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Reactive-cysteine profiling for drug discovery

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The recognition that only a small percentage of known human gene products are druggable using traditional modes of non-covalent ligand design, has led to a resurgence in targeted covalent inhibitors. Covalent inhibitors offer advantages over non-covalent inhibitors in engaging otherwise challenging targets. Reactive cysteine residues on proteins are a common target for covalent inhibitors, whereby the high nucleophilicity of the cysteine thiol under physiological conditions provides an ideal anchoring site for electrophilic small molecules. A chemical-proteomic platform, termed isoTOP-ABPP, allows for profiling cysteine reactivity in complex proteomes and is one of many techniques that can aid in two aspects of the covalent-inhibitor development process: (1) to identify functional cysteines that lead to modulation of protein activity through covalent modification; and, (2) to determine cellular targets and evaluate promiscuity of electrophilic fragments, small molecules, and natural products. Herein, we discuss recent advances in isoTOP-ABPP and potential applications of this technology in the drug-discovery pipeline.

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Introduction

Of the 20 proteogenic amino acids, cysteine is unique in its elevated nucleophilicity and redox sensitivity. Despite its low abundance, cysteine is highly conserved at functionally important sites [1••,2]. The high nucleophilicity and redox sensitivity of the cysteine thiolate facilitate key roles in several aspects of protein function [3]: (1) active-site nucleophiles in catalysis, or resolving residues in cellular redox buffering systems [4]; (2) protein structure stabilization through disulfide bonds, and metal coordination; and, (3) regulation of protein function through post translational modifications (PTMs), such as oxidation, nitrosation, and glutathionylation [5]. Diverse protein classes, including proteases, oxidoreductases, kinases, and acyltransferases, contain reactive and functional cysteine residues [3]. Thus, the high nucleophilicity and functional importance of cysteine render this amino acid an attractive chemical handle for the development of targeted and selective covalent ligands to modulate the function of diverse proteins.

Covalent inhibitors can be categorized as reversible or irreversible depending on the target residence time. Covalent irreversible inhibitors can be further classified as either residue-specific reagents, affinity labels, or mechanism-based inhibitors, as recently described by Tuley and Fast [6•]. Residue-specific reagents are reactive compounds with minimal non-covalent affinity to a particular binding site. General cysteine alkylating agents, such as iodoacetamide (IAA) and methylmethanethiosulfinate (MMTS), fall into this category. The potency of residue-specific reagents is generally dictated by the inherent reactivity of the electrophile, as protein modification does not rely on the formation of an initial non-covalent encounter complex. As a result, these compounds generally lack selectivity and inactivate multiple targets. By contrast, affinity labels typically form an initial non-covalent complex, which increases the effective molarity of the reactive group proximal to the nucleophilic residue, and are generally more selective [7]. Potency of affinity labels is defined by the second order rate constant of inactivation, that is, k_{inact}/K_I, which incorporates the affinity of the initial encounter complex. Optimizing the potency of an affinity label, therefore, involves maximizing non-covalent interactions and positioning an appropriate electrophile for optimal reaction with the nucleophilic residue on the protein. It is important to note that non-covalent interactions can contribute to the binding of some residue-specific reagents, and in a similar vein, affinity labels can display off-target effects driven solely by reactivity and not non-covalent affinity.

Covalent inhibition as a therapeutic strategy has been shown to demonstrate: (1) a long residence time and duration of action, which has been associated with efficacy [8]; (2) ability to target shallow binding pockets that are recalcitrant to non-covalent ligands [6•]; and, (3) potential to circumvent resistance mechanisms, as well as the ability to selectively target disease-associated mutants as has recently been shown for KRAS G12C [9•]. It is important to note that non-covalent inhibitors can also achieve the latter, as demonstrated for vemurafenib [10]. Disadvantages of covalent compounds include irreversible inhibition of off-targets leading to

toxicity, as well as potential immune-related idiosyncratic adverse drug reactions [11]. Cysteine-targeted inhibitors have been approved or are in various stages of the drug-discovery pipeline including, Tecfidera (dimethyl fumarate (DMF)) [12•,13], kinase inhibitors (Afatinib, Ibrutinib, Osimertinib, and Neratinib) [14•], and, inhibitors of KRAS G12C [15•,16,17].

