized, and in only two iterations the dibenzylamide analog (Figure 1, panel G) was found to have a 200-fold increase in affinity (IC_{50} = 0.125 μM). While the amino-oxazole moiety provided an "anchor" for binding to the ATP site, the dibenzylamine moiety interacted with a patch of hydrophobic residues on the enzyme to increase the affinity and generate antibacterial activity against Gram-negative organisms (Figure 1, panel H). The antibacterial effect was likely due to inhibition of biotin carboxylase because treated bacteria showed a decrease in fatty biosynthesis. A kinase screen also showed that the dibenzylamide analogue did not inhibit 40 human kinases or human biotin carboxylase.

While the work by Mochalkin *et al.* provides an excellent framework for using FBDD to develop new antibiotics, the work suggest several challenges for using biotin carboxylase as an antibiotic target. For instance, modifications of the fragments that are effective against Gram-positive bacteria need to be discovered. These compounds could be particularly revealing given the recent report that fatty acid synthesis cannot

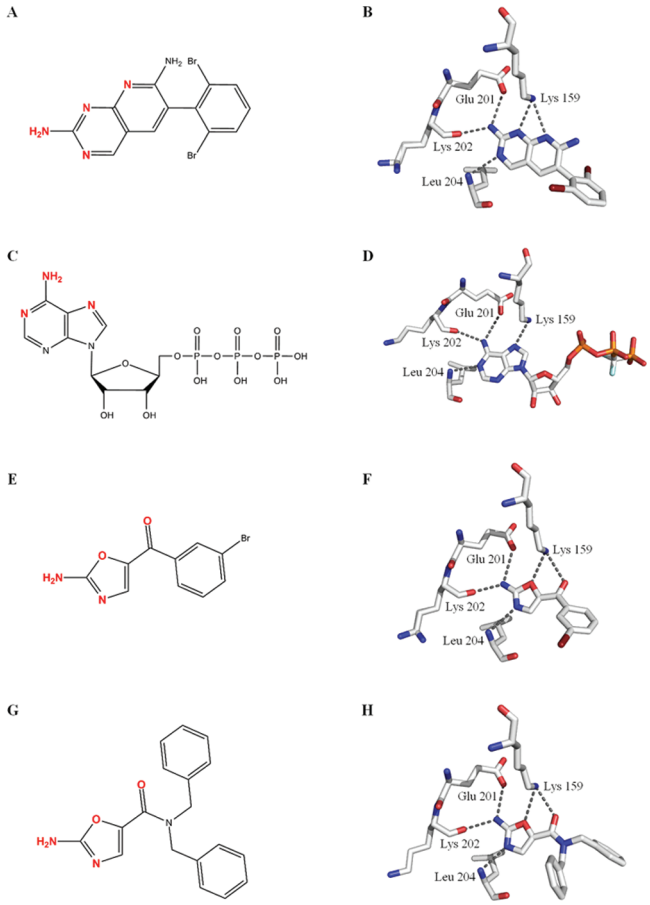


Figure 1. Biotin carboxylase inhibitors. Panels on the left-hand side (A, C, E, and G) show the structure of the inhibitors and the substrate ATP. The atoms that represent similar pharmacophore features and that interact with residues in biotin carboxylase are highlighted in red. The panels on the right-hand side (B, D, F, and H) show the interaction of the inhibitors with active site residues in biotin carboxylase. Panels A and B) Pyridopyrimidine; C) structure of the substrate ATP; D) the ATP analog ADPCF$_2$P bound to biotin carboxylase; E and F) amino-oxazole fragment detected by virtual screening; G and H) amino-oxazole fragment generated by fragment growing with dibenzylamine.

be targeted in Gram-positive bacteria (*10*). Moreover, can fragments with antibacterial activity be discovered that bind in the biotin binding site of biotin carboxylase? If so, then fragment linking with the molecules that bind in the ATP site can be done to perhaps make a very potent antibiotic. The research by Mochalkin *et al.* has laid the groundwork for addressing these challenges.

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