



Review paper

Small-molecule probes based on natural products: Elucidation of drug-target mechanisms in stroke



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ABSTRACT

Natural products (NPs) are an important source of new drugs for the treatment of stroke. Identifying cellular targets for bioactive molecules is a major challenge and critical issue in the development of new drugs for stroke. Small-molecule probes play a unique role in target discovery. However, drawbacks to these probes include non-specificity, unstable activity, and difficulty in synthesis. Small-molecule probes based on NPs at least partially compensate for these shortcomings. NPs feature rich chemical and structural diversity, biocompatibility, and unique biological activities. These features could be exploited to provide new ideas and tools for target discovery. Small-molecule probes based on NPs provide a precise and direct search for interacting protein targets of NPs-active small molecules. This review explores the properties of small-molecule probes based on NPs and their applications in mechanistic studies of stroke and other diseases. We hope that this review will bring new perspectives to the mechanistic study of NPs-active small molecules and accelerate the translation of these ingredients into drug candidates for the treatment of stroke.

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1. Introduction

Small-molecule probes specifically recognize and interact with biomolecules [1]. Natural products (NPs) possess specific “ligand-protein” adaptor structures and provide a more favorable structural basis than synthetic small molecules [2]. Therefore, small-molecule probes based on NPs are more suitable for screening drug targets for disease treatment. As a potential research tool, small-molecule probes based on NPs mainly consist of active small-molecule compounds extracted or modified from NPs, which have

promising applications in elucidating the mechanism of drug action. Systematic research on small-molecule probes of NPs has revealed their crucial roles in the mechanism of disease action, which, in turn, provides a scientific basis for the modernization of NPs and the development of new drugs.

Stroke is an acute cerebrovascular disease caused by the sudden blockage or rupture of a brain blood conduit that disrupts the blood supply to the brain tissue, leading to localized brain tissue damage [3]. There are two primary types of stroke depending on the etiology and pathological mechanisms: ischemic and hemorrhagic stroke [4]. The active ingredients of NPs exert long-term, multi-faceted, biomechanistic effects in the treatment of stroke. These effects include neuroprotective, anti-thrombotic, antioxidant, and anti-apoptotic effects; weakening of the blood-brain barrier; and improvement of microcirculatory disorders [5]. For example, *in vivo* experiments demonstrated that *Verbena officinalis* ethanol extract ameliorated middle cerebral artery occlusion (MCAO)-induced acute cerebral ischemia, and *in vitro* assays revealed the neuroprotection conferred by verbenalin, acteoside, luteolin, and apigenin [6]. NPs include icariin, which can ameliorate oxidative

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stress injury during cerebral ischemia-reperfusion by inhibiting the opening of the mitochondrial permeability transition pore [7]. Taohong Siwu Decoction can significantly decrease neurological dysfunction and blockade volume, reduce pathologic injuries, blood-brain barrier leakage, and cerebral blood flow discontinuation, and preserve and stimulate neovascularization [8]. NPs typically contain multiple active ingredients that potentially act through multiple signaling pathways. Their specific targets of action and molecular mechanisms remain unclear.

Incorporating small-molecule probes based on NPs into the investigation of stroke mechanisms enhances the accuracy and efficiency of the study of disease-related targets. This knowledge will contribute to evaluations of the therapeutic efficacy of NPs in stroke and facilitate the modernization and global use of NPs.

This review summarizes the characteristics of small-molecule probes of NPs, recent advances and their application in the discovery of stroke and other disease-related targets. Our goal is to provide scientific evidence for the target discovery of active ingredients of NPs in stroke therapy, in addition to promoting the transformation of these active ingredients into clinical drugs.

2. Characteristics of small-molecule probes based on NPs

The utilization of the active ingredients of NPs as small-molecule probes to reveal the mechanisms of action in the field of NPs research is complicated. Effective explorations require an understanding of how the active ingredients of NPs are capable of fulfilling the requirements of small-molecule probes. NPs used as small-molecule probes have some common properties, such as low molecular weight, modification potential, and biological activity, as shown in Table S1 [9–27].

(1) Higher biological activity: The essence of drug research lies in the uncovering of new mechanisms and targets, as well as the recognition of direct targets of active ingredients in NPs with known efficacy [28]. Therefore, the use of NPs as probes requires knowledge of their biological activities. Echinacoside (ECH) [29] has demonstrated neuroprotective effects in Parkinson's disease (PD) models by inhibiting the α -synuclein/toll-like receptor 2 (TLR2)/nuclear factor- κ B (NF- κ B)/nod-like receptor (NLR), and pyrin domain-containing protein 3 (NLRP3) axis in microglia and reducing neuroinflammation. ECH also effectively reduced α -synuclein expression and inhibited signaling pathway activation in an animal model of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD and a microglial cell model of lipopolysaccharide activation, showing the potential of natural compounds against PD [30]. Thus, the target protein casein kinase 2 α' subunit (CK2 α'), which has a favorable neuroprotective effect, was successfully screened in cell lysates using ECH as a probe for neuroprotection [9]. Furthermore, naringenin has demonstrated significant therapeutic effects on scopolamine-induced dementia in rats, with potential therapeutic value for the treatment of dementia through multiple pathways, such as memory enhancement, neurotransmitter modulation, attenuation of oxidative stress, and neuronal protection [31]. Naringenin was also successfully screened as a molecular probe for the target protein collapsin response mediator protein 2 (CRMP2) associated with Alzheimer's disease. The use of the active ingredients of NPs with high biological activity as probes will be beneficial for screening their target proteins [32].

(2) Appropriate molecular size: Small-molecule probes of NPs generally have a small molecular weight, which helps them penetrate cell membranes and bind to intracellular targets. For example, oridonin, with a molecular weight of 364.43 Da, a natural terpene compound from *Rabdosiae Rubescentis Herba*, can serve as a probe to screen for cancer-related target proteins [33]. Curcumin is mainly derived from *Curcuma aromatica* Salisb. and *C. longa* L. as probes

with a molecular weight of only 368.38 Da for screening target proteins related to colon cancer [34].

(3) Easily chemically modified: To achieve a higher affinity or selectivity of the probe, it is occasionally necessary to chemically modify the active compounds of NPs. Therefore, the original active ingredient is susceptible to these modifications, including the introduction of an alkyne group to the hydroxyl group C₁₄ of andrographolide. Clickable activity-based protein profiling (ICABPP) technology combined with isobaric tags for relative and absolute quantitation (iTRAQ) has been used to screen targets related to the inhibition of tumor metastasis [35]. Britanin and its derivatives were modified at the position of the C₁₁–C₁₃ double bond to screen for targets related to cerebral ischemia/reperfusion injury [10].

(4) Target binding specificity: Ideal small-molecule probes should specifically bind to their targets and reduce interactions with off-target molecules, thereby increasing the accuracy of their activity. Using the active components of NPs as molecular probes to screen disease-related targets makes full use of the specific binding effect of the effective substances of NPs and the target and screens out the specific binding proteins.

3. Recent advances in small-molecule probes based on NPs

3.1. Novel NPs candidates

NPs are an integral part of drug development for stroke therapy because of their chemical structure and medicinal potential. Tongxinluo is a neuroprotective agent with anti-inflammatory properties. A recent double-blind clinical trial including 2,007 patients who experienced acute ischemic stroke (AIS) demonstrated that patients who received tongxinluo within 72 h of symptom onset were more likely to have better functional outcomes than the placebo group [36]. A multicenter, randomized, double-blind trial indicated promising efficacy and safety of ginkgo diterpene lactone meglumine in the treatment of AIS. Rather than only improving the degree of functional recovery in patients, ginkgo diterpene lactone meglumine also reduced neurological deficits without significantly increasing the risk of adverse effects [37]. Another clinical trial demonstrated that Buyang Huanwu Tang combined with recombinant tissue plasminogen activator intravenous thrombolysis was effective in attenuating oxidative stress damage in the Kelch-like ECH-associated protein 1-nuclear factor erythroid 2-related factor 2/antioxidant response element (Keap1-Nrf2/ARE) pathway in ischemic stroke patients with qi deficiency and blood stasis. This combination approach reportedly facilitated the recovery of neurological function and improved overall treatment outcomes [38]. Qilong capsule (QLC) has been approved by the China food and drug administration for the treatment of ischemic stroke, and an interdisciplinary integration study revealed that the combination of QLC and conventional treatment is superior to conventional treatment alone in improving patients' functional independence at week 12. The potential mechanism of QLC in the treatment of ischemic stroke is to inhibit the myeloid differentiation factor 2 (MD 2)/TLR4/NF- κ B inflammatory signaling pathway, attenuate cerebral ischemia/reperfusion inflammatory injury, and promote neurological function recovery [39]. The available evidence indicates the beneficial role of NPs in stroke treatment and implicates NPs as promising candidates for stroke therapy.

3.2. Emerging technologies

Small-molecule probes that are typically developed from biologically active compounds have enabled significant progress in preclinical studies of biomolecular mechanisms, disease signaling

pathways, and drug targeting assays [40]. Multiple functional labels (radioisotopes, stable isotopes, fluorophores, etc.), modified small-molecule probes, and drug-target analysis technologies (imaging, proteomics, amplification, etc.) have been synergistically developed for translational and clinical applications [40]. By contrast, small-molecule probes based on NPs mainly serve to discover drug targets for disease treatment more directly. Interactions between proteins and small molecule probes are categorized into covalent and non-covalent bonding interactions (as illustrated in Fig. 1). The covalent bond binding is powerful, but non-covalent bond interactions are more prevalent between molecules and proteins. Non-covalent bonding interactions between drugs and target proteins primarily contain hydrophobic contacts, hydrogen bonds and π -stacking, and weak hydrogen bonds [41,42]. Mass spectrometry (MS) is an important method for protein identification [43]. The technical approach currently used is divided into two types of probes based on labeled and unlabeled types. Labeled small-molecule probes mainly adopt activity-based protein profiling (ABPP), ICABPP, bead-MS technologies, among others [35,44,45]. Unlabeled small-molecule probes employ methods that primarily include drug affinity responsive target stability (DARTS)-MS, cellular thermal shift assay (CETSA)-MS, stability of proteins from rates of oxidation (SPROX)-MS, thermal proteomics (TPP)-MS, and others [46–49]. Additionally, biophysical and biochemical techniques, as well as cellular assays, are often used for drug-target binding validation, and small-molecule probes based on NPs mainly use the first two for drug-target binding validation. Biophysical techniques primarily include computer simulation (molecular docking), surface plasmon resonance (SPR), microscale thermophoresis, biolayer interferometry, isothermal titration calorimetry (ITC), circular dichroism, nano differential scanning fluorimetry, and others [11–13,50–52]. Biochemical techniques include DARTS, SPROX, CETSA, TPP, and small-molecule pull-down [14,53,54]. The main cellular detection techniques involve bioluminescence resonance energy transfer and small-molecule immunofluorescence [55,56]. Whether for the discovery of drug targets based on NPs small-molecule probes or the validation of drug-target binding, a combination of approaches tends to be employed. This constitutes a comprehensive discovery and validation system that ensures the accuracy and reliability of drug-target identification and drug-target binding. These methods allow researchers to effectively identify and validate the interactions between potential drug candidate molecules and their targets, providing a scientific basis for drug development.