A reactive-cysteine profiling method, known as isoTOP-ABPP, has the potential to aid in two key aspects of covalent irreversible inhibitor discovery: (1) to identify ligandable cysteines for covalent modulation of protein function; and, (2) to evaluate both on and off-target cysteine engagement so as to minimize risks associated with off-target drug toxicity [18]. This review will discuss recent advances in cysteine-targeted isoTOP-ABPP, followed by an overview of potential applications of isoTOP-ABPP to covalent drug discovery.

Methods for the identification of reactive cysteines

Numerous chemical-proteomic methods currently exist for identifying reactive and functional cysteine residues. These include probes specific for cysteine PTMs, including *S*-sulfenylation [19–21], *S*-sulfination [22–24], *S*-nitrosation [25,26], and electrophilic lipid modifications [27], as well as probes selectively targeting cysteine residues on a defined protein class, including kinases [28], and cysteine proteases [29]. Here, we focus on a platform known as isoTOP-ABPP, which identifies a subset of reactive and functional cysteine residues independent of protein class or susceptibility to a particular PTM [1••].

The isoTOP-ABPP platform for reactive-cysteine profiling

IsoTOP-ABPP (Figure 1a) is a derivative of activity-based protein profiling (ABPP), a pioneering technology for interrogating protein activity directly in complex biological systems. In general, ABPP probes contain three elements: (1) a reactive warhead for covalently labeling target proteins; (2) a reporter tag for affinity purification or fluorescence detection; and, (3) a linker to minimize steric hindrance between the reporter and reactive groups [30,31]. Early ABPP methods utilized reactive warheads targeting a specific enzyme family, such as the fluorophosphonate probe for the serine hydrolases [32]. In latter iterations, more reactive and promiscuous electrophiles were utilized [33], culminating in the use of an iodoacetamide-alkyne (IA-alkyne) probe (Figure 1b) for modification of reactive cysteines in the proteome. The isoTOP-ABPP platform couples an IA-alkyne probe with an isotopically tagged cleavable linker, enabling the selective enrichment, release, and mass-spectrometry (MS)-based relative quantification of IA-labeled peptides from two samples. The isoTOP-ABPP platform involves the following steps: (1) treatment of lysates with IA-alkyne to label reactive cysteines; (2) conjugation of IA-labeled cysteines in control and experimental samples to isotopically differentiated cleavable biotin-azide tags (Figure 1c) using copper-catalyzed azide-alkyne cycloaddition (CuAAC) [34]; (3) enrichment of IA-labeled proteins on streptavidin beads, followed by on-bead trypsin digestion, and linker cleavage to release IA-labeled peptides; and, (4) analysis of the resulting isotopically heavy and light peptide pairs using LC/LC–MS/MS to quantify reactivity differences in two samples using light: heavy isotopic ratios [1••].

