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Targeted and proteome-wide analysis of metabolite–protein interactions

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Abstract

Understanding the molecular mechanisms of endogenous and environmental metabolites is crucial for basic biology and drug discovery. With the genome, proteome and metabolome of many organisms readily available, researchers now have the opportunity to dissect how key metabolites regulate complex cellular pathways *in vivo*. Nonetheless, characterizing the specific and functional protein targets of key metabolites associated with specific cellular phenotypes remains a major challenge. Innovations in chemical biology are now poised to address this fundamental limitation in physiology and disease. In this review, we highlight recent advances in chemoproteomics for targeted and proteome-wide analysis of metabolite–protein interactions that have enabled the discovery of unpredicted metabolite–protein interactions and facilitated the development of new small molecule therapeutics.

Introduction

Understanding the mechanisms of action of small molecules/metabolites from cellular metabolism and the environment (diet, microbiota, therapeutics) is important for dissecting fundamental pathways in biology and crucial for drug development. These small molecules/metabolites can interact with proteins covalently and non-covalently and are still challenging to biochemically characterize *in vivo*. Over the last two decades, the development of bioorthogonal reactions [1,2] and metabolite chemical reporters [3,4] has emerged as transformative technologies to discover and characterize specific metabolite–protein interactions (Figure 1). Notably, the functionalization of metabolites with a bioorthogonal detection tag (alkyne, azide and others) and a covalent crosslinker (electrophile or photoaffinity group) has afforded chemical reporters for profiling diverse metabolite–protein interactions (Figure 1a), ranging from covalent metabolite–protein modifications [3] to target identification of endogenous metabolites, natural products and synthetic drug candidates [5]. Whilst this approach enables the direct biochemical analysis of metabolite–protein interactions *in vitro* and *in vivo*, the installation of bioorthogonal detection tags and

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crosslinkers onto small molecules of interest may not be readily achieved and potentially interfere with activity. Moreover, direct metabolite modifications or binding to less abundant but key functional protein targets may be undetectable in large-scale unbiased proteomic studies. As a complementary approach, reactivity-based chemical reporters can profile proteins with unique activity and metabolite interactions [4]. Indeed, labelling of uniquely reactive amino acid residues combined with competition studies against native metabolites/synthetic compounds has uncovered the “ligandable” proteome and revealed unpredicted metabolite–protein interactions (Figure 1b) [6,7]. This approach derived from competitive activity-based protein profiling (cABPP), where mechanism-based reporters are used in metabolite competition studies to discover potential substrates and inhibitors of specific enzyme families [8]. Beyond amino acid reactivity-based and enzyme mechanism-based chemical reporters, functionalization of other bioactive metabolites and synthetic small molecules can afford more selective chemical reporters for specific protein families to characterize native ligands and drug candidates (Figure 1c). In this review, we highlight the recent development and application of these chemical strategies for targeted and proteome-wide analysis of small molecule/metabolite–protein interactions as well as their applications for drug discovery.

Chemical reporters for the direct analysis of metabolite–protein interactions

The development of chemical reporters that mimic diverse cellular metabolites have afforded new reagents to characterize many metabolite–protein interactions [3]. Here we focus on recent development and application of these approaches to lipid–protein interactions that have revealed new biological mechanisms and facilitated the characterization of small molecule drug candidates. Lipids perform a wide range of cellular functions from providing essential structural components of membranes to post-translationally modifying proteins and regulating cell signalling [9–11]. Given their hydrophobic properties and metabolism, lipid interactions with specific proteins are challenging to biochemically characterize. In this regard, chemical reporters of several lipid classes have been developed to facilitate their analysis of protein modifications and binding as well as trafficking in cells [12–14]. For example, the Lin group recently employed an alkyne-tagged fatty acid reporter (Figure 2a) to profile substrates of HDAC11, which was found to be an efficient defatty-acylase for SHMT2 and regulate type I interferon signalling [15]*. In addition, we have recently used alkyne-tagged fatty acid reporter labelling and proteomics of infected-mammalian cells to identify the fatty-acylated protein targets of secreted enzymes from intracellular bacterial pathogens in collaboration with the Alto group [16] and the Shao group [17]. Alternatively, the Tate group has used the alkyne-myristic acid reporter to profile N-myristoylated proteins in cells and characterized the specificity of different N-myristoyltransferase inhibitors [18]*. These studies nicely demonstrated how fatty acid chemical reporters can identify unpredicted metabolite–protein modifications regulated by specific enzymes and help determine the on- and off-target activity of inhibitors in cells [18]*. Similar approaches could also be employed for other classes of lipid–protein modifications and their regulatory enzymes [19,20], which highlights the general utility of these reagents and methods.

