SYMPOSIUM

Biology in a Gray Box: Targeting the Emergent Properties of Protein Complexes

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At the 2011 Yale Chemical Biology Symposium, Jason Gestwicki presented a novel yet intuitive approach to drug screening. This method, which he termed “gray box” screening, targets protein complexes that have been reconstituted in vitro. Therefore, the gray box screen can achieve greater phenotypic complexity than biochemical assays but avoids the need for target identification that follows cell-based assays. Dr. Gestwicki’s research group was able to use the gray box screen to identify myricetin as an inhibitor of the DnaK-DnaJ chaperone complex. This review will discuss Dr. Gestwicki’s approach to identifying DnaK-DnaJ inhibitors as well as where the gray box screen fits among traditional techniques in drug discovery.

INTRODUCTION

Biochemical and cell-based screens are two commonly practiced approaches in drug discovery. Each technique has unique advantages that make it amenable to different situations. Biochemical screens facilitate the discovery of molecules that directly target a protein of interest. However, biochemical assays alone cannot define the cellular phenotypes that result from a specific treatment. By contrast, cell-based assays identify effective small molecules exclusively based on a cellular phenotype. These assays are frequently termed “black

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†Abbreviations: FRET, Förster resonance energy transfer; HTS, high-throughput screen; HCV, Hepatitis C virus; ECIS, electric cell-substrate impedance sensing; Hsp70, Heat Shock Protein 70 kDa; NBD, nucleotide-binding domain; ECG, epicatechin-3-gallate; SARs, structure-activity relationships; SBD, substrate-binding domain.

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box” screens because the mechanism of action is largely unknown. In his “gray box” screening approach, Jason Gestwicki, Assistant Professor of Pathology at the University of Michigan, attempts to achieve a compromise between the biochemical and cell-based assays. The gray box screen takes advantage of the emergent properties of protein complexes. In this technique, multiple components of a protein complex are purified and reconstituted in vitro. While only a core enzyme in the complex has a measurable activity, the supplemental components of the complex lend a better approximation of the complex’s function in vivo. Here, I will review screening techniques as well as a gray box screen developed by Lyra Chang and colleagues in the Gestwicki group, which successfully identified the flavonoid myricetin as an inhibitor of the DnaK-DnaJ chaperone complex.

**BIOCHEMICAL SCREENS**

Biochemical screens identify molecules in an in vitro environment. The protein of interest must be purified and requisite assays must be performed to confirm the protein’s activity. This technique relies on a specific behavior of the protein as a read-out, such as its ability to cleave substrates, hydrolyze nucleotides, or modify protein targets with a small molecule or peptide. For example, screens for inhibitors of the molecular chaperone Hsp90 frequently target its ability to hydrolyze ATP in order to power its protein folding machinery [1]. Recently, increased attention has also been given to small molecule modulators of protein-protein interactions [2]. Protein-protein interactions are essential to many biological functions and therefore represent a large class of targets for screening [2]. A wide variety of methods can be utilized to measure these activities, including Förster resonance energy transfer (FRET†) and fluorescence polarization [3,4,5]. After effective molecules are identified, they can be moved to cell-based assays.

Biochemical assays leave no question about the target and reveal more details about the mechanism of action of a molecule than cell-based assays. Unfortunately, molecules that are active in vitro frequently lose their effectiveness in the complex environment of the cell. Biochemical assays often fail to predict how an interaction between a molecule and a protein can be complicated by a failure in delivery, toxicity, or a general loss of activity. For example, molecules may be processed in the cell or have its activity modulated by the properties that arise from unforeseen protein interactions. However, molecules that cannot be used for therapeutic purposes can still be effective probes for protein function.

**CELL-BASED SCREENS**

The cell-based screen is a useful technique in the identification of novel therapeutics because it incorporates the intracellular environment into the assay. It accounts for the presence of membranes and compartmentalization, as well as complex multi-protein interactions. These assays measure phenotypic changes in response to treatment. Common outputs include the activation or inhibition of signaling pathways and also protein interactions, which are often measured with the aid of reporters. Fluorescence can be effective in determining changes in protein activity and interactions. FRET is one tool that has been utilized with relative success [6]. For example, Yu et al. developed a high-throughput screen (HTS) to identify Hepatitis C virus (HCV) antivirals by employing an internally quenched peptide substrate of the HCV NS3 protease [7]. Bioluminescent reporters, such as luciferase, have also been used in HTS [8].

Fluorescence and bioluminescence can suffer from serious drawbacks. The modification of proteins with fluorescent probes can disrupt its function, and expression of reporters may also interfere with normal cellular behavior. Consequently, label-free approaches are often preferable when available. Label-free methods are minimally invasive and rely on inherent properties of the cell, such as its morphology, adhesiveness, and proliferative ability [6]. While these types of screens will require greater
development before extensive use, a number of techniques have been developed and suggested for screening applications. For example, electric cell-substrate impedance sensing (ECIS) is a potentially effective tool in quantifying cell adhesiveness and morphology [9].

Although cell-based assays measure the activity and toxicity of molecules in biologically relevant conditions, they often reveal little information about the target and mechanism of action. This adds an increased burden of biochemical experiments after an effective molecule is identified. Nevertheless, drug candidates have historically entered market despite a poorly elucidated mechanism of action [10], as long as they demonstrate low toxicity. This can unfortunately result in undetected side effects that become apparent only after the drug has reached market.