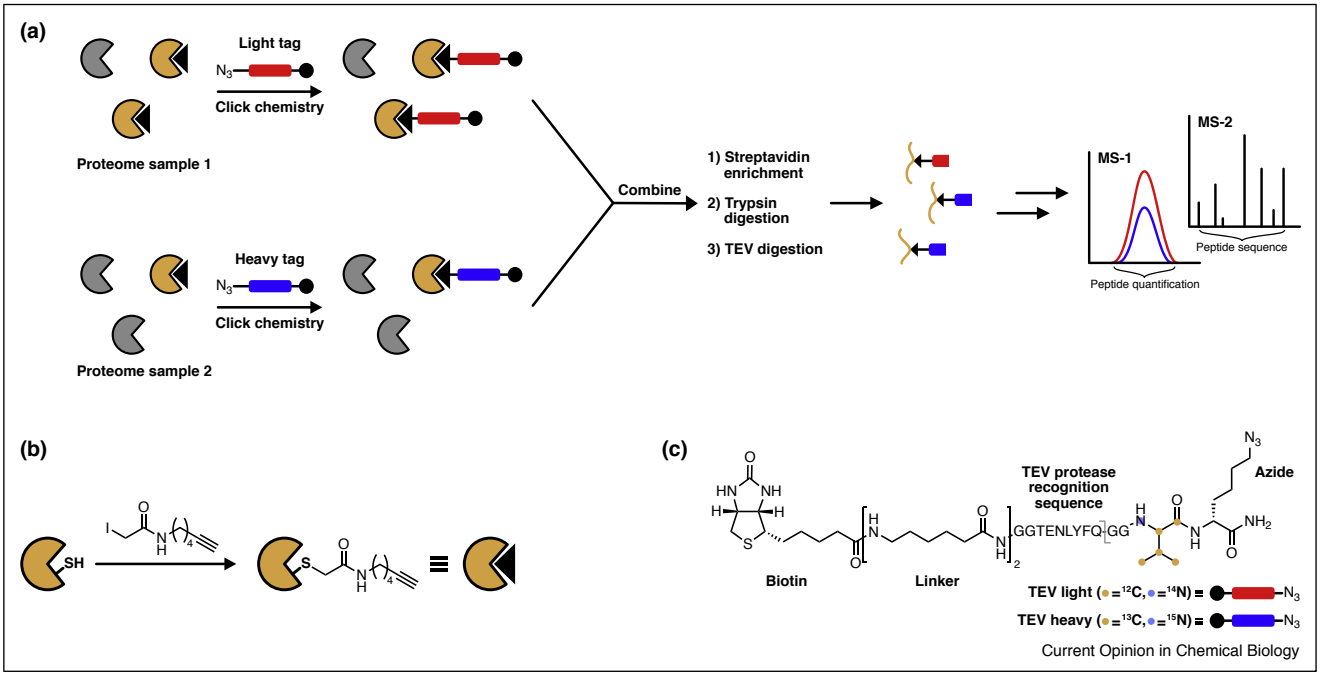
Limitations in the current isoTOP-ABPP platform include the low coverage of the cellular cysteinome. Since low concentrations (100 μM) of IA-alkyne are used for proteome labeling, only 1000–2000 cellular cysteines are identified in a typical analysis. The subset of cysteine residues identified are those that demonstrate high reactivity with the IA electrophile, and have been shown to be enriched in functional cysteines [1••]. However, some classes of functional cysteines remain intractable to IA labeling, and are, therefore, not typically captured in an isoTOP-ABPP analysis. Furthermore, reduced coverage of cysteine residues from low-abundant proteins, particular those localized within subcellular organelles, could limit the potential utility of isoTOP-ABPP for certain applications. Furthermore, cell lysates for isoTOP-ABPP are not typically treated with reducing agents, thereby limiting access to proteins that are highly susceptible to oxidation or aggregation. As discussed below, the development of new cysteine-reactive electrophiles, analytical methods, and cell-based profiling approaches, can serve to overcome some of these current limitations.

Recent advances in isoTOP-ABPP methods

Since the initial development of the isoTOP-ABPP platform, various iterations to the initial workflow have been reported, including variations to: (1) the cysteine-reactive electrophile; (2) the cleavable biotin-azide tag; and, (3) the mode of heavy isotope incorporation. These recent advances are summarized herein.

