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Streamlining Chemical Probe Discovery: Libraries of "Fully Functionalized" Small Molecules for Phenotypic Screening

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Small molecules are important tools for studying biology.[1] Consequently, the identification of suitable chemical probes represents one of the key subjects of current chemical biology research. Phenotypic screening has turned out to be a highly efficient approach for the discovery of small molecules with novel mode-of-actions.[2] In phenotypic assays, small-molecule compound libraries are screened for bioactive compounds that induce a particular phenotype in a living cell, tissue, or even organism. Such screening of phenotypic changes represents a biologically more meaningful readout than typical, target-directed screens, and allows screening for small-molecule modulators of whole biological pathways instead of only isolated proteins. These assets are, however, linked with one drawback: the additional task of identifying the target proteins responsible for the phenotypic effect.[3] Although target identification has undoubtedly seen significant new developments in the last years, it nevertheless remains a major challenge and therefore represents still one of the bottlenecks of phenotypic screening campaigns.[4] But why is this task so demanding? Several inherent factors complicate target identification: First, initial hits from phenotypic screens often bind to their target proteins with only low to moderate affinities. Therefore, unspecific binding events (background noise) often mask the interaction of the small molecule with its target. Additionally, most target identification methodologies rely on the attachment of the small molecule to a solid support, followed by affinity chromatography to enrich binding proteins prior to analysis. Thus, this method fails in those cases where the interaction of the small molecule with its protein target is only transient or strongly dependent on a native environment. These complications are in stark contrast to the relative ease of target identification of bioreactive small molecules that interact with proteins through formation of a covalent bond. Also in these cases, target identification relies on protein enrichment. The formation of the covalent bond however allows more stringent clean-up procedures that result in purer samples and thus more facile and unambiguous protein analysis.[5]

The Cravatt group has been a pioneer in the use of bioreactive small molecules for chemical proteomics applications, and has established several efficient, straightforward and reliable methodologies for the target elucidation of such bioreactive chemical probes.[6] Now, the same group has developed a new, very elegant approach that helps to overcome the shortcomings of target identification after phenotypic screenings.[7] The ingenious, yet simple idea behind the new approach is to perform phenotypic screenings with compounds that can form covalent bonds with their protein targets. This is, however, easier said than done. In fact, phenotypic screens with libraries of bioreactive small molecules have already been performed previously, and these studies demonstrated the feasibility of such an approach.[8] Nevertheless, these approaches are hampered by a lack of generality, as the required covalent bond formation occurs only in those cases in which a complementary chemical reactivity of the small-molecule probe and its protein target is found at the binding site. An alternative and well-established method to "induce" the formation of covalent bonds between small molecules and their protein targets is to use photoreactive groups attached to the bioactive small molecules. Upon UV irradiation, these chemical groups are able to form covalent bonds with all types of protein residues that are in close proximity. In their new approach, Cisar and Cravatt therefore propose to use libraries of so called "fully functionalized" small molecules in phenotypic screenings.[7] Such "fully functionalized" small molecules are based on 1) a photo-reactive group, such as the benzophenone moiety, integrated into the pharmacophoric core structure, and 2) an alkyne tag that allows the use of click chemistry after photo-affinity labeling of the protein targets, thereby enabling the use of standard protocols for protein enrichment (Figure 1 A). "Fully functionalized" compounds consequently contain already all necessary chemical residues to perform phenotypic screenings and subsequent chemical proteomics based target identifications of photo-affinity labeled proteins (Figure 1 B). Accordingly, such an approach should allow the elucidation of novel small-molecule probes in a highly streamlined fashion.

To provide a proof-of-concept for such an approach, Cisar and Cravatt synthesized two different compound libraries

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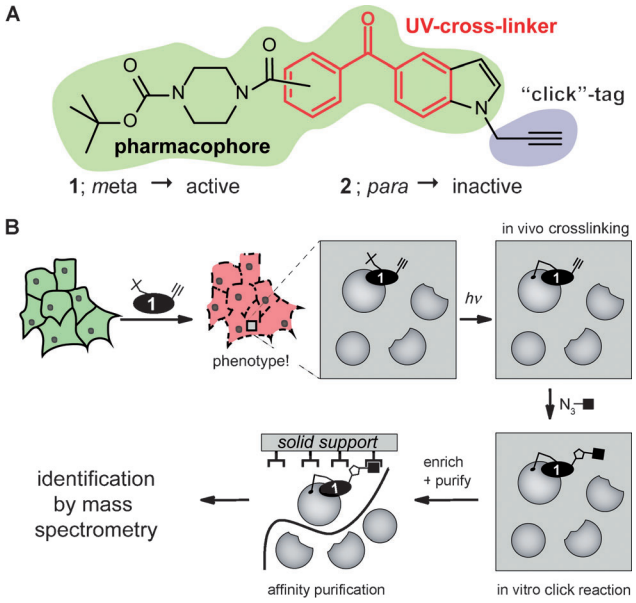

