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Chemical Biology Approaches in Drug Target Discovery: From Affinity Probes to Thermal Stability Analysis

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Abstract

Drug target discovery is a critical step in new drug research and development, and chemical biology techniques and theories provide powerful tools to advance this process. This review summarizes two major categories of drug target discovery methods—affinity probes and thermal stability analysis—and their recent research progress, highlighting the role of chemical biology approaches. It also aims to deepen the author's understanding of future directions for advanced study in chemical biology. The review focuses on activity-based protein profiling (ABPP) and affinity-based protein profiling (AfBPP) as representative affinity probe technologies, as well as label-free thermal stability techniques including DARTS, CETSA, and TPP, with extensions to related developments. Both categories of drug target discovery technologies have distinct advantages, and their combined use enables a complete workflow from theory to practice and from discovery to validation. Chemical biology methods play a positive role in drug target identification.

Keywords

chemical biology, drug target discovery, affinity probe, thermal stability analysis, ABPP, AfBPP, DARTS, CETSA, TPP

1. Introduction

Drug target discovery aims to elucidate the mechanisms by which drugs exert their effects, evaluate potential side effects, predict drug resistance, and constitutes a key component in the pipeline of novel drug research and development. Clearly identifying the direct protein targets of drug molecules not only helps define the drug's mechanism of action but also provides precise scientific guidance for clinical application and drug optimization. However, traditional target discovery approaches are largely hypothesis-driven and validated one by one, resulting in low efficiency and difficulty in addressing the diverse drug–protein interactions in complex biological systems. Statistics show that among the human proteome, approximately 4,000 proteins are disease-related, yet only about 500 are targeted by approved drugs. Many disease-associated proteins remain "undruggable" due to the lack of well-defined active sites, low ligandability, etc. These "undruggable targets" represent a critical bottleneck that traditional drug discovery urgently needs to overcome. Therefore, developing efficient and precise target discovery technology platforms has become a key pathway to break through multiple obstacles in drug development.

Chemical biology, as an interdisciplinary field, uses chemical tools to dissect biological processes and brings new methodological innovations to drug target discovery. Current technical systems in this field mainly advance along two routes: one is affinity/activity-based probe technologies, exemplified by activity-based protein profiling (ABPP), which offers high specificity and quantifiability but requires chemical modification of the drug; the other is label-free thermal stability-based techniques, represented by drug affinity responsive target stability (DARTS) and thermal proteome profiling (TPP), which require no drug modification and are particularly suitable for natural products that are difficult to chemically derivatize. In recent years, these two categories have increasingly been used in combination to build integrated research workflows. Against this background, this review systematically summarizes the principles, development progress, and application examples of these two major classes of chemical biology techniques in drug target discovery under the theme "From Affinity Probes to Thermal Stability Analysis." It compares their advantages and limitations, and looks forward to the potential value of emerging technologies—such as peptide-centric local stability assay (PELSA) and artificial intelligence-assisted prediction—in overcoming undruggable targets, with the hope of providing methodological references for researchers in chemical biology and drug discovery.

2. Target Discovery Technologies: Affinity Probes

2.1 ABPP Technology: Principles and Applications

2.1.1 Technical Principles

ABPP, or activity-based protein profiling [1,2], is a core method in chemical proteomics that uses small-molecule probes to profile active proteins. It excels at identifying direct targets of small-molecule drugs or toxicants. By precisely localizing these direct targets, researchers can gain deeper and more comprehensive insights into the pharmacological and toxicological mechanisms of small molecules. As a key chemical proteomics tool, ABPP focuses on direct interactions between small molecules and proteins. Its core mechanism involves specially designed small-molecule probes for targeted labeling, enrichment, and identification. These probes consist of a bioactive functional group, a linker connecting different modules, and a reporter tag that generates a detectable signal, enabling specific capture of proteins that directly interact with the drug molecule. Compared with traditional omics approaches that reflect macroscopic changes at the gene transcription or protein expression level, ABPP more sensitively and directly reveals the initial molecular targets of drug action, providing crucial clues for understanding drug mechanisms. To avoid potential interference of reporter groups with compound bioactivity, ABPP often incorporates bioorthogonal handles (e.g., alkyne or azide groups), facilitating efficient identification of covalently binding molecular entities.

2.1.2 Applications

Two representative examples illustrate the application of ABPP:

First, in studies of off-target effects of the anticancer drug mitocan family [1], previous understanding held that mitocans primarily act on DNA. However, ABPP revealed that they also directly bind to aldehyde dehydrogenase (ALDH). This discovery uncovered a novel mechanism of action and provided a theoretical basis for subsequent structural modifications to reduce toxicity.