IA-alkyne is widely used as the reactive warhead in isoTOP-ABPP [1••,35,36]; however, other electrophiles have also been incorporated into the isoTOP-ABPP workflow. A photocaged bromomethyl ketone (CBK) [37•] and iodomethyl ketone (CIK4) [38] (Figure 2a) were shown to have lower cytotoxicity compared to IA-alkyne and were used to profile reactive cysteines in live cells with high spatial and temporal control. CBK was used to monitor changes in cysteine reactivity in A431 cells in response to the epidermal growth factor (EGF) stimulated release of reactive oxygen species [37•]. Alternatives to halo acetamide electrophiles have also been developed, including aryl halides such *p*-chloronitrobenzene [39], and hypervalent iodine regents, such as ethynyl benziodoxolone (EBX) (Figure 2a) [40].

Figure 1



(a) General isoTOP-ABPP workflow. Reactive cysteine residues on two proteome samples are labeled with IA-alkyne, followed by CuAAC with an isotopically heavy or light biotin-azide cleavable linker. The two lysates are combined, biotinylated proteins are enriched on streptavidin-agarose beads, and subjected to an on-bead trypsin digestion. The IA-labeled peptides are released and analyzed by LC/LC–MS/MS. Heavy and light peptide pairs are quantified by their extracted MS1 peaks. **(b)** IA-alkyne structure and cysteine-labeling scheme. **(c)** The tobacco-etch virus (TEV) protease cleavable biotin-azide tag for isoTOP-ABPP.

The initial isoTOP-ABPP platform utilized isotopically labeled, protease-cleavable biotin-azide tags for CuAAC-mediated conjugation to probe-labeled proteins. Because of the advent of a wide-variety of cleavable chemistries, isotopically tagged variants of chemically cleavable [41] and photocleavable [42,43] biotin-azide tags have been developed and shown to be compatible with the isoTOP-ABPP workflow.

