DIRECTING DRUG EVOLUTION

Directed evolution is poised to change small molecule discovery and provide greater access to “drug space.” Sarah Webb looks into the evolving drug discovery landscape.

Credit: T. Shafee

Drug discovery research comes in two primary flavors. First there is the traditional medicinal chemistry approach where chemists use specialized reactions to carefully design and synthesize small molecule drug candidates that can then be tested against defined molecular targets. The second approach involves the use of directed evolution techniques, which have recently propelled drug discovery efforts for large molecules such as therapeutic antibodies.

But in between these two approaches, there exists a vast range of “drug space” that researchers haven’t been able to address, says medicinal chemist Andrew Davis of AstraZeneca in Mölndal, Sweden (1). Up to 80 percent of drug targets may actually reside inside cells rather than on their surfaces. These are targets involved in complex protein interactions or involve specialized proteins such as phosphatases that are currently considered undruggable.

Interdisciplinary research efforts are starting to bridge the gap between small molecule drug discovery and biology through the use of a range of new tools. And directed evolution approaches are showing real promise, unlocking novel and more efficient chemical transformations, providing ways to understand disease targets, and offering new options to probe drug space.

Tackling Targets

Before chemistry, structural biology and genomics moved drug discovery toward target-based approaches, it was phenotypic screens that were the primary way researchers found new drug targets. In fact, most antimicrobial targets were discovered using phenotypic screening, says Elizabeth Winzeler of the University of California at San Diego.

To identify an antimicrobial target, researchers studied organisms that developed resistance to a given agent. They would then do a range of plasmid mapping, adding back complementary pieces of plasmid DNA until finding the section that restored sensitivity to the antimicrobial compound.

“People who work in drug discovery really, really want to know the target, because there are so many tools for target-based drug discovery that just don’t exist for phenotypic screening,” Winzeler adds. But she and her colleagues have developed a strategy that streamlines the path from a resistance phenotype to target identification. They call it *in vitro* evolution and whole genome analysis.

Winzeler’s team subjects an organism to a molecule at lethal levels, which prompts some cells to develop resistance. In many cases, roughly half the time, genetic mutations show up in the direct molecular target, according to Winzeler. By finding and analyzing changes in the organism’s genomic sequence, it’s possible to isolate specific mutations and zero in on the molecular target.

The idea started with mapping traits in yeast, where they were able to demonstrate repeatedly that those genome changes indicated that organisms had acquired drug resistance. From there, Winzeler’s group applied the strategy to look at a variety of gene mutations conferring resistance in the malaria parasite, *Plasmodium* (2). Over time, they’ve even moved the strategy into looking at clinical trial candidates. The Bill and Melinda Gates Foundation funds a large consortium known as the Malaria Drug Accelerator (MalDA), that includes Winzeler, to explore a large collection of compounds and targets to characterize that disease’s resistome.

However, when possible, they like to use their strategy in yeast because yeast are easier to work with and provide faster results. Recently, they applied a yeast-based strategy to identify

Bryan Dickinson from the University of Chicago sees directed evolution tools being used in more real world drug discovery applications.
Credit: I.C. Hsiao.

drug targets for Chagas disease (3). The technique is also widely used in tuberculosis.

The strategy isn't limited to infectious disease though: resistance mutations also pop up with cancer drugs in human cells. Eventually researchers could develop a diagnostic test for resistance to a particular chemotherapy as a component of personalized medicine, Winzeler says. "This is something we deal with all the time in the malaria field," she says. "But you could also think about PCRing up genetic markers for Gleevec."

And then there is the possibility of identifying new cancer drug targets. When chemists find novel natural products in a sponge that stop cell division, such molecules can be incredibly difficult to synthesize. But with using an evolution-based strategy that reveals a target, researchers might not need to worry about complex synthetic chemistry. They could instead design other easier-to-synthesize molecules that interact with that target, according to Winzeler.