4. Small-molecule probes based on NPs in stroke target discovery

Considering the remarkable efficacy of the active ingredients of NPs for the treatment of stroke, the utilization of small-molecule probes of NPs to discover crucial targets in stroke treatment is important. Recently, small-molecule probes of NPs have been screened for a series of target proteins closely related to the pathological mechanisms of stroke by labeling or unlabeled, including cathepsin B (CTSB), high mobility group protein B1 (HMGB1), CK2 α' , and others (Table S2 [9–27,57]). The primary types of small-molecule probes for NPs are terpenoids, flavonoids, and alkaloids. Target discovery mainly involves labeling methods, such as biotin, beads, and microspheres, and unlabeled methods, such as TPP and molecular docking. Furthermore, there are probes for target screening in stroke that use a class of components or multiple components (formulae) in NPs as probes. Most of these are monomer compounds. Fig. 2 [11–13,18] illustrates the application of small-molecule probes of NPs in stroke target discovery,

providing a scientific reference for the potential mechanism of NPs in stroke treatment.

4.1. Labeled probes

Utilization of labeled small-molecule probes of NPs in the screening of stroke targets strengthens screening efficiency, reinforces targeting, and facilitates mechanistic research. Recently, several types of probes have been used to discover drug targets for stroke treatment, including biotin, beads (sepharose, magnetic), microspheres, azides, and nanoparticles. Biotin- (Bio-) and alkyne-labeled NPs structures are shown in Fig. 3.

Recent explorations of the neuroprotective mechanism of action of NPs formulae as a certain type or single ingredient by the research team of Zeng and Tu have achieved definite results. These researchers innovatively employed magnetic microspheres with photosensitive benzophenone groups bonded to the surface to enrich the chemical components of the NPs-modified Wuzi Yanzong pill through covalent bonding properties and identified 32 potential targets related to neuroprotective effects after incubation with neural cell lysates. Kyoto encyclopedia of genes and genomes analysis revealed that these targets were mainly involved in the hippo and cell cycle signaling pathways, suggesting that the modified Wuzi Yanzong pill may exert anti-dementia and neuroprotective effects by regulating neural stem cell proliferation and promoting neural cell differentiation. This suggestion offers a new approach for identifying the target groups of the complex components of NPs and is valuable for in-depth studies of the pharmacological mechanism of NPs [57]. Using the natural small-molecule sappanone A (SA) as a probe, human inosine monophosphate dehydrogenase 2 (IMPDH2) was affinity purified, followed by stable isotope labeling with amino acids (SILAC) and MS. Competitive pull-down assays, SPR analysis, and fluorescent labeling assays further validated the specific binding of SA to IMPDH2, with a dissociation constant (K_D) value of 3.944 nM. SA covalently conjugated to the Cys140 site of IMPDH2 reportedly had a modulatory effect and inhibited IMPDH2 activity, effectively suppressing neuroinflammation. SA has exhibited favorable anti-inflammatory effects in neuroinflammatory models, providing new ideas for treating neuroinflammation and related diseases [15]. A rat middle cerebral artery blockade model was established using the wire embolization method to verify the cerebral protective effects of phenylethanoid glycosides from *Cistanches Herba*. Target proteins associated with brain-protective effects were successfully captured from brain tissue lysates using specially designed solid-phase microspheres with surface-bonded phenylethanoid glycosides from *C. Herba*. Phenylethanol glycosides significantly attenuate cerebral ischemia/reperfusion injury in rats, as evidenced by a reduction in the ischemic area, improvement of behavioral scores, and elevation of superoxide dismutase levels. The target “hook-and-line” strategy revealed 18 target proteins, including heat shock protein 90 (Hsp90), which are involved in nine aspects, including antioxidant activity, ion channels, and immunomodulation [16]. In this study, the potential pharmacological mechanisms of phenylethanol glycosides in dementia, fatigue, tumors, and immunomodulation were investigated at the molecular level, and a new approach for analyzing the molecular pharmacology of NPs components was proposed [16]. The ECH probe was prepared by coupling ECH to epoxy-activated sepharose beads. The ECH probe was incubated with the cell lysate to bind ECH to intracellular target proteins. Bound target proteins were captured using the ECH probe and identified by silver staining and liquid chromatography tandem MS (LC-MS/MS) analysis. The study demonstrated that ECH could activate the Wnt/ β -catenin signaling pathway and promote the transcription and expression of the mitochondrial fusion-

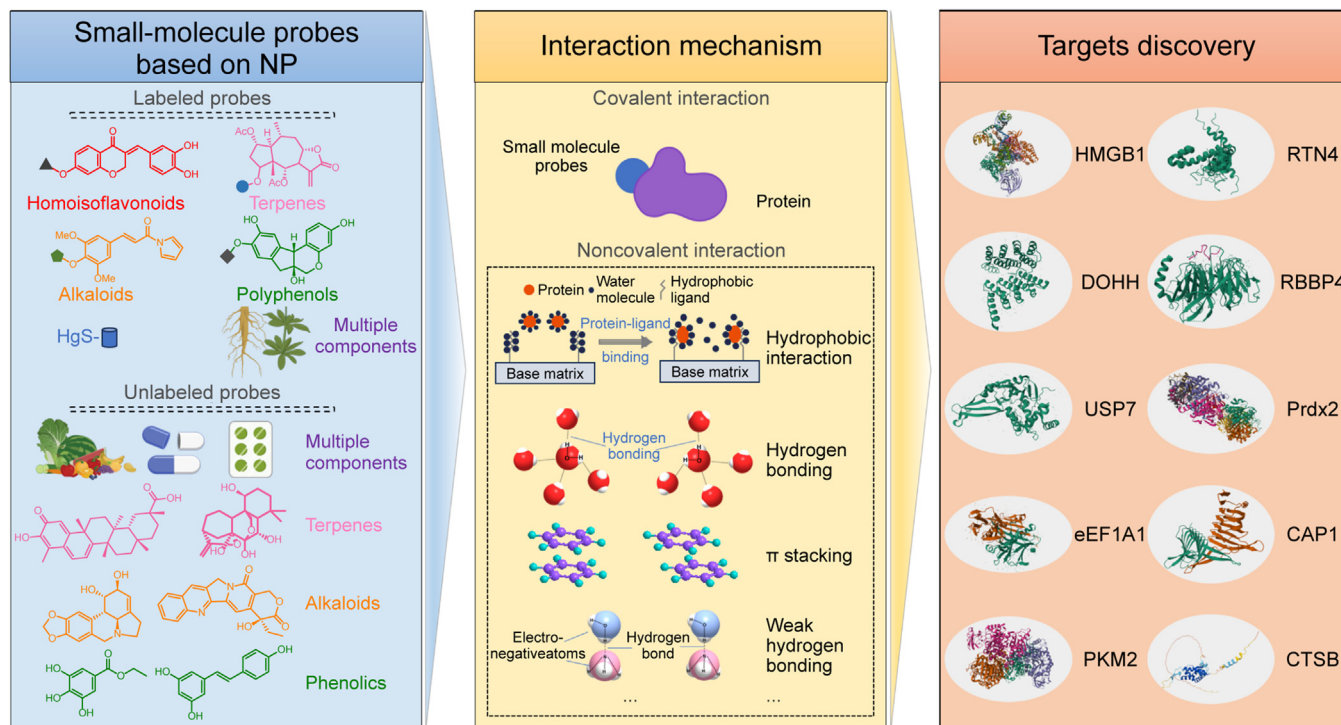


Fig. 1. Mechanisms of action of small-molecule probes based on natural products (NPs) for drug target discovery. HMGB1: high mobility group protein B1; DOHH: dihydroorotate dehydrogenase; USP7: ubiquitin specific peptidase 7; eEF1A1: eukaryotic elongation factor 1- α 1; PKM2: pyruvate kinase muscle isoform 2; RTN4: reticulon-4; RBBP4: retinoblastoma binding protein 4; Prdx2: peroxiredoxin-2; CAP1: cyclase associated actin cytoskeleton regulatory protein 1; CTSB: cathepsin B.

associated gene Mfn2 by binding to the CK2 α' subunit, promoting its conformational change, and recruiting basic transcription factor 3 (BTF3) to form a complex, finally exerting neuroprotective effects. In a mouse model of ischemic stroke, ECH significantly ameliorated brain injury and neurological deficits and increased the expression of Mfn2, further confirming its neuroprotective effects. These findings demonstrated for the first time the key role of CK2 in promoting mitochondrial fusion through a Wnt/ β -catenin-dependent pathway and indicated the therapeutic potential of CK2-targeted drug treatment strategies for ischemic stroke [9]. In this study, a biotin-labeled brazilin probe was used for target screening in a human proteomic microarray. Deoxyhypusine hydroxylase (DOHH) was successfully identified as a direct binding protein of brazilin, with the highest signal-to-noise ratio among 765 protein candidates (Fig. 2A) [11]. Subsequently, the binding affinity (K_D value of 1.4–5.8 μ M) and binding site (Cys232 site) of brazilin and DOHH were verified by various biochemical and biophysical techniques, including SPR, bio-layer interferometry (BLI), and ITC. Further studies have shown that brazilin binds to and enhances the enzymatic activity of DOHH, activating mitochondrial autophagy and exerting neuroprotective effects. The results provide new ideas for the development of novel ischemic stroke drugs, and brazilin, the first DOHH enzyme allosteric activator, has promising clinical applications [11].

Xu et al. [58] have made several important discoveries in their research on the mechanism of small-molecule probes of NPs for the treatment of stroke. TLR4 protein was screened as a target of RV01, a quinoline analog of resveratrol, in BV2 microglial cell and primary microglia models using techniques that included molecular docking, DARTS, CETSA, and SPR. RV01 displayed a high affinity for TLR4 protein ($K_D = 2.558 \times 10^{-5}$ M/L) and effectively inhibited the formation of the TLR4/myeloid differentiation protein-2 (MD-2) complex and NF- κ B signaling pathway, with anti-inflammatory

and neuroprotective effects. In a rat model of MCAO/reperfusion (MCAO/R) ischemic stroke, RV01 remarkably reduced infarct volume, improved neurological deficits, increased the duration of rotation, and reduced the number of right turns, indicating its potential for the treatment of ischemic stroke [58]. The PB-1 N-cinnamoylpyrrole derivative alkaloid exhibits significant anti-neuroinflammatory and anti-oxidative stress effects. Using ABPP and click chemistry ABPP (CC-ABPP) techniques, the researchers successfully identified eukaryotic elongation factor 1- α 1 (eEF1A1) as a functional target of PB-1 (Fig. 2B) [13]. PB-1 regulated NF- κ B and NRF2 signaling pathways by binding to eEF1A1, thereby inhibiting neuroinflammation and oxidative stress and ultimately ameliorating cerebral ischemic injury [13]. In an lipopolysaccharide (LPS)-induced BV2 microglia model, PB-1 was capable of significantly inhibiting the expression of inflammatory factors that included inducible nitric oxide synthase (iNOS), interleukin (IL)-1 β , IL-6, and tumor necrosis factor- α (TNF- α), and upregulating the expression of antioxidant enzymes, such as heme oxygenase-1 (HO-1) and nicotinamide adenine dinucleotide (phosphate) hydride quinone dehydrogenase 1 (NQO1). The ability of PB-1 to protect BV2 microglia from hypoxia/reoxygenation-induced cytotoxic injury in an oxygen-glucose deprivation/reperfusion (OGD/R) model. In a transient MCAO mouse model, PB-1 significantly attenuated neurological deficits, cerebral edema, and cerebral infarct volume and reduced neuronal injury and microglial cell activation. Thus, PB-1 has the potential to be a novel drug for the treatment of ischemic stroke [13].