To evaluate non-covalent metabolite binding to proteins, chemical reporters can be further functionalized with photocrosslinkers such as diazirine to capture potential low-affinity and

dynamic lipid–protein interactions [21,22]. After administration of photoaffinity chemical reporters to lysate or live cells, UV irradiation induces covalent crosslinking of functionalized metabolites with target proteins [21,22]. For example, Holthuis and co-workers developed a palmitic acid photoaffinity reporter (Figure 2b) that enabled the photocrosslinking and proteomic analysis of known and new candidate fatty acid-binding proteins in cells [23]. In addition, our laboratory has shown that metabolic incorporation of these fatty acid photoaffinity reporters onto proteins allows selective photocrosslinking of fatty-acylated protein–protein interactions in cells [24]*, which has been further employed to characterize other fatty-acylated protein complexes [25,26]. To facilitate temporal control of fatty acid–protein crosslinking and minimize metabolism, caged photoaffinity reporters (Figure 2c) have been developed that allow a “release and catch” approach for time-resolved fatty acid–protein photocrosslinking in cells [27]**. These studies highlight the utility of metabolic labelling and caging strategies for capturing lipid–protein interactions in cells with photoaffinity reporters, which can be further applied to phospholipids [28,29] and sphingolipids [28,30].

Unsaturated fatty acids exhibit diverse biological activities but have been challenging to identify specific protein binding partners and dissect mechanisms of action [31]. To characterize the unsaturated fatty acid neurotransmitter anandamide protein targets in cells, the Cravatt group developed photoaffinity reporters and performed large-scale analysis of their candidate protein targets (Figure 2d) [32]**. Notably, the two anandamide photoaffinity reporters engaged with many similar protein targets but also interacted with a number of reporter-specific targets, indicating subtle changes in photoaffinity reporter structure can yield distinct lipid–protein interactions in cells. Besides the recovery of known lipid transport proteins (SCARB1, FABP5, SLC25A20) and metabolic enzymes (FASN, PNPLA2, ACADs and HADHA), unannotated lipid-interacting proteins (NUCB1 and NENF) were identified as anandamide targets. Interestingly, arachidonic acid competed anandamide reporter crosslinking, suggesting these proteins may be general targets for unsaturated fatty acid binding and activity [32]**. Given that the protein targets and mechanisms of action for many active unsaturated fatty acids are still unknown [31], further application of these approaches may illuminate their roles in cancer cell metabolism, inflammation and neuronal activity.

Beyond the lipids described above, photoaffinity reporters have also been developed for sterols that build upon classical radiolabelled derivatives. For example, initial cholesterol photoaffinity reporters from the Cravatt group enabled proteomic analysis of many known sterol-binding proteins and revealed new candidate receptors/regulators (Figure 2e) [33]. Other photoaffinity sterol reporters have also been developed to more closely resemble the structural features and activities of cholesterol (Figure 2f) [34,35]. In addition, photoaffinity reporters of bile acids have also been developed to identify and characterize their unique receptors, transporters and regulatory enzymes [36]. Based on the general structure of cholic acid, the Lei group synthesized three bile acid reporters, each of which possesses a diazirine group and an alkyne group (Figure 2g) [18]. They chose C-3 and/or C-24 positions for modification to minimize perturbations of biological activity and used amide linkage to improve metabolic stability [18]. The proteomic analysis of these three different bile acid photoaffinity reporters revealed many shared protein targets but also recovered unique

proteins including the well-characterized bile acid receptor FXR [18], suggesting the modification of both C-3 and C-24 positions afforded the optimal bile acid mimic. Application of this bile acid photoaffinity reporter with methyl cholic acid competition revealed many known bile acid-interacting targets (NTCP, SLC25A6, PLD3) and novel binding proteins (CPT1A, ADPGK, COMT). In addition to bile acid photoaffinity reporters, an activity-based reporter has been developed to profile bile acid processing enzymes from gut microbiota (Figure 2h) [37]. Further development of sterol photoaffinity and activity-based reporters should facilitate the analysis of their protein targets and regulatory enzymes in eukaryotes and microbes, which is an exciting area of research given the emerging roles of bile acid interactions with microbiota, host metabolism and immunity [38].