Evolving enzymes

Drug discovery has been relying on chemical synthesis to create screening libraries of molecules and to refine hits. Chemical space could encompass a mind-boggling 10^{60} chemical combinations, yet existing natural products only cover a small fraction of this. Synthetic organic chemistry can't keep pace with current demands for molecules, even with increasing automation and virtual screening approaches.

Molecular biology and evolutionary tricks have expanded the toolkit when it comes to synthesizing small molecule libraries and drug candidates. Directed evolution is one strategy that AstraZeneca's Davis thinks could have a significant impact in drug discovery.

Caltech chemical engineer Frances Arnold pioneered the use of directed evolution to develop enzymes for chemical catalysis,

and she thinks the field has made amazing progress. "We have added whole new bonds and even new chemical elements to Nature's biocatalytic repertoire." Her group developed enzymes that form bonds between carbon and silicon, as well as carbon and boron, and specialized reactions that produce moieties that lock in useful stereochemistry in complex chemical cores (4).

As industry strives to reduce waste, improve quality, and cut costs, enzymes are replacing synthetic chemistry in drug manufacturing. Though not all molecules can be made with enzymes or costs might not add up yet, she says "That will change." In addition, enzymes can actually be employed to perform useful tasks for small molecule drug developers such as late stage modifications, many of which couldn't be achieved using traditional organic synthesis methods.

Researchers would also like to harness the muscle of complex biosynthetic enzyme systems to augment chemical libraries. Multi-domain enzymes or complexes known as polyketide synthases produce an array of interesting and diverse natural products in bacteria, fungi and plants. The products of these enzymes are notoriously difficult to synthesize using organic chemistry, and researchers have struggled to engineer chimeric or hybrid enzymes that could refine these products or make entirely new ones, says chemist Gavin Williams of North Carolina State University.

Last year his group applied a limited evolution approach to the problem, developing a polyketide synthase that preferentially swapped in a segment with a terminal alkyne, rather than its natural substrate (5). This moiety is valuable to synthetic chemists because they can then use click chemistry to hook an enzyme produced chemical fragment to another fragment with an azide. "That provides a platform for more efficiently producing new semi-synthetic derivatives," Williams says.

Williams and his team have also developed biosensors for evolving and screening vast libraries of polyketide synthases,

Phage-assisted Continuous Evolution (PACE) system developed in the lab of David Liu for protein evolution studies. Credit: D Liu.

and his team even developed a sensor based on a transcription factor that turns on either a fluorescent signal or an antibiotic selection marker when a desired polyketide product is present. By using these genetically encoded biosensors, his team can screen and select from libraries of polyketide synthases and variants (6). “We’re able to screen millions of mutants for their ability to produce a natural or unnatural polyketide,” Williams explains. Using these tools, he says, they’re setting the stage for evolving polyketide biosynthesis pathways for the first time.

Generation after generation

The jump from evolving hundreds of variants to evolving millions or even billions has come about through the development of in vivo evolution techniques that take the time-consuming steps out of the hands of researchers. Though well-developed, many classic evolution techniques such as phage display and mRNA display require hands-on work meaning evolution experiments can only proceed at one generation per week or even per month.

“The real challenge was to figure out a way to replace all of those steps that required human intervention with steps that a biological entity could perform by itself,” explains David Liu, a chemist at Harvard University.

A decade ago, Liu and then graduate student Kevin Esvelt set out to solve this problem, eventually developing an in vivo strategy known as Phage Assisted Continuous Evolution, or PACE, which is now one of several in vivo strategies available for speeding directed evolution (7). Continuous evolution strategies like PACE, and in vivo continuous evolution (ICE) (8) developed in yeast, “offer the potential for deeper, subtler and faster exploration of structure-activity space,” notes Davis of AstraZeneca.