Wu et al. [10] performed target screening of britanin on the DiscoverX's KINOMEScan platform and found that it did not directly bind to protein kinases. Further studies revealed that britanin can inhibit Keap1-mediated Nrf2 ubiquitination by forming a covalent bond with the BTB structural domain of Keap1, thereby activating the Nrf2 pathway, significantly reducing the level of

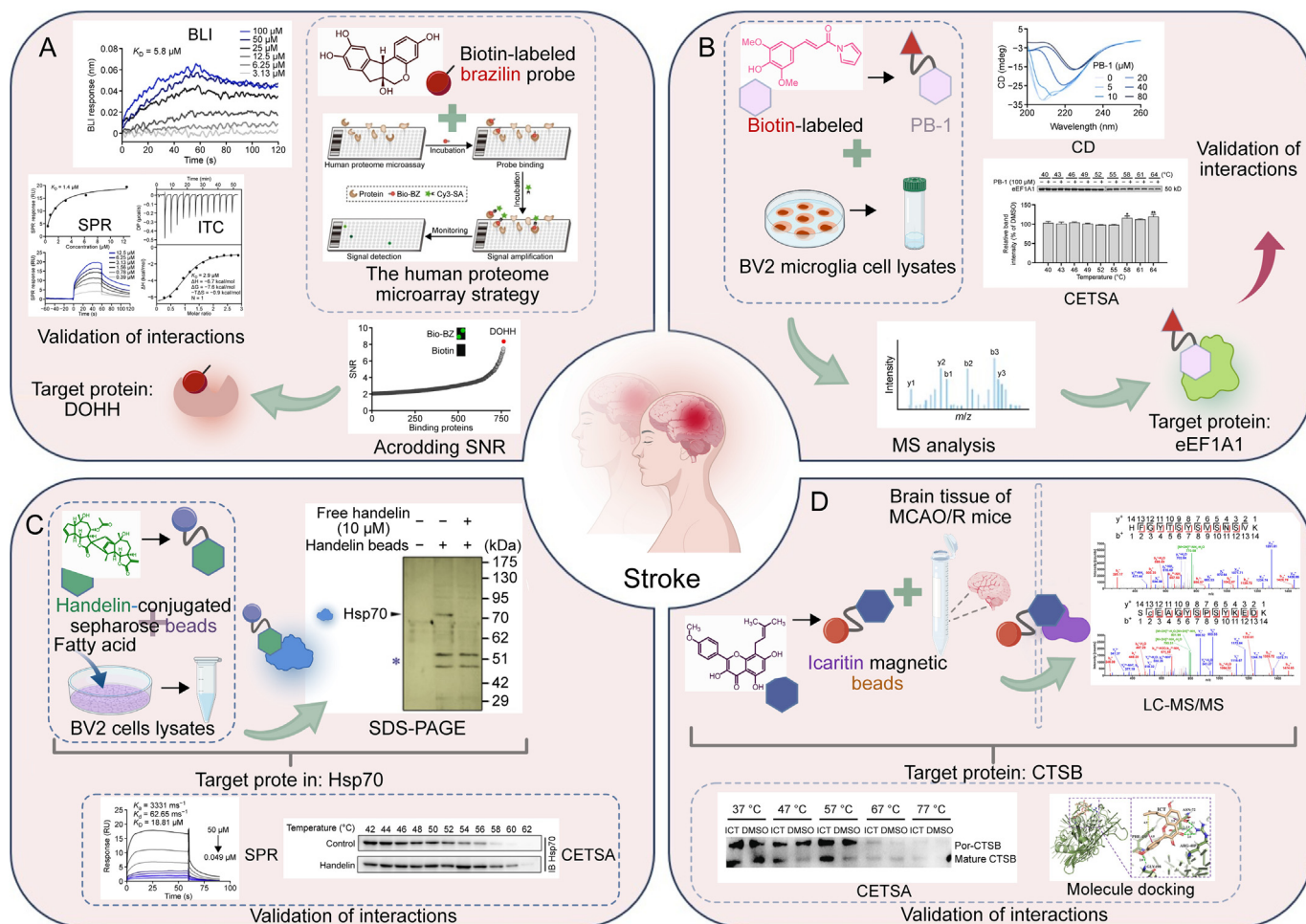


Fig. 2. Application of small-molecule probes based on natural products (NPs) in stroke target discovery. (A) HuProt™ human proteome microarray identifies deoxyhypusine hydroxylase (DOHH) as a key target for ischemic stroke treatment with brazilin. Reprinted from Ref. [11] with permission. (B) PB-1 emerges as a promising therapeutic agent for ischemic stroke by targeting eukaryotic elongation factor 1-α (eEF1A1). Reprinted from Ref. [13] with permission. (C) Handelin participates in the treatment of neuroinflammation through the cysteine residue of heat shock protein (HSP) 70. Reprinted from Ref. [12] with permission. (D) Icaritin prevents ischemia-reperfusion injury in mice by triggering inflammatory regression by targeting cathepsin B (CTSB). Reprinted from Ref. [18] with permission. DOHH: dihydroorotate dehydrogenase; SNR: signal-to-noise ratio; Keap1: Kelch-like ECH-associated protein 1; MS: mass spectrometry; BLI: bio-layer interferometry; SPR: surface plasmon resonance; ITC: isothermal titration calorimetry; CETSA: cellular thermal shift assay; MCAO/R: middle cerebral artery occlusion (MCAO)/reperfusion; LC-MS/MS: liquid chromatography tandem MS; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CD: circular dichroism; DMSO: dimethyl sulfoxide.

intracellular reactive oxygen species, and inhibiting apoptosis. These collective activities are neuroprotective. Britanin has shown significant neuroprotective effects in cortical neurons cultured *in vitro* and *in vivo* in a rat model of MCAO/R model and is expected to be a novel drug for the treatment of cerebral ischemia/reperfusion injury and other diseases [10]. Wang et al. [12] using handelin as a chemical probe, heat shock protein 70 (Hsp70) was demonstrated to be a direct target of handelin by affinity chromatography and MS. The powerful binding ability of handelin to Hsp70 ($K_D = 18.81 \mu\text{M}$) was demonstrated by SPR (Fig. 2C) [12]. CETSA and DARTS analyses further validated the direct interaction between handelin and Hsp70. Gene silencing experiments demonstrated the critical role of Hsp70 in handelin-mediated anti-inflammatory effects. Handelin has demonstrated significant anti-inflammatory activity *in vitro* and *in vivo* and has been used in the treatment of neuroinflammatory disorders, among others [12].

Gao et al. [17] synthesized a highly efficient and low-toxicity benzoxazine derivative, which is considered a potential lead compound for targeting pyruvate kinase muscle isoform 2 (PKM2) for the treatment of inflammation-associated diseases, compared to the known PKM2 inhibitor viologin. The introduction of a

photoaffinity labeling group and reporter group based on the structure of the benzazine derivative allowed for irreversible binding to the target protein and facilitated subsequent detection. The probe was co-incubated with cells and activated by ultraviolet light. The target protein labeled with the probe was observed by fluorescence imaging. The bound target proteins were enriched using probes, and proteomic analysis was performed to screen for PKM2 as a possible target. The binding kinetics and affinity of compound 10i (benzoxazine derivative) for PKM2 were investigated using CETSA and SPR. The anti-inflammatory effect of 10i was verified in cell and animal experiments by inhibiting PKM2 activity. This compound also displays anti-neuroinflammatory effects *in vitro* and *in vivo* by inhibiting PKM2-mediated glycolysis and NLRP3 activation, suggesting that PKM2 is a novel target for neuroinflammation and related brain diseases. Other researchers used a photochemically induced coupling reaction system to couple icaritin (ICT) with magnetic nanoparticles, mixed it with brain tissues from a mouse model of MCAO/R, and screened 119 proteins bound to ICT (Fig. 2D) [18]. The binding of ICT to CTSB was confirmed by CETSA, SPR, molecular docking, and enzyme activity assays. CETSA showed that ICT could protect the CTSB

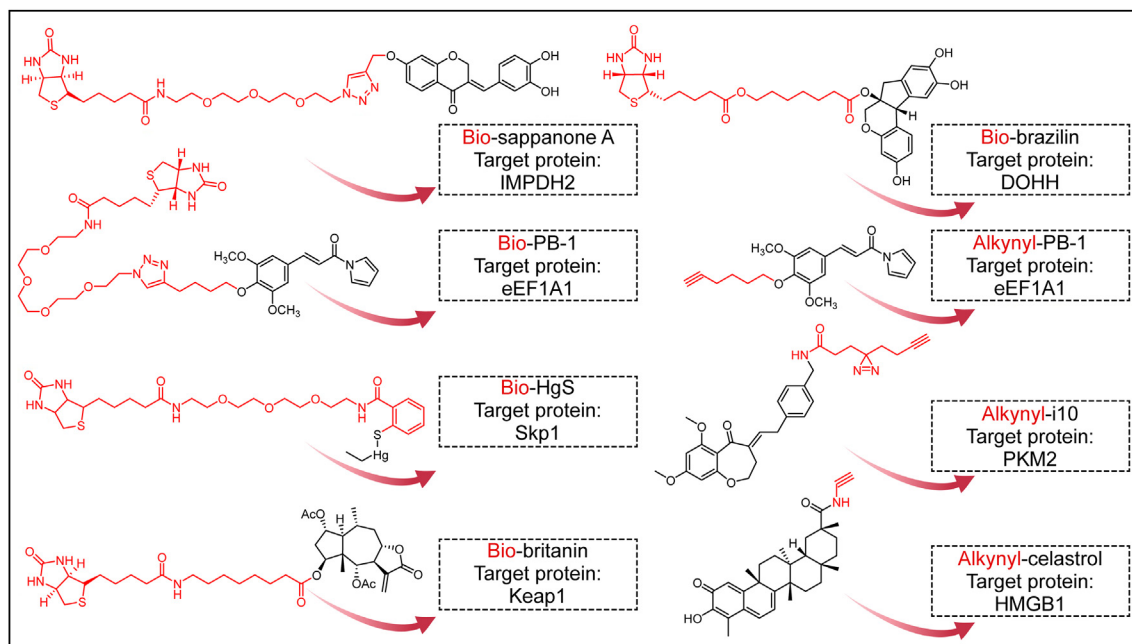


Fig. 3. Biotin- (Bio-) and alkyne-labeled small-molecule probes based on natural products (NPs). IMPDH2: human inosine monophosphate dehydrogenase 2; DOHH: dihydroorotate dehydrogenase; eEF1A1: eukaryotic elongation factor 1- α 1; Skp1: S-phase kinase-associated protein 1; PKM2: pyruvate kinase muscle isoform 2; Keap1: Kelch-like ECH-associated protein 1; HMGB1: high mobility group protein B1.