Second, in off-target effect research on the marketed anti-obesity drug orlistat [1], orlistat inhibits lipase to reduce calorie intake. Researchers used ABPP to systematically "fish" for potential protein targets of orlistat. Three alkyne-bearing probes were designed and incubated with live cells, followed by enrichment and identification, yielding 8 proteins that interact with the drug. In addition to the known fatty acid synthase (FAS), newly identified targets included ribosomal proteins L7a, 14, and S9, which are highly expressed in many cancer cells. These findings provide important clues for further exploring orlistat's antitumor effects and off-target mechanisms.

2.2 AfBPP Technology: Principles

AfBPP (affinity-based protein profiling) shares similarities and differences with ABPP [1]. Key distinctions and advantages/disadvantages include:

(1) Probe preparation: ABPP requires only the attachment of bioorthogonal groups (e.g., alkyne or azide) to the target molecule; AfBPP additionally requires incorporation of photoaffinity labeling groups such as benzophenone, aryl azide, diazirine, or aryl tetrazole. Among these, diazirine is currently the most widely used due to its small size and high photocrosslinking efficiency.

(2) Protein capture workflow: In ABPP, after incubation with cells or tissue lysates, direct enrichment and purification of labeled proteins can proceed; in AfBPP, UV irradiation is required after incubation to trigger covalent crosslinking between the photoaffinity group and nearby proteins before subsequent enrichment.

(3) Target identification: ABPP captures only proteins that form covalent bonds with the probe; AfBPP can identify both covalently and non-covalently interacting proteins (via hydrogen bonds, van der Waals forces, etc.), offering broader coverage and a more comprehensive reflection of the compound's targets. However, this comprehensiveness also increases the risk of nonspecific background interference, complicating result interpretation.

2.3 Emerging Developments in Probe Technologies

Beyond ABPP and AfBPP, photoaffinity labeling represents another frontier in probe-based methods [3]. Photoaffinity probes, as core tools in chemical proteomics, rely on exquisitely designed molecular architectures to capture transient protein interactions in cells. These probes typically comprise four functional modules: (1) a target recognition module that specifically binds to the active or affinity site of the target protein, ensuring selectivity; (2) a photoreactive trigger unit (e.g., diazirine, benzophenone, aryl azide) that, upon specific light excitation, generates highly reactive intermediates and forms covalent bonds with nearby amino acid residues via radical-mediated mechanisms (see Table 1 for detailed comparison of photoreactive groups); (3) a linker module that modulates overall probe conformation and flexibility (e.g., simple alkane chains, PEG, or peptide chains) to optimize binding efficiency and labeling yield—for instance, Winter's team screened 407 candidates from the Enamine database and selected a six-carbon alkane linker after considering steric hindrance and hydrophobicity to effectively capture diverse small-molecule fragment–protein interactions; and (4) a reporter tag module, usually containing a bioorthogonal handle that enables highly specific enrichment and sensitive detection of labeled products via click chemistry. Using carefully designed photoaffinity probes in cell lysates or live cells, specific wavelengths trigger the photoreactive group, covalently anchoring the probe to interacting target proteins. The reporter tag then undergoes efficient click chemistry with biotin-azide, and streptavidin-coated magnetic beads exploit the high-affinity biotin–streptavidin interaction for specific capture and enrichment of labeled proteins. Enriched products are identified by mass spectrometry, enabling real-time tracking and precise identification of dynamic cellular molecular interactions. This approach provides innovative solutions and new perspectives to overcome limitations of traditional methods.

3. Target Discovery Technologies: Label-Free Thermal Stability Analysis

3.1 DARTS Technology and Applications

3.1.1 Technical Principles

Drug affinity responsive target stability (DARTS) is widely used for identifying and screening drug targets [2,4]. Its core principle is as follows: the target drug (or equivalent volume of vehicle control) is incubated with protein samples, followed by limited proteolysis with a protease. After digestion, samples are separated by SDS-PAGE. In the gel, proteins specifically bound and protected by the small-molecule drug are more resistant to protease degradation, resulting in stronger band intensity compared with the vehicle control. DARTS can be applied to purified proteins or whole-cell lysates. After SDS-PAGE separation, depending on research goals, Western blotting or mass spectrometry can be used for qualitative and quantitative detection of differential protein expression between drug-treated and control groups. A major advantage is that no drug modification is required, making it particularly suitable for natural products.