To develop PACE, Liu and Esvelt tested a variety of strategies but ultimately developed a bacteriophage system that linked a desired feature with phage survival. Overall, PACE is at least 100 times faster than earlier protein evolution strategies. The

trick, Liu says, was to figure out exactly how to make phage propagation dependent on a particular molecular activity you’re trying to evolve. The value of the approach can be seen in the diversity of Liu’s work. Using PACE, Liu’s laboratory has evolved proteins ranging from Bt toxins and biosynthesis genes to plastic synthesizing enzymes and more recently proteases. It’s surprisingly general, according to Liu, because of the many possible ways to link activities of interest to gene expression.

In vivo evolution strategies not only allow researchers to create diversity more quickly, they also provide an opportunity to access previously undruggable targets, particularly intracellular ones with ill-defined binding sites, which just happen to represent a tantalizing 80 percent of so-called “drug space.”

Liu’s recent protease study represents one step toward this goal (9). His team evolved a test protease, changing the peptide sequence that it recognizes by six out of seven amino acids over thousands of generations of evolution. The new target sequence is in IL-23, a cytokine implicated in human inflammatory disease. It’s the furthest that a protease has been evolved in a laboratory. Though such a system is a long way from producing a human therapeutic protease, it demonstrates the potential power of the idea.

Still, as Liu notes, antibodies have proved a more powerful class of therapeutics than proteases in large part because the immune system works to evolve large proteins that go after largely extracellular targets. But antibodies have shortcomings that proteases could address. “Antibodies mostly hug their targets to death,” Liu says, while proteases offer other ways to disrupt disease: the ability to catalytically destroy protein targets as well as alter their post-translational modification and even localization within a cell.

“Evolution is, in my opinion, most powerful for problems where we just don’t understand the fundamentals well enough to go after a particular target,” says chemist Bryan Dickinson of the University of Chicago. It allows researchers to search for function and even fitness. “It’s really agnostic to structure,” he notes.

Classic display technologies used in directed evolution select for binding to a target. Though useful, these leave out many targets currently considered “undruggable”, such as protein-

RNA polymerase biosensors can be used to study protein-protein interactions. These biosensors offer genes as catalytic outputs which can be directly sequenced. Credit: B. Dickinson.

protein interactions, where the biophysical details of the contact between binding partners are complicated and potentially unknown. But Dickinson is envisioning selection systems that allow the disruption of a key protein-protein interaction that spurs a cell to become cancerous, linking that activity to the overall fitness of an in vivo directed evolution system such as PACE. “Our focus is on how do you link that fitness advantage to interesting targets,” he says, “beyond just molecular interactions.”

For protein-protein interactions Dickinson’s lab has developed a RNA-polymerase based biosensor, which offers an immediate advantage because its catalytic output is a gene that can be sequenced. To examine protein-protein interactions they’ve built on a popular imaging strategy that splits an indicator protein into two domains that become active in response to molecular binding. Their RNA-polymerase biosensors created a network of biosensors that split a set of enzymes into two domains that can then be fused to protein binding partners of interest. If partners bind, they produce a fully functioning polymerase that transcribes a nucleic acid barcode highlighting the interactions (10).

“Even short of drug discovery, what evolution will allow us to do is really look for, again, how do molecules actually have these target activities,” he says. Just by evolving molecules that change protein-protein interactions, researchers can use those structures as tools to learn about mechanisms, such as allosteric regulators or other interactions that could disrupt a critical

molecular interaction while not directly interacting with a known hotspot on the surface of a protein.

Dickinson thinks the available directed evolution tools have finally reached a critical turning point in drug discovery: instead of focusing developing tools and methods, researchers are now moving toward real applications. In his lab, researchers are working on at least two projects involving specific inhibitors of targets using already developed tools. “The time is right to actually translate that into something meaningful for medicine,” he says.

As a medicinal chemist, AstraZeneca’s Davis encourages others focused on small molecules to bridge the chasm between their field and the large molecule field. It’s here he thinks that small molecule researchers can use, adapt and develop concepts from directed evolution to overcome some of the remaining challenges. “In essence, we wish to see ourselves as drug hunters, who have at our disposal all the most relevant technologies so we can develop a drug for every target.”

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