protein from degradation. The K_D of ICT and CTSB was determined to be 10.28 μM by SPR, which indicated that the two were strongly bound to each other. Molecular docking predicted that ICT could bind to CTSB through hydrogen, π - π , and π - σ bonding. An assay of enzyme activity demonstrated that ICT significantly inhibited the activity of CTSB with an IC_{50} of 1.22 μM . This study revealed the potential of ICT as a small-molecule targeting CTSB in the anti-inflammatory effects of neurological disorders and provided a new therapeutic strategy for the treatment of cerebral ischemia/reperfusion injury [18]. Ginsenoside CK, the main metabolite of ginsenoside Rd, was studied for its neuroprotective effects against cerebral ischemia/reperfusion injury [59]. The authors found that ginsenoside CK has a high affinity for protein tyrosine phosphatase 1B (PTP1B) with a K_D value of 0.89 mM by using TargetNet bioinformatics prediction and screening by the SPR technique. CETSA further confirmed that ginsenoside CK increased the thermal stability of PTP1B. CETSA further confirmed that the ginsenoside CK increased the thermal stability of PTP1B. In neuroscreen-1 cells and a rat model of MCAO, ginsenoside CK inhibited the phosphatase activity of protein tyrosine phosphatase 1B (PTP1B), thereby restoring the tyrosine phosphorylation level of insulin receptor substrate 1 (IRS1) and activating the phosphoinositide 3-kinase (PI3K)-Akt signaling pathway. In addition, ginsenoside CK reduced neuronal apoptosis induced by PTP1B overexpression. These results suggest that ginsenoside CK exerts neuroprotective effects by inhibiting PTP1B-mediated IRS1 tyrosine dephosphorylation and activating the PI3K-Akt signaling pathway [59]. Liu et al. [19] successfully identified high mobility group box 1 (HMGB1) as the direct binding target of celastrol (cel) in cerebral ischemia/reperfusion injury using quantitative chemical proteomics. The results showed that cel probe (cel-p) has high affinity for HMGB1 and that cel can completely compete for cel-p binding to HMGB1. In addition, the CETSA results showed that cel stabilized HMGB1 and reduced its degradation. In both the primary rat cortical neuronal model of OGD and an adult rat model of MCAO, cel exhibited neuroprotective effects and up-regulated the

expression of Hsp70 and down-regulated the expression of NF- κB p65. These results suggest that cel inhibits the pro-inflammatory activity of HMGB1 by directly binding to it and exerts neuroprotective and anti-inflammatory effects by targeting Hsp70 and NF- κB [19]. Ma et al. [20] revealed that the Seventy Flavors Pearl Pill (SFPP) achieves neuroprotective effects by regulating the oxidative phosphorylation (OXPHOS) pathway. Proteomic analysis revealed that the abnormal expression of key OXPHOS proteins, including ATP synthase peripheral stalk subunit D (ATP5PD), NADH: ubiquinone oxidoreductase subunit B4 (NDUFB4), and cyclooxygenase 15 (COX15) in the model group was reversed by SFPP treatment. Reductive dimethyl labeling with tandem orthogonal proteolysis-ABPP technology confirmed that SFPP regulates OXPHOS proteins. Inhibition of OXPHOS by SFPP promoted cell proliferation in a dose-dependent manner. Ultra-performance liquid chromatography-MS (UPLC-HRMS) identified SFPP components and found that isosalantolactone promoted the proliferation of SH-SY5Y cells, initially confirming the involvement of SFPP in the OXPHOS pathway. SFPP and isosalantolactone exert neuroprotective and cell-proliferation-promoting effects through the OXPHOS pathway [20]. Finally, another study verified that the key component of the Huangxiong formula, α -asarone, has a strong binding ability to PI3K proteins through network pharmacology, molecular docking, DARTS, and CETSA analyses, and confirmed its neuroprotective effect through the OGD/R cell model [60]. The findings provide new insights into the mechanism of action of the Huangxiong formula during ischemic stroke treatment, as well as possible targets for the development of novel curative drugs [60].

4.2. Unlabeled probes

Labeled small-molecule probes of NPs may damage the pharmacological activity of the parent small molecules of NPs and suffer from defects in complex synthesis methods and inapplicability of trace components. Therefore, label-free small-molecule probes for

NPs have been introduced for screening stroke targets. In recent years, these probes have mainly been utilized for target screening using TPP, molecular docking, and affinity chromatography.

Targeted screening was performed using costunolide (CTL) to investigate its mechanism of action in LPS-induced neuroinflammation. Cyclin-dependent kinase 2 (CDK2) was identified as a direct binding target protein for CTL through filtration of the scPDB protein structure collection through a receptor reverse virtual screening technique based on the principle of molecular docking [21]. SPR, CETSA, and DARTS analyses were used to validate the affinity of the target proteins. The results showed that the binding of CTL to CDK2 was at the micromolar level ($K_D = 32.02 \mu\text{M}$). Follow-up experiments in an LPS-induced BV2 microglial cell model demonstrated that CTL inhibited the AKT/inhibitory kappa B kinase beta (IKK β)/NF- κ B signaling pathway through the CDK2 target, thus exerting anti-neuroinflammatory effects [21]. In the present study, anti-silencing factor 1a (ASF1a) was screened as a direct target of artone in a model of LPS-induced neuroinflammation using TPP technology. CETSA and SPR analyses confirmed the binding of artone to ASF1a with a binding affinity of $K_D = 5.52 \mu\text{M}$. Further studies demonstrated that artone inhibited the NF- κ B signaling and pro-inflammatory pathways by modulating the levels of ASF1a-dependent histone H3 acetylation and trimethylation, thus exerting anti-neuroinflammatory effects. These findings provide new ideas for the development of anti-neuroinflammatory drugs targeting ASF1a and the possibility of an effective treatment of inflammation-related neurodegenerative illnesses [22]. The possible cellular target of murrayafoline A (MA), a natural carbazole alkaloid, was screened using TPP in an LPS-induced BV2 microglial cell model as specific protein 1 (Sp1). Further SPR analysis revealed that MA binds to Sp1 with a binding affinity of $11.34 \mu\text{M}$, indicating a strong interaction between the two. In addition, CETSA and DRATS techniques confirmed that MA could inhibit the degradation of Sp1 protein, thus exerting anti-neuroinflammatory effects. MA inhibits the NF- κ B and mitogen-activated protein kinase (MAPK) signaling pathways by directly targeting Sp1, ultimately exerting neuroprotective effects [23]. Kellerin, a sesquiterpene coumarin compound derived from *Ferula sinkiangensis* K. M. Shen, protects the brain from ischemic damage. Kellerin is important in diseases such as stroke. Research incorporating molecular docking, DARTS, and CETSA analyses discovered the ability of kellerin to bind to Akt proteins and activate the Akt/Nrf2 pathway. These events inhibit ferroptosis, mitochondrial generation of reactive oxygen species, producing neuroprotective effects [14]. The study findings implicated kellerin as a potential drug for the treatment of neurological disorders such as stroke [14]. Chen et al. [27] identified five protein targets of ginsenosides in the brain by affinity “fishing” and MS: 14-3-3 protein, actin, creatine kinase type B, ATP synthase, and an unknown protein. Among them, the 14-3-3 protein was validated by BLI and confirmed to have direct interaction with ginsenosides, and its *in vivo* metabolites, proto-ginsenodiol and proto-ginsenotriol, showed the strongest binding affinity (K_D values: 7.8×10^{-4} and 5.62×10^{-4} , respectively). These findings implicated that the 14-3-3 protein is a critical target of total ginsenosides within the brain and that the protein might be activated by protopanaxadiol (PPD) and protopanaxatriol [27].

The discovery of drug targets for stroke is critical. However, current research remains limited. Phosphodiesterase type 1 (PDE1) is widely distributed in both the central nervous and cardiovascular systems, and is important in the regulation of cerebral blood flow and neurological function. NPs include vinpocetine, which is a selective PDE1 inhibitor. Vincristine tablets and injections have been developed for the treatment of stroke [61]. Endothelin B receptor facilitates neurovascular remodeling in adult ischemic stroke, and

Pharmazz's peptide agonist Sovateltide (Tycamzzi™), a peptide agonist of endothelin B receptor, has been approved for the treatment of acute cerebral ischemic stroke [62,63]. Nevertheless, there have been few clinical drug developments based on therapeutic targets for stroke treatment. Recent genetic studies have provided valuable insights into stroke pathogenesis. Coagulation factor XI, apolipoprotein(a), and scavenger receptor class A, member 5 (SCARA5) were identified as biomarkers for ischemic stroke treatment using Mendelian randomization analysis [64]. A genome-wide association study implicated F11, KLKB1, PROC, GP1BA, LAMC2, and VCAM1 as potential stroke drug targets [50]. However, the discovery of more precise drug targets for NPs therapy in patients with stroke is imminent. Small-molecule probes based on NPs provide critical information for drug discovery and development by accurately identifying and binding potential drug targets via specific interactions with biomolecules. Nevertheless, small-molecule probes based on NPs are in the stage of *in vitro* and *in vivo* experiments, which are limited because they cannot completely replicate the physiological environment. Moreover, the lack of clinical trials to determine the safety and efficacy of these probes in humans has restricted their clinical application.

5. Application of small-molecule probes based on NPs in the discovery of other disease targets

Small molecule probes based on NPs are applied to a wide range of diseases in addition to their application in stroke target discovery. The type of NPs contains terpenoids, alkaloids, flavonoids, phenolic compounds, and others. Terpene components have the greatest number of applications as small-molecule probes of NPs in disease mechanisms, mainly for identifying cancer and tumor-related targets. Probes for disease-related target discovery are mainly categorized into labeled and unlabeled probes. Labeled probes account for the majority of probes, which are primarily labeled with biotin, alkynes or azides, photosensitive moieties, and microspheres. In contrast, unlabeled probes are unmodified active ingredients of NPs that allow direct incubation with cell or animal lysates to discover target proteins by determining changes in protein stability, oxidation rates, and other aspects. Table S3 [32–35,44–47,49,51,52,54,56,65–105] presents data on the utilization of small-molecule probes of NPs in the discovery of different disease-related targets in recent years. Fig. 4 demonstrates the general procedure for small-molecule probes of NPs used for target discovery.

5.1. Labeled probes

The labeled small-molecule probes of NPs are designed to enhance the specificity of the binding of small molecules of NPs to the target by introducing specific labeling molecules, facilitating subsequent enrichment, detection, and analysis. The primary labeled substances included biotin, alkynes, azides, photosensitive moieties, and microspheres. The advantages of these probes include improved specificity, easier enrichment and detection, and adaptability to complex systems. These taggants enable researchers to utilize small-molecule probes of NPs more effectively to exploit the mode of action and prospective medical applications of active ingredients [106,107].

5.1.1. Biotin labeling

Streptavidin is a protein derived from *Streptomyces avidinii*. The tetrameric protein resembles avidin in its structure [108]. The binding between streptavidin and biotin exhibits an extremely high affinity (K_D approximately 4×10^{-14}) and remains highly specific. Given the small size of biotin (244 Da), covalent links easily form with molecules, a process that scarcely influences its biological

activity [109]. Consequently, biotin labeling is a commonly used approach for designing small-molecule probes.