Figure 1. A) Chemical structure of "fully functionalized" compounds **1** (active) and **2** (inactive) identified by phenotypic screening.[7] B) Workflow of phenotypic screening using fully functionalized probes.

with an integrated photo-reactive benzophenone moiety as the scaffold. These efforts resulted in the generation of a small library of 5-benzoyl indoles (BzIndoles, Figure 1 A) and of 7-benzoyl-benzo-1,4-diazepin-2,5-diones (BzBDs).[7] In order to test, if the synthesized compounds displayed sufficient chemical diversity to target different protein subsets in living cells, they then performed a gel-based proteome-wide profiling of their chemical library of fully functionalized compounds in MDA-MB-231 cells.[7] They observed a diverse labeling pattern, which demonstrated that the compound library might indeed have different bioactivities in a phenotypic screen. To test this hypothesis, they investigated the antiproliferative activity of the fully functionalized compounds in presence of high and low glucose levels. Intriguingly, only one library compound (**1**, Figure 1 A) showed selective cytotoxicity under low glucose levels, while the structurally related compound **2** (Figure 1 A), which differed from **1** only by a shift of a *tert*-butyloxycarbamoylpiperazine carbonyl group from a *meta* to a *para* position, was found to be inactive. To identify the phenotype-causing molecular targets of probe **1**, Cisar and Cravatt then employed SILAC (stable isotope labeling with amino acids in cell culture) target identification methods. They treated cells grown in the presence of heavy amino acids with active **1** or inactive **2**, irradiated the cells briefly with UV light, and enriched labeled proteins by affinity purification mediated by click chemistry. Subsequent mass spectrometry-based analysis of these proteins resulted in the identification of two protein targets, epoxide hydrolase-1 (EPHX1) and the mitochondrially encoded NADH dehydrogenase 1 (MT-ND1), a subunit of the mitochondrial complex I. Additional gel-based profiling experiments confirmed that two proteins matching the size of EPHX1 and MT-ND1 were labeled preferentially by the antiproliferative compound **1**, whereas the inactive compound **2** labeled only epoxide hydrolase-1 (EPHX1). Finally, tests with submitochondrial particles corroborated that the observed antiproliferative effect of compound **1** is due to inhibition of complex I, an important protein of mitochondrial oxidative phosphorylation. While compound **1** inhibited complex I in the upper nanomolar range, compound **2** was ten-times less active, thus confirming the labeling selectivity observed in the gel-based assays. Although the authors did not further investigate if EPHX1 plays an additional role for cancer cell viability, these findings strongly indicate that the small-molecule modulation of MT-ND1 contributes to the observed antiproliferative phenotype. Taken together, the authors demonstrated that the use of fully functionalized small molecules may reduce the experimental efforts and timelines for phenotypic screening and subsequent target identification, leading to a straightforward process that can be easily implemented and standardized for high-throughput screening campaigns.

However, despite the advantages of the new approach, not all the problems associated with phenotypic screens have yet been solved. First, much larger compound libraries of fully functionalized small molecules are required. Because this new approach is not adaptable to already prepared "classical" compound libraries, intense (and expensive) synthetic efforts are required to generate more compound libraries of fully functionalized small molecules. Second, and even more limiting, the number of different photo-reactive cross-linking groups, mainly benzophenone or trifluoromethyldiazirine derivatives, is currently low. As these moieties represent an integral part of the small-molecule structure, they have a direct impact on the chemical diversity of the generated compound libraries. Additionally, the photo-reactive groups have a limited chemical stability and are often incompatible with many reaction conditions regularly applied in compound library synthesis. These limitations call for the development of novel, chemically diverse, and more stable photo-reactive groups to expand the scope of libraries of fully functionalized compounds. Finally, it should be noted that the efficiency of photo-cross-linking, and thus of target identification, is strongly dependent on a selective binding mode. This requirement might lead to cases in which, despite the use of fully functionalized probes, target identification will again represent a challenge. Therefore, the study of Cisar and Cravatt is not the endpoint for the design of chemical libraries, it rather opens new opportunities and may serve as a guide for future synthesis of compound libraries. The next step will be to use these opportunities and to advance the pilot study to a general application in chemical-probe discovery.

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