We have also applied this approach to other microbiota-associated metabolites and recently demonstrated that peptidoglycan fragments such as γ -d-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl-dipeptide (MDP) directly bind their respective innate immune receptors NOD1 and NOD2 in cells [39]*. Interestingly, we discovered that MDP also binds to GTP-Arf6 small GTPase in NOD2-expressing cells and forms a ternary complex with NOD2, which may be crucial for sensing MDP and downstream signalling [39]*. These studies highlight the utility of generating specific metabolite chemical reporters for the direct and unbiased identification of their protein targets in cells. This approach is not limited to metabolites described above and has even been applied to more complex natural products can be readily derivatized, which is beyond the scope of this minireview and recently reviewed elsewhere [40,41].

Competitive chemoproteomic profiling of metabolite–protein interactions

As highlighted above, the modification of metabolites with bioorthogonal detection tags and photocrosslinkers can reveal direct small molecule–protein targets. Many bioactive metabolites, especially natural products, however, are still difficult to derivatize due to their structural complexity or chemical stability [40,41]. Moreover, derivatization of metabolites may perturb their biological activity. The development of broadly reactive chemical reporters and quantitative chemoproteomic methods by the Cravatt lab has provided a powerful approach to explore unpredicted metabolite–protein interactions (Figure 1b) [42,43]**. This chemoproteomic platform, also known as ‘competitive’ isotopic tandem orthogonal proteolysis-activity-based protein profiling (isoTOP-ABPP), uses activated chemical reporters to label uniquely reactive amino acids such as cysteine and lysine, which can reveal “ligandable” hotspots in proteins with metabolite competition [6,7]. These activated chemical reporters are analogues of classical protein labelling reagents such as iodoacetamide (IA), N-hydroxysuccinimide (NHS) or sulfotetrafluorophenyl (STP) esters and can be employed for proteome-wide analysis of metabolite-reactive protein hotspots (Figure 3) [6,7]. For example, chemoproteomic profiling of cysteines with iodoacetamide-alkyne (IA-alkyne) (Figure 3a) was used to evaluate reactive lipid metabolites such as 4-hydroxy-2-nonenal (HNE), 15-deoxy-^{12,14}-prostaglandin J₂ (15d-PGJ₂) and 2-*trans*-hexadecenal (2-HD) [44]*. These highly reactive lipid metabolites contain α,β -unsaturated carbonyls that can compete with IA-alkyne labelling, which revealed selective protein targets and sites of modification for HNE (C22 of ZAK, C369 of PHGDH, C1001 of RTN4 and C41 of EEF2) and 15d-PGJ₂ (C55 of CHRA1, C244 of CCT8 and C848 of MMS19) [44]*.

Other recent applications of this approach include target identification of reactive oncometabolites such as fumarate [45] and its derivatives [46-48] as well as microbiota-derived metabolites, pyrazinones and dihydropyrazinones [49]. In addition, the Nomura group has recently employed this IA-based reporter to characterize the protein targets of two bioactive small molecules, a synthetic compound EN6 [50] and the natural product nimbolide (Figure 3a) [51]*. From these studies, they discovered that EN6 covalently targets C277 of Vacuolar H⁺-ATPase (ATP6V1A), leading to inhibition of mTORC1 signalling and activation of autophagy [50]. Alternatively, nimbolide impairs breast cancer cell proliferation through targeting C8 of E3 ubiquitin ligase RNF114, resulting in inhibition of ubiquitination and degradation of tumour suppressors [51]**. Furthermore, modification of nimbolide enabled the repurposing of this specific covalent metabolite–protein interaction for targeted protein degradation applications [51]**.

Besides cysteine proteome profiling, additional activated chemical reporters have been developed for other uniquely reactive amino acids. Lysine is another attractive amino acid due to its intrinsic reactivity and abundance in proteins. Indeed, the Cravatt group discovered that N-hydroxysuccinimide (NHS) and sulfotetrafluorophenyl (STP) ester reporters can function as selective lysine-proteome profiling reagents (Figure 3b) [52]. STP-alkyne reporter proteomic profiling revealed over 300 highly-reactive lysine residues in three human cancer cell lines (MDA-MB-231, Ramos and Jurkat cells) [52]. Competitive metabolite profiling with a small library of amine-reactive electrophilic fragments identified several small molecule-sensitive lysine hotspots such as K100 of PNPO and K155 of SIN3A [52]. Interestingly, the analysis of NHS-ester-alkyne reporter (Figure 3c) by the Nomura group showed that NHS-esters primarily target lysine residues but also label other nucleophilic amino acids such as serine, threonine, tyrosine, arginine and cysteine residues [53]. Based on these observations, two compounds equipped with NHS-ester reactive moiety were synthesized. Compound **3** labelled R53 of Bpifb5, K497 and T489 of DPYD, S69 on Hba5, K55 on Atp5l, and K48 on Slc25a42, whereas compound **4** showed selective interactions with four targets, including S151 of Rpl6, K639 of Zfp318, K211 of ALDH2, and K71 of GSTT1 [53]. These studies highlight the utility of proteome-wide analysis of metabolite-reactive hotspots in proteins, which suggests further applications and development of other amino acid-selective labelling reagents [54-57] can significantly expand the scope of this approach for ligand discovery and drug development.