α -Mangostin (α -MG) elicits cell pyroptosis. Zhao et al. [65] designed a biotin-labeled α -MG probe designated biotin- α -MG. The high-affinity interplay between biotin and streptavidin was employed by the researchers in a human proteomic microarray screen by binding biotin to α -MG. The strategy captured a protein that binds to α -MG that was designated reticulon-4 (RTN4). Research findings have established that α -MG induces ubiquitination degradation of RTN4, remodels the curvature of the endoplasmic reticulum, promotes the fusion of the ER with the plasma membrane, activates the caspase-3/gasdermin E (GSDME) signaling pathway, and induces cellular focal death. α -MG upregulates the expression of programmed death-ligand 1 in tumor cells and enhances the anti-tumor immune response. In an osteosarcoma mouse model, α -MG significantly inhibited tumor growth and activated immune responses. Immunohistochemical analysis revealed that high RTN4 expression was associated with osteosarcoma metastasis and death. The collective results implicate RTN4 as a potential target for anti-tumor therapy and identify α -MG as a novel anti-tumor immunotherapy candidate [65]. In addition, affinity capture and MS analyses involving biotinylated triptolide (biotin-TL) identified peroxiredoxin I (Prx I) as the target of TL. TL and its derivatives, such as celastrol and withaferin A, specifically bind to the cysteine residue of Prx I and inhibit its molecular chaperone activity, demonstrating their therapeutic potential. Given the role of Prx I in cancer and inflammation, these findings suggest that TL analogs may be novel molecular targeting agents for the treatment of related diseases [110]. Data obtained from mice fed a high-fat diet have demonstrated that artemether (ART) protects thyrotropin-releasing hormone neurons and improves hepatic lipid metabolism by inhibiting the activation of hypothalamic microglia

[66]. The researchers further demonstrated that biotin-labeled ART acts on protein kinase C δ (PKC δ) in microglia, preventing zinc finger DHHC-type palmitoyltransferase 5 from binding to PKC δ , and inhibiting PKC δ palmitoylation and neuroinflammatory signaling. Specific knockdown of PKC δ reportedly impaired the metabolic ameliorating effect of ART. Finally, clinical data revealed that PKC δ levels in activated microglia of hyperlipidemic patients were positively correlated with lipids [66].

5.1.2. Alkynyl or azide labeling

Among the diverse pathways for exploring drug targets, the combination of alkyne and azide groups is widely recognized as a classical strategy in click chemistry. The small molecular weight of the alkyne or azide groups has little influence on the activity of the ligand moiety without affecting cell permeability, making it easy to obtain highly active probe molecules [111]. The demonstrated high efficiency and selectivity of this chemical reaction, as well as the mild reaction conditions, have indicated its potential for application in the targeted discovery of active ingredients in NPs [112]. Therefore, modification of the active ingredients of NPs with alkynes or azides as click chemical probes allows the effective capture of their targets of action.

Click chemistry plays an instrumental role in the exploration of anti-malarial mechanisms. Multiple studies have shown that click chemistry effectively recognizes the targeting proteins of artemisinin-related compounds during the anti-malarial process, enhancing our understanding of the anti-malarial mechanism of artemisinin. We screened 58 target proteins in a *Plasmodium falciparum* 3D7 malarial parasite model by designing artemisinin analog probes containing alkyne or azide moieties and utilizing their specific alkylation using ABPP technology. Using glycolysis, hemoglobin degradation, and other processes, these proteins were

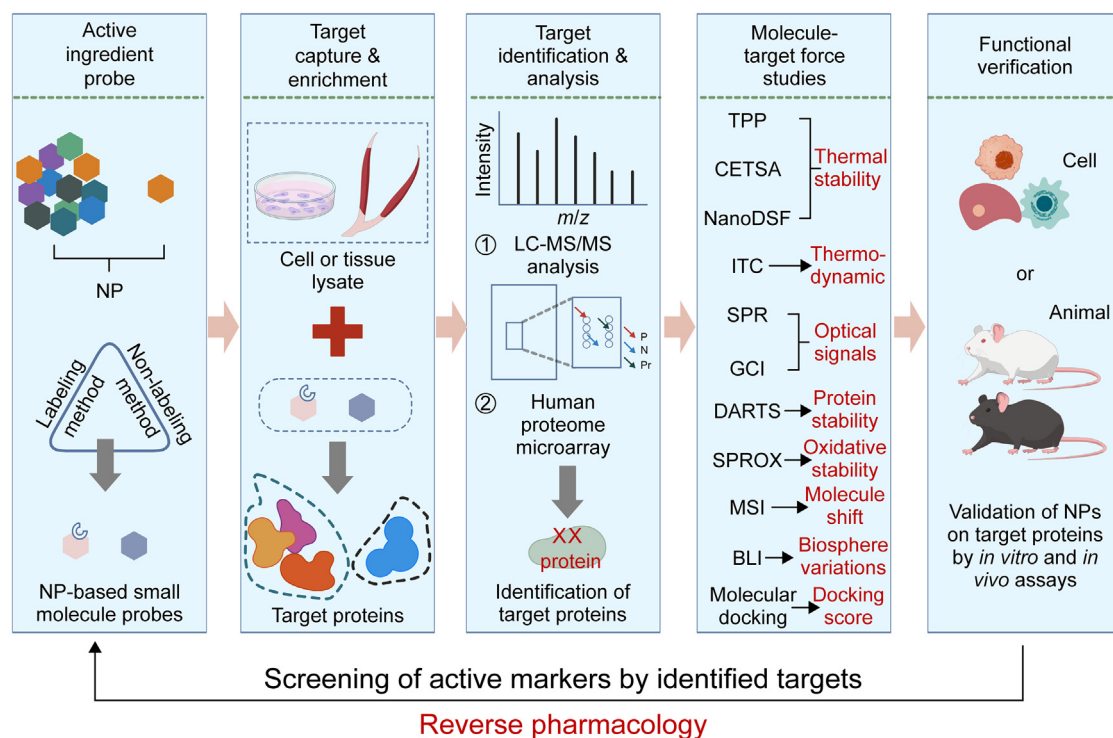


Fig. 4. Fundamental steps of small-molecule probes based on natural products (NPs) applied to screening of target proteins. LC-MS/MS: liquid chromatography tandem MS; TPP: thermal proteomics; CETSA: cellular thermal shift assay; NanoDSF: nano-differential scanning fluorimetry; ITC: isothermal titration calorimetry; SPR: surface plasmon resonance; GCI: grating-coupled interferometry; DARTS: drug affinity responsive target stability; SPROX: stability of proteins from rates of oxidation; MSI: mass spectrometry imaging; BLI: bio-layer interferometry.

characterized by LC-MS/MS. The demonstrated strong affinity for artemisinin analogs (IC_{50} values of 43.86–30.26 nM) revealed a novel mechanism of artemisinin action [67]. Wang et al. [68] designed an artemisinin-based probe designated AP1 linked to a kynurenine tag, using chemical proteomics techniques. Using click chemistry and affinity purification combined with MS analysis, the researchers identified 124 *P. falciparum* protein targets covalently bound to AP1 whose involvement in key biological processes was also identified. *In vitro* and *in vivo* experiments confirmed that AP1 binding to target proteins is influenced by the artemisinin dose and that heme is the major iron source for AP1 activation, with its source varying with the developmental stage of *P. falciparum*. The multi-target mechanism of artemisinin was revealed, and the high efficiency and low toxicity of artemisinin were elucidated, establishing a cornerstone for the development of new anti-malarial drugs [68]. Artemisinin analogs modified with an alkyne group at the carbon-10 position were screened for 124 *P. falciparum* protein targets using click chemistry and biotin labeling via chemical proteomics. MS analysis confirmed that these target proteins were critical for *P. falciparum* survival. *In vitro* experiments have verified that some proteins bind specifically to artemisinin, revealing a novel mechanism through which artemisinin broadly targets proteins. Artemisinin is covalently conjugated with several key proteins to disrupt the biochemical environment of *P. falciparum* and efficiently and selectively fight malaria, providing a basis for optimizing treatment and drug resistance [69].

Click chemistry is also crucial for investigating tumor mechanisms. Specific target proteins were screened and labeled in HCT116 colorectal cancer cells using the ABPP technology with a curcumin derivative and an alkyne group probe (Cur-P). iTRAQ quantitative analysis revealed that curcumin significantly affected metabolic enzymes and cell cycle proteins with a higher average affinity than the control, implying that its anti-tumor mechanism may be associated with metabolic and cell cycle regulation, providing a new idea for the treatment of colorectal cancer [70].

In addition, small-molecule probes of NPs involved in click chemistry can also be crucial in elucidating the mechanism of function in other diseases. The research team of Tu and Zeng performed a chemical proteomics study involving photoaffinity labeling by synthesizing a cell-penetrating PPD probe using a bifunctional alkyne group-containing azide photocrosslinker [71]. Retinoblastoma binding protein 4 (RBBP4) was identified as a key cellular target of PPD and was found to significantly reduce RBBP4-dependent lysine trimethylation at histone H3 27. The results revealed that PPD can inhibit cell proliferation and migration. RNA silencing of RBBP4 reversed this inhibitory effect [71].

In another study, magnetic microspheres were modified by the addition of the natural C-glycosylated flavonoid mangiferin using click chemistry. These microspheres were used to screen for two key target proteins, 70 kDa heat shock protein family A member 5 (Hspa5) and tyrosine 3-monooxygenase (Ywhae), in a mouse model of acute lung injury induced by LPS. These target proteins were identified by MS, and their affinities were verified by molecular docking at respective binding energies of -9.50 and -7.63 kcal/mol [72]. These findings have contributed to unraveling the molecular mechanism of the anti-inflammatory effects of mangiferin and have provided new targets for the treatment of acute lung injury. Other researchers demonstrated that the Rv3852 protein is the target of agrimophol in *Mycoplasma tuberculosis* using a reactive group strategy and affinity chromatography [51]. Following click chemistry to introduce biotin in combination with affinity chromatography to enrich the protein, Western blot, ITC, and electrophoretic mobility shift assay analyses confirmed that agrimophol binds specifically to Rv3852 with binding constants of approximately 34 and 22 μ M and that it interferes with the

binding of Rv3852 to DNA. These results indicating that Rv3852 may regulate pH homeostasis of *M. tuberculosis* could provide a new direction for the development of drugs active against tuberculosis [51]. In the other study [113], target screening was performed using an azide-modified glycolphoric acid derivative (azide-terminal GA (ATGA)). Through a click chemistry reaction, researchers combined ATGA with a quantum dot-modified alkyne group and used this for protein screening in HEK293 cells. Data from confocal microscopy and competition experiments confirmed the specific binding of ATGA to β_2 AR, providing novel vehicles to investigate the molecular mechanisms of asthma [113]. Han et al. [44] recently developed an alkyne-based probe for the coumarin analog columbianadin, whose specific target was identified as vimentin MH7A cells stimulated by TNF- α by reactive group proteomics. Molecular docking confirmed the avid binding of columbianadin and vimentin and that the columbianadin probe effectively inhibited the VAV2/Rac-1 signaling pathway mediated by vimentin, effectively alleviating the symptoms of arthritis in collagen-induced arthritis mice, including the reduction of swelling, inhibition of erosion, and reduction of inflammation. Thus, this study provides a new direction for the development of anti-inflammatory drugs [44].

5.1.3. Photoaffinity group labeling

Photosensitive photoaffinity groups constitute the core of photoaffinity probes. These groups are responsible for the formation of covalent bonds with target proteins in the presence of light. Small-molecule probes of NPs labeled with the photosensitive moiety allow more avid binding to proteins, which compensates for the deficiency of some probes that lack functional groups covalently modified with proteins. The selection and application of photosensitizing moieties is crucial for the efficiency and specificity of small-molecule probes for NPs. Typical photosensitizing moieties include benzophenones, tetrazoles, and aryl azides. When constructing small-molecule probes, photoaffinity groups that feature high reactivity, high specificity, longer light wavelengths, less protein damage, and higher affinities for target proteins are usually selected [114].