‘GRAY BOX’ SCREENING

In a gray box screen, a protein complex is purified and reconstituted in a biochemical environment. The complex is then used to specifically screen for molecules that target the emergent properties that arise only when the complex is formed. Ideally, only one core enzyme possesses a measurable activity, while the accessory proteins modulate this activity. The gray box approach exploits features of both previously discussed approaches by providing a more physiologically relevant environment than the biochemical screen but reducing the need for additional assays to determine the target of effective molecules.

Lyra Chang and colleagues from the Gestwicki group coined the term “gray box” screening to define their approach in identifying inhibitors of the DnaK-DnaJ complex [11]. The molecular chaperone DnaK, a member of the Heat Shock Protein 70 kDa family (Hsp70), plays an essential role in protein homeostasis in *Escherichia coli* [12]. DnaK consists of a 41 kDa nucleotide-binding domain (NBD) and a 26 kDa substrate-binding domain (SBD) [13]. The NBD is itself made up of four subdomains, IA, IIA, IB, and IIB, which form an ATP-binding cleft [14]. The NBD is responsible for hydrolyzing ATP, thereby providing energy for the chaperone machinery. DnaJ, a member of the Hsp40 family, is a co-chaperone which binds to the IA and IIA subdomains of DnaK [15]. This interaction stimulates the ATPase activity of DnaK. In an ADP-bound form, DnaK binds tightly to its protein substrate, but loses this affinity when ADP is replaced by ATP [16].

Chang et al. purified DnaK and DnaJ and established a high-throughput ATPase assay for DnaK that utilizes the inorganic phosphate chelator malachite green as a reporter [17]. In their current work, they combined DnaK and DnaJ in an optimized ratio and screened for inhibitors at high concentrations of ATP to limit the discovery of nucleotide competitive inhibitors. They then screened through a collection of extracts from 36 commercial spices and crude plant materials. Chang et al. justified this approach citing the well-defined chemical components of these materials and also a motivation to determine whether natural products produced by plants could inhibit Hsp70-Hsp40 family members, commonly found in plant pathogens. They identified six extracts (allspice, black tea, cocoa, cinnamon, cloves, and white tea) that inhibited ATPase activity by greater than 30 percent. Chang et al. proceeded to isolate and confirm epicatechin-3-gallate (ECG), a member of the flavonoid family, as the major active component in white tea.

Based on this evidence, the Gestwicki group studied the structure-activity relationships (SARs) of flavonoids on the DnaK-DnaJ complex and ultimately identified myricetin as the most effective inhibitor of DnaK ATPase activity among flavonoids. Through extensive structural biology studies, they discovered that myricetin does not competitively inhibit ATP or DnaJ binding but instead binds to the IB and IIB subdomains of DnaK. In fact, they showed that myricetin has no effect on the intrinsic ATPase activity of DnaK. Furthermore, they demonstrated that myricetin can bind to the subdomains only when ATP-binding causes
a DnaK conformational change. This interaction prevents contacts between DnaK and DnaJ allosterically, thereby reducing DnaJ’s ability to stimulate ATPase activity.

The power of this screen lies in its ability to identify molecules that modulate an emergent property that is only apparent through the formation of a protein complex between DnaK and DnaJ. Because their output is the signal that arises from the co-stimulatory effect of DnaJ, their screen is designed to specifically identify molecules that disrupt its interaction with DnaK. Not surprisingly, they identify a molecule that exerts an effect on enhanced ATPase activity allosterically to the NBD. An essential criterion to their approach is to use high concentrations of ATP, thereby reducing the possibility of identifying competitive inhibitors. This is important because the high concentrations of intracellular ATP make the development of a competitive inhibitor more difficult. Therefore, Chang et al. have developed a screen that can identify inhibitors that might have otherwise gone undetected by alternative screening techniques.

Nevertheless, the gray box screen can suffer from drawbacks similar to the biochemical screen. Like biochemical assays, the gray box method is contingent on an initial understanding of a protein’s activity. Of course, this requires the purification of the protein of interest and its accessory proteins followed by in vitro reconstitution as well as assays to characterize its activities. While DnaK and DnaJ form a relatively simple two-component complex, purification and reconstitution of some larger complexes is hardly a trivial task. Moreover, the gray box method may be unfeasible if the phenotypic complexity of the reconstituted system is too great. Chang et al. were able to focus on one output and one modulator, but other complexes may have multiple activities. Finally, the gray box is ultimately still a biochemical environment and therefore may not be the most efficient screen for therapeutics. Molecules discovered in a gray box will still need to be moved to a cell-based assay to gain a greater understanding of its activity and toxicity in vivo.

CONCLUSION

Chang et al. identified and characterized myricetin as an inhibitor of the DnaK-DnaJ interaction, demonstrating that the gray box method can be a viable screening approach. They then suggested that it could be an appropriate method of studying Hsp70 members, as well as other proteins that are regulated by multiple accessory proteins, particularly molecular chaperones. They recognized that this approach may be less effective in studying systems that are difficult to reconstitute in vitro or that demonstrate exceedingly complex phenotypes. Consequently, a fairly exhaustive understanding of the complex of interest is necessary before a gray box screen can be applied, as the screen is not appropriate for all situations. Nonetheless, the Gestwicki group has high expectations for this approach, anticipating that it might soon be used to study multi-protein systems like the mTor complex, chromatin-remodeling complexes, and the exocyst. These studies are certainly not beyond the realm of possibility. The gray box screen has the potential to be useful as both a tool to study protein function and as one to identify drug candidates.

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