Selective chemical reporters for targeted analysis of metabolite–protein interactions

The profiling of uniquely reactive amino acids enables large-scale analysis of metabolite–protein hotspots; however, some protein families may be expressed at low levels and/or not labelled by simple activated chemical reporters. In this regard, the repurposing and modification of selective small molecules provide new opportunities for targeting specific protein families for chemoproteomic profiling and metabolite competition analysis. This approach was originally developed for several enzyme classes such as serine hydrolases, cysteine proteases, and metalloproteases [4,8], but considerable progress has now been made towards other protein families including new enzyme families and receptors. Moreover, target-agnostic approaches that focus on a subset of proteome dictated by affinity with chemical reporters have also emerged.

The discovery of many chemical probes from high-throughput screening and medicinal chemistry studies offers new opportunities for the development of selective photoaffinity reporters. For example, kinases are prime targets for chemoproteomic analysis given their significant roles in cell signalling relevant to basic biology and many diseases. Initial studies of different cell-permeable kinase inhibitor-based reporters demonstrated these reagents could be developed for proteome-wide competitive metabolite profiling [58-60]. Notably, recent studies from the Taunton group showed pyrimidine 3-aminopyrazole-based reporters have expanded kinome coverage [61]**. Based on co-crystal structures of pyrimidine 3-aminopyrazoles bound to various kinases, the Taunton lab appended a phenylsulfonyl fluoride warhead through short linkers such that it would be positioned near the catalytic lysine [61]. Indeed, LC-MS/MS analysis and X-ray co-crystal structures confirmed a new compound XO44 site-specifically modified the catalytic lysine of SRC and EGFR kinases (Figure 4a). In live cells, XO44 targeted 133 endogenously expressed kinases. With this cell-permeable reporter in hand, they assessed selectivity of dasatinib, an approved drug for chronic myeloid leukaemia, and noted previously unappreciated ability of dasatinib to partially discriminate among related SRC kinases [61]. This chemical reporter and other broadly active kinase photoaffinity reporters are excellent new tools to explore other metabolites and drug candidate interactions with kinases in live cells.

Selective chemical probes for other protein families have also been developed into chemical reporters. For example, the van der Stelt group has developed the chemical reporter for type 2 cannabinoid receptor (CB₂R) LEI121 (Figure 4b) [62], based on their previous structure–activity and docking studies of a selective ligand LEI101. The ring opening of the hydantoin and functionalization with diazirine and alkyne afforded CB₂R photoaffinity reporter LEI121, which enabled the photocrosslinking and target engagement studies of endogenous CB₂R. A chemical reporter has also been developed for adenosine A_{2A} receptor [63]. Alternatively, the Brennan and Kessler groups have developed a chemical reporter for bromodomains, called bromotriazine (Figure 4d) [64]*. To obtain a reporter with broad activity across the bromodomain family, they selected the broad-spectrum inhibitor, bromosporine, as the starting point. The methyl sulfonamide group was replaced with the lysine-reactive warhead, chlorotriazine, as sequence alignment and docking studies suggested the methyl sulfonamide group pointed to one of three conserved lysine residues that are potentially nucleophilic. LC-MS/MS analysis and co-crystallization confirmed covalent modification of target lysine residues in BRD3 and BRD4 as well as unexpected tyrosine residue in BRD9, which has tyrosine in place of lysine. Subsequent chemical proteomics experiments of cell lysate showed bromotriazine indeed labels several endogenous bromodomain-containing proteins [64]. A photoaffinity reporter based on the same scaffold has also been reported recently [65]. In addition, we have recently developed the chemical reporters for nuclear receptors such as peroxisome-proliferator activated receptors (PPARs) [66]*. We designed a PPAR reporter (*m*-alk-GW9662) (Figure 4d) based on the known antagonist, GW9662, which covalently modifies a cysteine residue in ligand binding site [66]. Experiments with mutant constructs confirmed site-specific modification of PPAR γ as well as the other subtypes PPAR α and PPAR β/δ . A series of competition experiments with synthetic PPAR γ ligands confirmed that *m*-alk-GW9662 quantitatively measures the occupancy of ligand binding site. We further demonstrated the utility of PPAR

reporter for target engagement studies in cell lysate and live cells by screening microbiota-associated metabolites for candidate ligands of PPAR γ . Notably, we discovered previously unappreciated difference in potency among conjugated linoleic acid isomers, which may help identify specific microbiota modulators of nuclear receptors in the future [66]. These studies highlight the potential development of new selective chemical reporters for other protein families and their application towards ligand discovery.