Yu et al. [73] demonstrated that the mode of action of ethyl gallate, a molecule with potential activity in the treatment of ulcerative colitis, involves phosphatidylethanolamine-binding protein 1 (PEBP1) using a chemical biology strategy involving photoaffinity labeling. The study findings indicated that ethyl gallate influences the phosphorylation of PEBP1 by forming a hydrogen bond with a specific site, which in turn regulates the NF- κ B inflammatory pathway and inhibits inflammation. Silencing the expression of PEBP1 using a specific small interfering RNA reversed the anti-inflammatory effect of ethyl gallate, emphasizing the key role of NF- κ B signaling, revealing PEBP1 as a new target for the treatment of ulcerative colitis therapy, and implicating ethyl gallate as a chemical tool to study PEBP1 function [73]. The researchers also synthesized a photoaffinity labeling probe for halofuginone. Use of the probe with extracted *P. falciparum* proteins along with LC-MS and click chemistry analyses identified proteins to which the probe bound. Direct binding of halofuginone to *P. falciparum* ubiquitin carboxyl-terminal hydrolase 3 was confirmed by SPR and grating-coupled interferometry (GCI) techniques. Molecular docking was performed to analyze the mode of binding. Protein profile comparison screened *P. falciparum* ubiquitin carboxyl-terminal hydrolase 3 as a potential target of halofuginone. The avid binding of halofuginone was confirmed by competition experiments using SPR and GCI [74]. Dai et al. [75] used baicalin as a photoaffinity probe by introducing a benzophenone photo-crosslinking group and an alkyl reporter group through a carboxyl group. Baicalin activated carnitine palmitoyltransferase 1A (CPT1A) using

SILAC-ABPP technology. The strength and activity of binding were verified through structural and functional experiments. SILAC-ABPP screening verified CPT1A as the key target of baicalin. The binding strength and activity of baicalin were verified by structural and functional experiments. This binding-mediated activation promoted fatty acid β -oxidation, which ameliorated obesity and related metabolic diseases in diet-induced obese mice.

5.1.4. Labeling with beads

Although compound formulae of NPs have demonstrated definite therapeutic efficacy in clinical applications, understanding the synergistic effects of these formulae still relies mainly on traditional experience, as their active ingredients and mechanisms of action are not yet completely clear. To provide clarity, in-depth explorations of the multi-target mode of action in the complex system of NPs sought to determine the mechanism of efficacy and identify new targets [115,116]. Microspheres have been employed to extensively study targets of ingredient groups of NPs, including total saponins from *Albizia julibrissin* (TSAJ), Baoyuan decoction, Shouhui Tongbian Capsules, Optimized Yinxieling Formula (OYF), and Shenqi Jiangtang granules. Other formulae have been targeted for application in different diseases. In a 2017 study, the researchers constructed solid-phase microspheres surface-modified with TSAJ. These microspheres specifically enriched target proteins in human hepatocellular carcinoma cell lysates. High-resolution MS profiling and recognition of the target proteins revealed the potential molecular pharmacological mechanism of TSAJ and offered a new research avenue to decipher the multi-component and multi-target mechanisms of action of NPs [45]. In another study, the same researchers bound chemical components of the Baoyuan decoction to solid-phase microspheres modified with photosensitive benzophenone by a photochemical coupling reaction. The microspheres were incubated with heart tissue lysate to enrich the corresponding binding proteins, which were identified by HR-MS. Bioinformatic analysis of the function of each binding protein elucidated the molecular mechanism of the cardioprotective activity of Baoyuan decoction [117]. Based on the innovative preparation of magnetic microsphere extracts of Shouhui Tongbian Capsules, the researchers successfully captured and analyzed the top 10 target proteins that were more potent than inherent capsule proteins and demonstrated their clinical efficacy in promoting intestinal motility and lessening inflammation, enhancing the intestinal cell barrier, optimizing water secretion, and regulating intestinal microbial ecology [105]. Shouhui Tongbian Capsules can promote intestinal peristalsis, lessen inflammation, enhance the intestinal cellular barrier, optimize water secretion, and regulate intestinal microbial ecology, and ultimately achieve the clinical efficacy of laxatives [105].

Following the precise localization of the action targets of NPs components in tissue and cell lysates, a reverse pharmacology strategy was developed to efficiently screen a series of biologically active compounds that function as targets, using the targets as a starting point [76]. The researchers developed OYF microspheres and used them to deduce the target of action from LPS-induced macrophages by target fishing. Seventy-six potential target proteins were identified using LC-MS/MS, and bioinformatics analysis revealed that fused in sarcoma proteins are essential for the anti-psoriasis activity of OYF. Using the fused in sarcoma protein affinity capture approach in combination with LC-MS and SPR analyses, they screened and identified five active ingredients from OYF that bound to the proteins and inhibited the expression of IL-23. The combination of these five components significantly decreased the area and severity index scores of psoriasis and reduced the expressions of IL-23 and Ki67 in an imiquimod-induced psoriasis mouse model [76]. Other researchers described the use of

microspheres of NPs combined with BLI technology for the precise capture of drug targets from complex biological systems [118]. The nonselective immobilization and acquisition of small drug molecules and proteins were achieved using multilayer-structured microspheres. Twenty-six small molecules were verified to interact with GNAS using BLI reverse capture. Of these, seven displayed significant binding activity in *in vitro* experiments, which confirmed that these compounds could effectively activate the cyclic adenosine-3', 5' -monophosphate (cAMP) pathway, demonstrating the potential application of this strategy for drug-target discovery [118].

This series of innovative research revealed the molecular mechanism of NPs prescription for treating diseases and has provided a solid scientific basis and potential new small-molecule drug candidates for the therapy of various diseases.

5.2. Unlabeled probes

Although labeled small-molecule probes of NPs provide advantages, their limitations include a time-consuming labeling process and the possibility of altering the structure of small molecules. The latter drawback could trigger nonspecific binding and produce false-positive results, affecting the accuracy of the study. Consequently, unlabeled small-molecule probes for NPs have been developed. The probes enable the screening of protein targets based on changes in the physiological properties of proteins (e.g., thermal stability and oxidation rate) before and after binding to the proteome. Typical target discovery approaches for small-molecule probes of unlabeled NPs, as reviewed below, include DARTS, SPROX, CETSA, TPP, solvent-induced protein precipitation [119], and others.

5.2.1. DARTS

The DARTS technology enables the discovery and characterization of the interactions of small-molecule drugs with their biological targets. The technology is based on the premise that certain small-molecule drugs adhere to proteins and protect them from protease-mediated degradation. The procedure is simple. The drug is mixed with the target protein candidate or cell lysate and incubated, followed by protease digestion. Digestion is terminated by an inhibitor. The resulting protein bands are detected by electrophoresis and staining. The advantages of DARTS are notable; no drug modification is required, the sample source is flexible, and it can be synergized with proteomic analysis, especially for studying membrane proteins that are difficult to purify. Despite being a qualitative technique, DARTS can be combined with quantitative techniques, such as microscale thermophoresis, allowing the comprehensive analysis of drug-target interactions. This compensates for the protease degradation of insensitive proteins [120,121].

The target of the anti-angiogenic alkaloid voacangine was successfully identified as vascular endothelial growth factor receptor 2 (VEGFR2) using DARTS and matrix-assisted laser desorption ionization-MS imaging techniques. DARTS assay data implied that the stability of VEGFR2 was considerably enhanced after the binding of voacangine to VEGFR2, suggesting that voacangine could directly bind to VEGFR2. *In vivo* experiments have demonstrated that voacangine effectively suppresses the growth and angiogenesis of glioblastoma with low toxicity, suggesting that voacangine acts as a VEGFR2 inhibitor in the treatment of angiogenesis-related illnesses [77]. In another study, daurisolone significantly suppressed the proliferation of pulmonary cancer cells and tumor development [46]. The direct binding of daurisolone to Hsp90 disrupts the interaction of Hsp90 with β -catenin, thus promoting the ubiquitination-mediated degradation of β -catenin, as demonstrated by DARTS, ITC, and other hormones. These results implicate

daurisolone as a promising new inhibitor of Hsp90 and provide new research avenues for the treatment of lung cancer [46]. Endosidin2 small molecules were screened for the target *Arabidopsis thaliana* EXO70A1 protein using DARTS technology [120]. The significant binding of ES2 to EXO70A1 was verified, suggesting that endosidin2 may affect plant cell membrane transport by specifically binding to EXO70A1. The findings provide new molecular targets and lead compounds for plant growth regulation and disease treatment [120]. Another study [78] explored the action mechanism of resveratrol, an active ingredient with life-extending effects. The findings demonstrated the binding of resveratrol to eukaryotic translation initiation factor 4A (eIF4A) and ribosomal proteins in yeast and the subsequent protection from degradation using DARTS and MS analyses. By inhibiting eIF4A-dependent translation initiation, resveratrol extended nematode longevity. This effect disappeared in eIF4A-knockdown nematodes, confirming eIF4A as the key target for longevity [78]. To explore the molecular mechanism of the anti-tumor effect of oridonin, other researchers used DARTS and chemical proteomics to screen its target protein and validated the findings by SPR and CETSA techniques [33]. Oridonin combined with Hsp70 and nucleolin, with a higher binding affinity evident for nucleolin ($K_D = 3.8 \pm 1.2$ nM). Oridonin interferes with multiple cellular signaling pathways and displays anti-tumor effects by simultaneously inhibiting Hsp70 and nucleolin. The study findings implicate oridonin as a potential anti-tumor drug [33]. Other researchers evaluated the target protein of naringenin, CRMP2, using DARTS and immunoprecipitation in conjunction with LC-MS [32]. After naringenin treatment, DARTS analysis showed that the CRMP2 protein bands were thinned, and the immunoprecipitation-conjugated LC-MS analysis confirmed that naringenin bound to CRMP2. In addition, naringenin reversed amyloid β -induced axonal atrophy and ameliorated memory deficits and pathological changes in five familial Alzheimer's Disease (5XFAD) mice. The findings indicate that naringenin targets CRMP2, inhibits its phosphorylation, and promotes axonal growth, and thus could have therapeutic effects in Alzheimer's Disease [32]. Another study [79] characterized grape seed extract (GSE) target proteins, including glucose regulatory protein 78 (GRP78), protein disulfide isomerase A3 (PDIA3), and heat shock cognate 71 kDa protein (Hsp7c) in human colorectal cancer cells using DARTS in combination with LC/MS and MASCOT databases. GSE inhibited cancer cell growth by inducing endoplasmic reticulum stress, inhibiting the PI3K-Akt-mammalian target of rapamycin (mTOR) pathway, downregulating the expression of translated proteins, and promoting oxidative modification, particularly affecting methionine residues. The findings demonstrate that the multi-targeted inhibition of colorectal cancer has the potential to be developed as a preventive and therapeutic drug [79]. Finally, a study [80] analyzed the neo-lathyrane diterpene laurifolioside extracted from *Euphorbia laurifolia* using DARTS and proteomics. The target was identified as clathrin heavy chain 1. Confocal microscopy and molecular modeling confirmed that laurifolioside affects clathrin heavy chain 1-mediated endocytosis by binding to its β -helical domain, suggesting the therapeutic potential of laurifolioside in inhibiting cancer cells or viruses [80].