3.1.2 Applications

One example is the identification of tanshinol sodium target proteins using DARTS [5]. Tanshinol sodium may exert cardiovascular protective effects by directly binding to the target protein CD44. This study

provides a scientific basis for deeper mechanistic investigation and new drug development. In this work, DARTS acted as a "label-free fishing rod," successfully identifying direct drug targets from complex cell lysates without altering the natural structure of the compound, followed by confirmation through subsequent experiments.

Another example is the discovery of the brain protein target AK1 for ginsenoside AK1 using DARTS, with validation by BLI [6]. DARTS served as a label-free target screening tool, successfully "fishing" potential targets of ginsenoside from the complex brain tissue proteome, followed by BLI and molecular docking to complete the full target discovery workflow from "screening" to "validation," highlighting DARTS's practical value in studying mechanisms of complex traditional Chinese medicine components.

3.2 CETSA and TPP Technologies and Applications

3.2.1 Technical Principles

Cellular thermal shift assay (CETSA) [4,7] is based on the principle that specific binding of a small-molecule drug to its target protein induces conformational changes that enhance the protein's thermodynamic stability, making it more resistant to thermal denaturation. In experiments, intact cells or cell lysates are exposed to a temperature gradient. Unbound proteins, with lower structural stability, readily denature and form insoluble aggregates upon heating, precipitating out; in contrast, drug-bound target proteins retain solubility due to higher thermal stability. A major advantage is the ability to monitor drug–target interactions in live cells or near-physiological conditions in real time and directly measure binding affinity. However, CETSA relies heavily on the availability of specific antibodies, limiting its use to validation of known targets or a small number of candidates and making high-throughput screening of unknown proteins difficult.

Thermal proteome profiling (TPP) [4,7] shares the same core mechanism as CETSA—enhanced thermal stability upon drug–target binding—but represents a major breakthrough by expanding from validation of known targets to discovery of novel targets. By performing unbiased, system-wide analysis of the entire proteome's thermal stability, TPP not only precisely identifies direct drug targets but also reveals downstream regulatory proteins or complexes affected by target functional changes.

Both techniques rely on the principle that drug binding enhances target protein thermal stability. CETSA, as a foundational method, focuses on antibody-based precise validation of specific known proteins. TPP, by replacing antibodies with mass spectrometry, extends the application from a few targeted proteins to comprehensive proteome-wide coverage, achieving unbiased discovery of novel targets in a high-throughput manner.

3.2.2 Applications

In a study identifying TH588 target proteins [7], CETSA was first used to optimize experimental conditions, followed by TPP for proteome-wide scanning, successfully mapping the action profile of TH588 and demonstrating the powerful complementary nature of these two techniques in discovering novel drug targets and elucidating complex mechanisms without modification.

In research on the therapeutic mechanism of Erigeron breviscapus extract for encephalopathy [8], TPP was successfully applied to a multi-component traditional Chinese medicine system. Without isolating individual compounds, TPP captured overall action targets, highlighting its unique advantage in natural product studies. The study went beyond target identification to integrate "target–pathway–downstream molecule–disease" layered analysis, linking TPP-discovered targets to clinical diseases. This scientifically explained traditional efficacy (e.g., treatment of Alzheimer's and Parkinson's) and prospectively predicted new indications (anti-glioma and neuroregeneration).

3.3 Brief Introduction to Other Label-Free Techniques

3.3.1 SPROX

Stability of proteins from rates of oxidation (SPROX) [3,4] is based on the principle that drug binding stabilizes target protein structure, increasing resistance to chemical denaturants (e.g., urea or guanidine hydrochloride). This stability change is reflected in methionine (Met) residues: folded-state Met is resistant

to hydrogen peroxide oxidation, while denatured-state Met is readily oxidized. By detecting the extent of Met oxidation, one can infer whether a protein binds the drug.

3.3.2 SIP

Solvent-induced protein precipitation (SIP) [4] relies on the principle that drug binding increases target protein stability, conferring greater resistance to organic solvent-induced denaturation and precipitation (e.g., acetone–ethanol–acetic acid mixtures). Cell lysates are treated with varying solvent concentrations, and soluble proteins in the supernatant are quantified by mass spectrometry. Shifts in precipitation curves between drug-treated and control groups identify target proteins. Advantages include no chemical modification, simple operation, use of common reagents, and complementarity with CETSA and SPROX. Limitations include applicable only to cell lysates, not live cells.

3.3.3 LiP

Limited proteolysis (LiP) [4] is based on the principle that drug binding stabilizes target protein conformation, protecting certain flexible regions from short-term hydrolysis by nonspecific proteases (e.g., proteinase K). After initial limited digestion, samples are fully digested with trypsin, and differential peptides are quantified by mass spectrometry to infer drug-binding proteins and potential binding regions. Advantages: provides domain-level structural information, clearly delineating binding regions; good applicability to complex proteomes. Limitations: requires exogenous protease, limited to lysates, multiple steps, and complex sample composition.