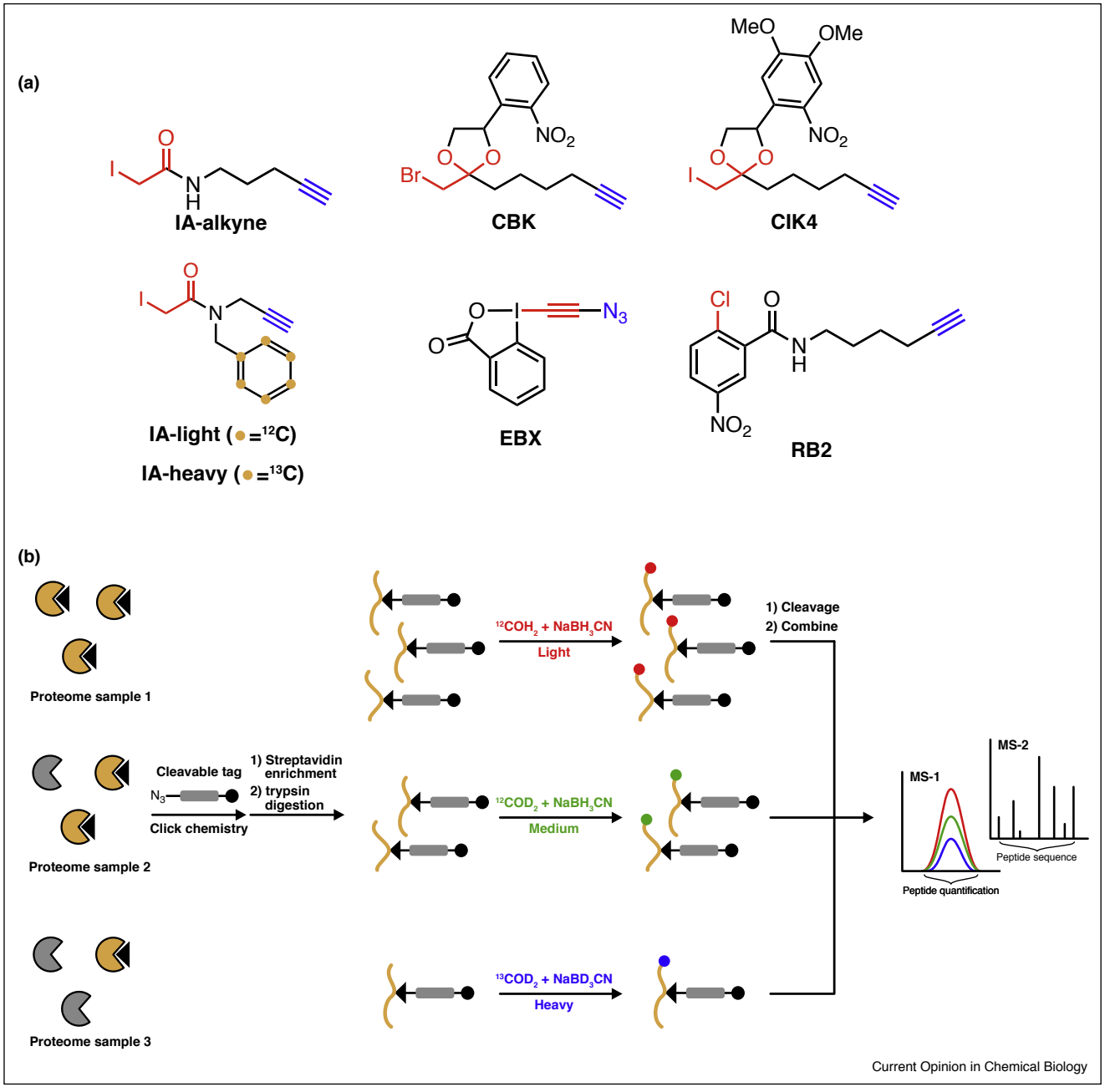
Lastly, approaches to incorporate isotopic labels into the isoTOP-ABPP workflow have been explored, including: (1) stable isotope labeling by amino acids in cell culture (SILAC) to incorporate heavy isotopes into the proteomes under evaluation; (2) isotopically tagged cysteine-reactive probes; (3) isotopically light and heavy biotin-azide linkers; and, (4) post-digest peptide labeling using reductive demethylation (ReDiMe) or isobaric tags (iTRAQ, TMT). Incorporation of isotopic labels into the cysteine-reactive probe was accomplished through synthesis of isotopically differentiated iodoacetamide-alkyne probes containing a $^{12}C_6$ or $^{13}C_6$ benzyl moiety, termed IA-alkyne light (IAL) or IA-alkyne heavy (IAH), respectively [44] (Figure 2a). IAL and IAH can be obtained through a more facile synthesis, and using less expensive starting materials compared to the isotopic biotin-azide tags [44]. IAL and IAH also support the profiling of reversible cysteine modifications within the same sample in a work flow similar to the OxICAT method [45]. Combining TOP-ABPP with reductive dimethylation (ReDiMe) results in a method called rdTOP-ABPP [42] (Figure 2b). rdTOP-ABPP was shown to be comparable to several commercially available linkers for site of identification, and has the added benefit of supporting triplex quantitative experiments [42]. Multiplexed thiol reactivity profiling (MTRP) with isobaric tags for relative and absolute quantitation (iTRAQ) labeling allows for analysis of up to eight samples in parallel [43].

The availability of multiple chemical probes, linkers and quantification methods, allow for tailoring the isoTOP-ABPP workflow for each desired application. Further advancements in chemical-probe and linker development, coupled with improvements in MS instrumentation and data-analysis software, will serve to increase the number of cysteine identifications from a complex proteome.

Applications of reactive cysteine profiling in drug discovery

To expand the protein targets amenable to covalent inhibition, it is necessary to globally interrogate the

Figure 2



Recent modifications to the isoTOP-ABPP platform. **(a)** New cysteine- reactive chemical probes. Electrophiles are highlighted in red, reporting handles in blue, and isotopic labels in orange. **(b)** Triplex rdTOP-ABPP workflow.

proteome for reactive cysteines that upon covalent modification, afford a functional outcome. Furthermore, given the toxicity risks associated with covalent and irreversible modification of off targets, it is imperative to extensively evaluate target engagement of covalent ligands within physiologically relevant proteomes. As described below, isoTOP-ABPP can aid in both these aspects of covalent ligand development.