5.2.2. SPROX

The stability of proteins has been analyzed by SPROX over a wide range of temperatures. The data provide information about the folding state of proteins. This information is valuable for examining the interactions between proteins and small molecules, which can reveal drug targets [121]. SPROX is frequently used to screen target proteins with the aim of revealing targets of small-molecule probes for NPs. The complexity of SPROX and the potential need in some cases for more extensive technology and resources can be challenges. For some membrane proteins or proteins under extreme

conditions, it might be difficult to obtain accurate results using SPROX [121].

In one study [53], Hsp90 was successfully screened as a target of amantadine in MCF-7 cell lysates using SPROX. The K_D value of amantadine binding to Hsp90 was $0.03 \mu\text{M}$, measured by the SPROX technology, demonstrating the strong binding affinity of amantadine to Hsp90, providing strong evidence for its application in breast cancer treatment [53]. A protein-ligand interaction research platform was developed and applied to screen the target proteins of β -nicotinamide adenine dinucleotide (NAD^+) and resveratrol in yeast lysates using SPROX technology and quantitative proteomics [47]. NAD^+ recognized 11 target proteins, including seven dehydrogenases and two Hsp70 family members. Resveratrol recognized seven target proteins, of which cytoplasmic aldehyde dehydrogenase was a known target and four were related to translation. The K_D of cytoplasmic aldehyde dehydrogenase and resveratrol was $10 \mu\text{M}$, and that of alcohol dehydrogenase and NAD^+ was $6 \mu\text{M}$. The findings indicate the biological functions of NAD^+ and resveratrol, providing a theoretical basis for the development of therapeutic drugs, especially for target proteins involved in metabolism and related to disease [47]. Finally, using iTRAQ-SPROX and SILAC-PP technologies, 28 potential target proteins of manassantin A (flavonoid) were screened in MDA-MB-231 breast cancer cells, including filamin A and elongation factor 1α [81]. The researchers verified that filamin A binds to manassantin A with a K_D value of approximately $8.8 \mu\text{M}$. The findings suggest that manassantin A may exhibit anti-tumor effects by affecting the folding stability of target proteins, providing a molecular basis for anti-breast cancer therapy [81].

5.2.3. CETSA

CETSA provides information on protein stability and drug-binding capacity within cells. The technique may be suitable for studies under physiological conditions. CETSA is relatively simple, easy to implement, and does not require complex sample handling. Hence, this method can contribute to target discovery of small-molecule probes for NPs. However, there may be limits to the application of CETSA, as it may be less accurate in detecting proteins with low or high melting points, and the analysis of the stability of membrane proteins may be challenging [122,123].

Two-dimensional gel electrophoresis-based proteome-wide CETSA (2DE-CETSA) is a proteomics technology that combines CETSA and 2D gel electrophoresis to analyze changes in the thermal stability of proteins due to compound-protein binding. The 2DE-CETSA technique has been used to filter NPD10084 from an NPs library; subsequent experiments by the authors confirm pyruvate kinase M2 as its target [48]. Techniques that included CETSA and SILAC established collagen I as the direct target of protocatechualdehyde in H9c2 cardiomyocytes ($K_D = 41.82 \mu\text{M}$). In another study [82], protocatechualdehyde attenuated isoprenaline-induced cardiac fibrosis in a mouse model of heart failure by covalently modifying lysine residues in collagen I and inhibiting its conformational dynamics and cross-linking. These activities reduced myocardial fibrosis induced by isoprenaline in a mouse model of heart failure. Thus, protocatechualdehyde can regulate collagen dynamics and may have a therapeutic role in the treatment of myocardial fibrosis, providing a new approach for the treatment of heart failure [82]. The role of the direct binding of bruceine D in the inhibition of β -catenin and T-cell factor in HepG2 and Huh7 cell models was confirmed by CETSA and microscale thermophoresis (MST) analyses [52]. Bruceine D bound with a K_D value of $33 \mu\text{M}$. The findings indicate that by blocking the interaction between T-cell factor and β -catenin, bruceine D promotes the degradation of β -catenin and then inhibits the expression of hypoxia-inducible factor-1 α , which ultimately inhibits the

proliferation of hepatocellular carcinoma cells and the growth of tumors. Therefore, bruceine may be harnessed as a therapeutic drug for hepatocellular carcinoma. Its mechanism of action may involve the regulation of the β -catenin/hypoxia-inducible factor-1 α [52]. Finally, usenamine A, a novel NPs, was demonstrated to effectively suppress the growth of human hepatocellular carcinoma cells by targeting myosin-9/actin cytoskeletal remodeling [83]. The findings provided the first evidence of the potential value of myosin-9 as a potential target for the treatment of hepatocellular carcinoma. Immunoblotting, SPR, CETSA, and other techniques demonstrated the significant anti-tumor effects of usenamine A in HepG2 and SK-HEP-1 cell lines and its direct action on myosin-9 [83].

5.2.4. TPP

TPP enables highly sensitive and high-throughput identification of potential drug targets and effector proteins in the cellular environment without assuming a target and is an essential tool for drug discovery [124]. This technique can be applied to *in vitro*, *in situ*, and *in vivo* studies and covers a wide range of proteins, enabling the analysis of their stability under different conditions. TPP has been successfully used for drug-target identification and protein interaction studies, providing new perspectives for understanding proteins in their native states and biological processes [29,122]. Thus, this approach plays an invaluable role in the target discovery of small-molecule probes for NPs.

Iso citrate dehydrogenase 1 (IDH1) was identified as a target of lycorine using unbiased TPP in HCT116 colon cancer cells [54]. Multiple techniques (CETSA, SPR, MST, and DARTS) verified the binding of lycorine to IDH1 with K_D values of 25.2 and 15.83 ± 2.54 μ M, and molecular docking confirmed that lycorine interacted with the C-terminal structural domain of IDH1. The results show that lycorine has anti-colon cancer effects by directly targeting IDH1 and triggering reactive oxygen species-mediated imbalances in mitochondrial dynamics [54]. Another study described the discovery of gambogic acid using the TPP strategy and the demonstration using ascorbate peroxidase 2-based proximity labeling that gambogic acid induced thermotropin-like cell death in prostate cancer cells by directly targeting the canopy fibroblast growth factor signaling regulator, implicating this signaling regulator as a new therapeutic target for prostate cancer treatment [84].

Other studies have applied TPP technology to analyze drug-target interactions. Thermoproteomic analysis data revealed glutathione peroxidase 4 (GPX4) to be a key target of conophylline-induced autophagy, which results from the inhibited activity of GPX4, leading to the accumulation of lipid peroxides, and possibly acting through mechanisms other than the mTOR signaling pathway [49]. In another study, nucleolin 14 was specifically targeted in Jurkat human acute lymphoblastic leukemia cells by affinity-based protein profiling using the myxobacterial product, vioprolide A, and validated by TPP [125]. Vioprolide A attenuates the direct binding of nucleolin 14 to EMG1 in a dose-dependent manner. The significant anti-tumor effect of vioprolide indicates its potential value as an anti-cancer agent, especially in human acute lymphoblastic leukemia models, providing scientific support for the development of novel therapies [125].

5.2.5. Solvent-induced protein precipitation (SIP)

SIP enables high-resolution proteomic data for the analysis of complex samples. The method excels in quantification, allowing the precise measurement of the abundance of different proteins. Consequently, small-molecule probes for NPs target discovery rely on this method. Nevertheless, the experimental cost of SIP is relatively high, requiring the use of expensive isotope-labeled reagents

and equipment. In addition, the implementation of SIP requires skilled and experienced laboratory personnel, limiting its application in some laboratories [121,122].

Sun et al. [85] repeatedly applied SIP combined with TPP to successfully screen the target proteins of the active ingredients in NPs. Using this approach, kurarinone was identified as a potential target for soluble epoxide hydrolase (sEH). The binding of kurarinone to sEH was further verified using enzyme kinetic assays, SPR, and co-crystallization techniques. The K_D was determined to be 1.45 μ M. *In vivo* experiments demonstrated that kurarinone stabilized MPTP-induced levels of epoxyeicosatrienoic acids in a mouse model of PD by inhibiting sEH activity, thereby reducing neuroinflammation and ameliorating disease symptoms. Kurarinone stabilized MPTP-induced levels of epoxyeicosatrienoic acids by inhibiting sEH activity, thereby reducing neuroinflammation and ameliorating symptoms of PD [85]. In addition, the target protein of shikonin, NF- κ B essential modulator (NEMO), which inhibits the NF- κ B signaling pathway, underwent successful screening using this technology and demonstrated a significant inhibitory effect on colorectal cancer. *In vitro* and *in vivo* experiments confirmed that shikonin was able to effectively reduce tumor size and weight and inhibit inflammatory markers, demonstrating its potential as an inhibitor of the NEMO/IKK β complex and providing a strong scientific basis for the treatment of colorectal cancer [86].

5.2.6. Other methods

In addition to the frequently adopted approaches for target discovery of small-molecule probes for unlabeled NPs, there are several other approaches, including molecular docking, quantitative proteomics, phage technology, affinity chromatography, and protein microarrays.

Phage technology is applicable to bioactive peptide screening and the identification of small-molecule probe targets in NPs. The biological functions and therapeutic potential of small molecules can be revealed through specific binding peptide screening. A biotinylated paclitaxel derivative was used in a study by Rodi et al. [87]. Human Bcl-2 protein was successfully identified as a potential target of paclitaxel using a phage-displayed peptide library screening technique. Sequence similarity analysis and enzyme-linked immunosorbent assay (ELISA) experimental validation demonstrated that paclitaxel may exert its therapeutic effect by inducing apoptosis in tumor cells by directly binding to the Bcl-2 protein, providing a theoretical basis for the development of a new generation of anti-tumor drugs targeting Bcl-2 [87]. Takakusagi et al. [88] successfully prepared the biotinylated camptothecin derivative CPT-20-B. Screening of human leukocyte complementary DNA (cDNA) libraries using T7 phage display technology identified a calcium-binding protein sequence, NSSQSARR, which can bind specifically to CPT-20-B. Forward and reverse affinity assays confirmed the high affinity of calcium-binding protein for CPT-20-B, suggesting that it may be a new therapeutic target for camptothecin drugs, which provides a new direction for the research and development of anti-tumor drugs [88]. The potential target of ginsenoside Rh2 ((20S) G-Rh2) was identified as Annexin A2 by phage display technology [89]. The results of ITC showed that its binding constant was 1.13×10^{-5} M^{-1} , indicating moderate affinity. The observations that (20S) G-Rh2 inhibited Annexin A2-NF- κ B p50 subunit interaction, blocked NF- κ B activation and anti-apoptotic gene expression, and promoted apoptosis of cancer cells reveal a new strategy for the treatment of hepatocellular carcinoma and demonstrate the natural anti-cancer potential of (20S) G-Rh2 [89].