4. Technical Comparison and Integration Strategies

4.1 Comparison of Advantages and Limitations

The two major categories—affinity probe and thermal stability analysis—each have strengths and weaknesses, as summarized in Table 1.

Table 1: Comparison of the Two Categories of Technologies

Technology Type	Representative Techniques	Advantages	Limitations	LimitationsApplicable Scenarios
Affinity Probes	ABPP, AfBPP	High specificity, quantitative	Requires chemical modification, complex probe design	Known active sites, covalent drugs
Thermal Stability	DARTS, TPP	No modification needed, proteome-wide screening	Insensitive to weak binding, limited for membrane proteins	Natural products, difficult-to-modify drugs

4.2 Combined Applications

The study identifying methylenetetrahydrofolate dehydrogenase 1-like protein (MTHFD1L) as the binding target of natural product pseudolaric acid A (PAA) [9] exemplifies the integrated use of affinity probe and thermal stability technologies:

Phase 1: Preliminary screening with DARTS

PAA was incubated with HeLa cell lysates, followed by limited proteolysis. Drug-bound proteins are protected from protease degradation. MS identified 42 potential binding proteins (overlapping from two replicates).

Phase 2: Cross-validation with ABPP

Alkyne-bearing PAA activity probes were used for ABPP, with a competition group pre-incubated with excess free PAA. Probe-labeled proteins are specifically enriched if free PAA competitively inhibits labeling. MS identified 18 potential binding proteins (overlapping from two replicates).

Phase 3: Intersection to lock in the most likely target

Intersection of 42 DARTS proteins and 18 ABPP proteins yielded only one overlapping protein: MTHFD1L. Conclusion: MTHFD1L is the most likely target of PAA.

Phase 4: In vitro biophysical validation

NMR saturation transfer difference (STD) confirmed direct binding between PAA and MTHFD1L, identifying key binding groups (e.g., C4-acetoxy) consistent with structure–activity relationship studies. Surface plasmon resonance (SPR) measured binding constants, further confirming direct interaction.

Phase 5: Functional validation

Gene silencing of MTHFD1L in HeLa cells significantly reduced cell viability, phenocopying PAA treatment, confirming MTHFD1L mediates PAA's antitumor activity. Mechanistic studies showed PAA treatment induces reactive oxygen species (ROS) accumulation, with transcriptomic analysis revealing related pathway changes.

5. Challenges and Prospects

Although chemical biology-based target discovery has advanced significantly and multi-technology integration is maturing, methodological systems still face challenges, primarily stemming from technical limitations such as insufficient sensitivity for low-abundance targets and poor applicability to insoluble systems like membrane proteins.

Emerging technologies offer hope for overcoming these hurdles:

First, upgraded applications of peptide-centric local stability assay (PELSA) [10]; second, in situ live-cell applications of photoaffinity labeling [2]; third, deep integration of artificial intelligence with chemical proteomics.

In the future, chemical biology target discovery is expected to trend toward multidimensional integration, combining precise chemical probe design, biophysical in situ detection, high-sensitivity mass spectrometry, and AI-driven high-throughput prediction. This will build a comprehensive technical framework from in vitro screening to live-cell validation, and from single-target analysis to full interaction network mapping. With breakthroughs in these emerging directions, many previously "undruggable" targets are likely to gain new therapeutic opportunities, and chemical biology will play an increasingly pivotal role in the era-defining challenge of precise drug discovery.

6. Conclusion

Drug target discovery technologies are advancing toward multidimensional integration. Affinity probe-based methods (e.g., ABPP) enable specific enrichment and quantification of target proteins, while label-free thermal stability techniques (e.g., DARTS, TPP) allow unbiased proteome-wide screening. These two categories are complementary in principle. Single technologies struggle to balance sensitivity and accuracy, making multi-technology integration the mainstream strategy. A typical workflow involves preliminary screening with label-free methods, cross-validation with probe-based techniques, in vitro biophysical confirmation, and cellular functional validation—forming a closed "discovery–validation" loop that effectively reduces false positives. The target identification of pseudolaric acid A exemplifies successful integration of DARTS and ABPP.

Current technologies still face challenges such as insufficient sensitivity for low-abundance targets, limited applicability to membrane proteins, and difficulty capturing transient/weak interactions. Emerging directions including PELSA, live-cell photoaffinity labeling, and AI-assisted prediction continue to develop and are likely to enable chemical biology to make greater contributions to overcoming undruggable targets.

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