To explore additional metabolite–protein interactions, metabolite photoaffinity reporters can also be used for competitive metabolite and drug candidate profiling. For example, the Cravatt lab has used their anandamide photoaffinity reporters (Figure 2d) to evaluate bioactive compounds [32]. Interestingly, their competitive profiling of lipid photoaffinity reporters revealed KDT501 targeting of PTGR2 (prostaglandin reductase 2) and the adaptor protein BAIAP2 as well as miconazole targeting of CLPP, SGPL1 (sphingosine-1-phosphate lyase 1), EPHX1, and TMEM97. The application of these chemical reporters may reveal the protein targets of other bioactive small molecules, as lipid-binding domains contain hydrophobic patches in proteins for potential drug binding. In addition, the Cravatt lab has generated fragment-based (Figure 4e) [67]** photoaffinity reporters and performed competition studies with synthetic compounds in cells to identify new ligands and druggable proteins. To expand upon this approach, the Kelly lab has developed latent chemical reporters with electrophiles such as aryl fluorosulfate and conducted small molecule competition studies to identify druggable proteins (Figure 4f) [68,69], which they have termed “Inverse Drug Discovery”.

In summary, the chemical strategies summarized above highlight powerful approaches for targeted and proteome-wide strategies for characterizing metabolite–protein interactions in cells. These approaches and new classes of chemical reporters should enable the dissection of endogenous and environmental metabolite functions in biology and facilitate the development of new therapeutics towards previously undruggable protein targets.

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Figure 1. Strategies for targeted and proteome-wide analysis of metabolite–protein interactions. (a) Direct analysis. Metabolites of interest are functionalised to provide chemical reporters that maintain their physicochemical properties and bioactivity. Functionalization of metabolites with small chemical tags such as terminal alkynes (alk) facilitates the analysis of covalent or photocrosslinked metabolite–protein interactions *in vitro* and *in vivo*. Addition of photocrosslinkers such as diazirines enable photocrosslinking of non-covalent metabolite interactions. Subsequent bioorthogonal or click chemistry labelling [1,2] with azide-modified reagents facilitates the fluorescent detection, affinity enrichment and identification of metabolite–protein targets. These reporters are used to directly pull-down and identify candidate protein targets from the entire proteome [3,4]. (b) Large-scale competitive profiling. Metabolites of interest are competed against broadly reactive-chemical reporters that sample a subset of proteome [8]. (c) Targeted analysis. The workflow is similar to large-scale competitive profiling. Chemical reporters for targeted analysis, however, are structurally more complex, less reactive, or selective towards a protein family, hence the smaller size of sampled proteome.

Figure 2. Chemical structures of reporter molecules used for direct analysis of metabolite–protein interactions.

(a) Fatty-acylation reporters [15–18]. (b) Fatty acid photoaffinity reporters [23–26]. (c) Caged fatty acid photoaffinity reporter [27]. (d) Anandamide photoaffinity reporters [32]. (e) Sterol photoaffinity reporter [33]. (f) Sterol photoaffinity reporter [34–35]. (g) Bile acid photoaffinity reporter [36]. (h) Bile acid hydrolase reporter [37].

Figure 3. Chemical structures of reactivity-based reporters and their competitive metabolites from cellular metabolism, synthetic libraries and natural products.

(a) IA-alkyne labels cysteine residues [42-44] and is widely used to profile targets of covalently acting small molecules [49-51]. (b) STP-alkyne labels lysine residues [52]. (c) NHS-ester-alkyne is more broadly reactive towards nucleophilic residues. STP-alkyne and NHS-ester-alkyne were used to map hotspot residues sensitive to electrophilic small molecules such as compounds 1 – 4 [52,53].

Figure 4. Chemical structures of selective chemical reporters for targeted analysis of metabolite–protein interactions.

(a) Kinase reporter [61]. (b) Cannabinoid receptor reporter [62]. (c) Bromodomain reporter [64]. (d) Nuclear receptor (PPARs) reporter [66]. (e) Fragment reporter [67]. (f) Aryl fluorosulfate reporter [68, 69].