Protein microarray is a high-throughput protein analysis tool. Microarrays are constructed by arranging a large number of protein samples on a solid-phase surface to detect protein-molecule

interactions and study protein functions and networks. A rat model of acute gouty arthritis was constructed. The expression levels of 90 proteins in synovial tissues of the rats were detected by using RayBio® human label-based antibody array I protein microarray. Proteins, such as the tumor necrosis factor-related apoptosis-inducing ligand and neuropilin-2, were upregulated in the rats, and the expression of these proteins could be downregulated by *Dioscorea dioscorea*, indicating the therapeutic potential of this NPs [90]. Another study used human proteomic microarrays and multiple validation methods to confirm that the target of silibinin was ferritin heavy chain 1 (FTH1). FTH1 regulates cellular iron metabolism, and silibinin inhibits nuclear receptor coactivator 4-FTH1-mediated ferritinophagy, reducing ferroptosis and effectively attenuating acute kidney injury caused by ischemia/reperfusion injury, demonstrating its potential as a treatment for ischemia/reperfusion injury and acute kidney injury [56].

Affinity chromatography is a separation technique based on specific intermolecular interactions that utilizes the high affinity between the ligand in the stationary phase (affinity matrix) and the target molecule (e.g., proteins, nucleic acids, and small molecules) to achieve separation. In one study, nearly 40 target proteins from ginsenosides were screened and validated using affinity chromatography, which confirmed that 20(S)-PPD binds directly to muscle-type creatine kinase (CK-MM), with binding constants of $2.53 \pm 0.12 \times 10^{-5}$ M and $1.9 \pm 0.30 \times 10^{-6}$ M. This study showed that PPD activated CK-MM, elevated phosphocreatine levels, optimized energy metabolism, and reduced lactate accumulation during exercise. In a weight-bearing swimming experiment, PPD significantly enhanced the exercise endurance of mice, confirming its effect of relieving fatigue. The finding opens a new avenue for the development of CK-MM activators based on dammarane-type triterpene structures [91]. Another study identified peroxiredoxin-2 (Prdx2) as the target protein of Cel using biotinylated Cel affinity capture combined with MS in BGC-823 and SGC-7901 gastric cancer cells [92]. The high affinity of binding (K_D , approximately 6.9 nM) indicates that Cel directly targets Prdx2 and induces apoptosis mediated by reactive oxygen species. These findings indicate the therapeutic potential of Prdx2 for gastric cancer and provide a new target for the development of drugs to treat gastric cancer [92].

Inflammation-associated proteins of the MAPK, NF- κ B, and FoxO1/3 signaling pathways were identified in LPS-stimulated RAW264.7 cells using label-free quantitative proteomics [93]. The authors reported that carnosis acid significantly inhibited the phosphorylation of extracellular signal-regulated kinase 1/2, c-jun N-terminal kinase, p38 MAPKs, and NF- κ B pathway proteins and prevented NF- κ B activation. Carnosis acid also inhibited the expression of FoxO1/3 and promoted their degradation. The findings suggest that carnosis acid has therapeutic potential as an effective anti-inflammatory agent by inhibiting the aforementioned signaling pathways [93]. MT quantitative proteomics was used to analyze differentially expressed proteins in the serum of an adenovirus-TSH289-induced Graves' disease [126] mouse model [94]. FAS-associated death domain protein was identified as a key target of Xiehuo Xiaoying decoction in the treatment of Graves' disease. Immunohistochemistry and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining further confirmed that Xiehuo Xiaoying decoction promoted the apoptosis of thyroid cells in the mouse model of Graves' disease by upregulating the expression of FAS-associated death domain protein, caspase 8, and caspase 3, with a therapeutic effect on Graves' disease [94].

Oridonin, an ent-kaurene diterpenoid with anti-inflammatory activity, specifically inhibits the assembly and activation of the NLR, and NLRP3 inflammasome by covalently binding to Cys279 in the NACHT structural domain of the NLRP3 protein, with anti-

inflammatory effects through targeted mutagenesis. The findings implicate oridonin as a potential drug for treating NLRP3-related inflammatory and metabolic diseases [95].

With the increasing interest in complex biological systems and multi-component drugs, researchers need a method to identify the specific targets of action in these multimolecular systems. Although traditional target identification techniques are mainly used for single compounds, it is difficult to deal with complex mixtures of molecules, such as herbal extracts. The Zhao-Yao strategy has emerged to overcome the limitations of the existing techniques. This strategy is an innovative multimolecular systematic target identification method that immobilizes molecules on nanoparticles via photochemical cross-linking to capture target proteins in cell lysates. Using SPR technology, six anti-inflammatory target proteins (UBE2I, peroxiredoxin-6 (Prdx6), and RHOA) were rapidly screened from *Dendranthema indicum* extracts. Thirty-three major compounds had strong affinities for these target proteins in an LPS-induced BV2 microglial cell model, suggesting that the *D. indica* extract and its compounds have considerable therapeutic potential for inflammatory diseases [96]. The authors then constructed a photocrosslinking platform utilizing gold nanoparticles and 4,4'-dihydroxybenzophenone to accurately identify cellular targets in ultra-complex molecular systems, crosslinked phytochemicals to gold nanoparticles using an ultraviolet photocatalytic reaction, identified neuroprotective targets from brain tissues, and screened and verified the neuroprotective effects of target-binding compounds using Hsp60 as a target [24]. These findings provide an efficient strategy for the exploration of the pharmacological mechanism and active ingredients [24].

Other screening methods include molecular docking and SPR screening of TLR4, the target protein of the flavonoid baicalein in colorectal cancer cell lines, providing a new strategy for the use of novel TLR4-targeted therapeutic agents for the treatment of colorectal cancer [97]. SPR, cellular assays, and molecular docking techniques were utilized to screen for the binding of rutaecarpine to Keap1. Rutaecarpine significantly altered the symptoms in a model of colitis induced by dextran sulfate sodium, suggesting its therapeutic potential for the treatment of inflammatory bowel disease [98]. Functional and structural screening techniques demonstrated that osthole significantly relieved airway constriction induced by acetylcholine and enhanced the diastolic effect of salbutamol, suggesting that it may be a new drug for the treatment of asthma [127]. Virtual screening and a main protein inhibitory activity assay based on fluorescence resonance energy transfer revealed that oridonin effectively inhibited the main protein activity of severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) as well as SARS-CoV-2 polymerase and papain-like protease activities at high concentrations [128]. The findings demonstrate the broad-spectrum antiviral activity of oridonin and provide a theoretical and experimental basis for its use as a novel anti-SARS-CoV-2 drug [128]. Wang et al. [129] prepared magnetic nanoparticles enriched with surface benzophenone, which could effectively couple different drug molecules. The authors also demonstrated the potential application of this method in the preparation of NPs ingredient microarrays and targeted "hook fishing" [129].

6. Conclusion and future perspectives

Research on bioactive molecules is crucial for drug discovery and development. Target identification is especially critical. Other reviews explored photoaffinity-labeled small-molecule probes and chemical proteomic approaches for screening target proteins [106,114], labeling and label-free target recognition techniques for NPs [130], and advances in small-molecule probe structure design

and imaging [40]. The present review fills the gap of using NPs as small-molecule probes to discover targets by exploring the application of small-molecule probes based on NPs in target discovery.

Small-molecule probes based on NPs have been used in a variety of diseases, such as liver cancer, colorectal cancer, and malaria, in addition to stroke-related drug-target screening. Terpenoids account for the largest proportion of these probes, followed by alkaloids and flavonoids. Terpenoids play an essential role in stroke therapy. Furthermore, labeled small-molecule probes are more widely employed than nonlabeled probes, probably because of their improved target enrichment and capture. Nevertheless, the unlabeled type has unparalleled advantages and is expected to be widely used in stroke-related target research in the future.

During the development of novel drugs based on NPs, target identification provides significant benefits for clarifying the mechanism of action, toxicity, and adverse effects of the drug, accelerating the development of a molecule from discovery to marketing. With continuous progress in the implementation of small-molecule probes for target revelation, small-molecule probes originating from NPs have gradually become a popular tool in disease target discovery by virtue of their clear therapeutic efficacy and natural origin. Small-molecule probes of NPs provide a new approach for the development of new drugs based on NPs that are more targeted, more efficacious, and safer, as they allow the direct identification of the targets of the therapeutic effects of components of NPs and better correlation with disease targets.

Small-molecule probes of NPs have significant advantages in studying stroke mechanisms (mainly target discovery), especially in the context of the existing multiple difficulties. First, the mechanisms of stroke are complex and have not been fully elucidated. While traditional drug development methods often rely on single-target studies, small-molecule probes of NPs permit more comprehensive explorations based on multiple targets and multiple pathways, thus better revealing the multiple pathological mechanisms of stroke. This multi-target property makes small-molecule probes of NPs particularly important in dealing with complex diseases because stroke involves vascular problems and is also intimately associated with multiple biological processes, such as neuroprotection and inflammatory responses [131]. Secondly, the rapid screening ability of small-molecule probes for NPs has obvious advantages over traditional methods [132]. Traditional methods typically require a step-by-step screening of targets using cellular experiments and animal models. This process is time-consuming, laborious, and suffers from inaccurate models, poor reproducibility, and other problems. However, small-molecule probes of NPs combined with modern technologies can efficiently identify stroke-related targets and analyze their mechanisms of action within a brief period, which greatly enhances the efficiency and effectiveness of the study. In addition, the use of small-molecule probes of NPs allows for better penetration of the blood-brain barrier, a physiological structure that is a major challenge in stroke treatment. Small-molecule probes of NPs can enter the central nervous system more easily because of their smaller molecular weight, thus demonstrating superior performance in drug action and target binding [133].

Despite these advantages, some issues that need to be emphasized. The first issue is the screening of active ingredients. Many NPs have therapeutic effects on stroke, and rapid screening of highly active small molecules from NPs as probes for drug-target screening is crucial. The second issue is the selection of suitable targets. NPs can interact with multiple targets, and the best possible drug targets must be screened appropriately. The third issue is maintenance of biological activity. The labeling process of small-molecule probes may affect the biological activity of these

molecules, and it is important to find a suitable probe labeling method to maintain biological activity.

Several goals need to be met to overcome these challenges. The first is to establish an efficient screening system, such as screening highly active small molecules by constructing stroke-related cell models and testing cell survival rates, collecting small molecules known to treat stroke, and using computer simulation techniques (e.g., molecular docking) to dock small molecules with target proteins to predict the binding energy. The second is research on the relationship between the structure and activity of small molecules, and the type of potential target based on structural features, and determination of the best target by functional validation of the screened potential target in cells or animal models using knockout or overexpression techniques. The third is to assay small-molecule probes based on NPs for bioactivity immediately after labeling and adjustments to remedy reduced activity. Switching to a mild labeling technique (unlabeled type) maintains small-molecule bioactivity. In conclusion, the application of small-molecule probes based on NPs for drug target discovery in stroke treatment provides a beneficial perspective for the development of new drugs.

CRedit authorship contribution statement

Xingyue Jin: Writing – review & editing, Writing – original draft, Investigation, Data curation. **Suyi Liu:** Writing – original draft, Supervision, Investigation. **Shujing Chen:** Writing – review & editing, Supervision, Investigation. **Rui Han:** Writing – review & editing, Supervision, Investigation. **Xingyi Sun:** Writing – review & editing, Supervision. **Mingyan Wei:** Writing – review & editing, Supervision. **Yanxu Chang:** Writing – review & editing, Supervision, Conceptualization. **Lin Li:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Han Zhang:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

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