Identification of reactive and functional cysteines for covalent targeting

Despite the wealth of genetic information acquired from genome sequencing efforts, only ~2% of predicted human gene products are currently targeted by small-molecule drugs, and only 10–15% of human gene products are thought to be druggable [46•]. ABPP, and associated technologies, can be powerful tools to identify functionally

important and ligandable sites present in different disease states [43]. In particular, isoTOP-ABPP has been utilized to identify reactive cysteines that modulate protein function upon covalent modification. One of the first applications of the isoTOP-ABPP platform was to identify and rank cysteines by reactivity, thereby demonstrating that cysteine reactivity is highly predictive of functionality [1••]. Although active-site cysteines on proteases and oxidoreductases are well annotated, allosteric regulatory cysteines within proteomes are poorly characterized. IsoTOP-ABPP has been applied to identify non-active-site cysteines that are susceptible to PTMs, including oxidation [47], *S*-nitrosation [48], modification by lipid derived electrophiles [36], and zinc chelation [35]. Biochemical characterization of these non-catalytic cysteine residues has shown that modification results in modulation of protein function. The ability of these cysteines to regulate protein function render them putative target sites for designing covalent inhibitors. However, since modification with a small molecule is not a direct mimic of a specific cysteine PTM, covalent binding may not necessarily phenocopy the function of the endogenous PTM.

IsoTOP-ABPP can also identify cysteine residues whose reactivity is elevated under disease conditions. For example, Bar-Peled *et al.* applied isoTOP-ABPP to identify druggable cysteines in KEAP1-mutant non-small-cell lung cancers [49•], and Martell *et al.* applied isoTOP-ABPP to identify changes in cysteine reactivity associated with impaired insulin signaling in *Caenorhabditis elegans*. These studies lay the groundwork for future applications of isoTOP-ABPP to compare cysteine-reactivity changes in healthy and diseased systems and aid in identifying upregulated cysteine residues that could be explored as potential targets for covalent inhibitor development.

Screening the potency and selectivity of covalent ligands

Covalent ligands of various modalities have been reported, including: (1) low-molecular weight reactive fragments; (2) drug-like small molecules with embedded electrophiles; and, (3) structurally complex electrophilic natural products. IsoTOP-ABPP provides a potential tool to evaluate the protein targets of covalent ligands.

Covalent fragment-based screening

Fragment-based ligand discovery (FBLD) utilizes low-molecular weight fragments that generally conform to the rule of three [50]; molecular mass >300 Da, up to three hydrogen bond donors and acceptors, and calculated logP ≤ 3. Early covalent FBLD screens used covalent tethering approaches in which a target protein containing a reactive cysteine reacts with a library of disulfide-containing fragments [51]. This disulfide-tethering approach was recently used to identify covalent ligands for the oncogenic KRAS G12C mutant, which allosterically attenuates GTP affinity [15•]. In a more global and untargeted approach, Backus *et al.* used isoTOP-ABPP to assess the proteome reactivity of a 52-member fragment library containing chloroacetamide and acrylamide electrophiles [52••]. The analysis was performed in a competitive format, whereby a proteome is treated with a covalent fragment before treatment with IA-alkyne, and a decrease in IA-alkyne labeling is indicative of ligand binding. Of the 700 ligandable cysteines identified, 535 were found on proteins which had no known ligands in DrugBank, representing classes of proteins classically considered to be undruggable, including transcription factors, and adaptor proteins [52••]. Among the ligands screened were two fragments that covalently modified pro-caspases [52••] (Figure 3a). Although the identified fragments are typically promiscuous and show low affinity, further chemical elaboration has the potential to yield potent and selective small molecules for these traditionally undruggable targets.

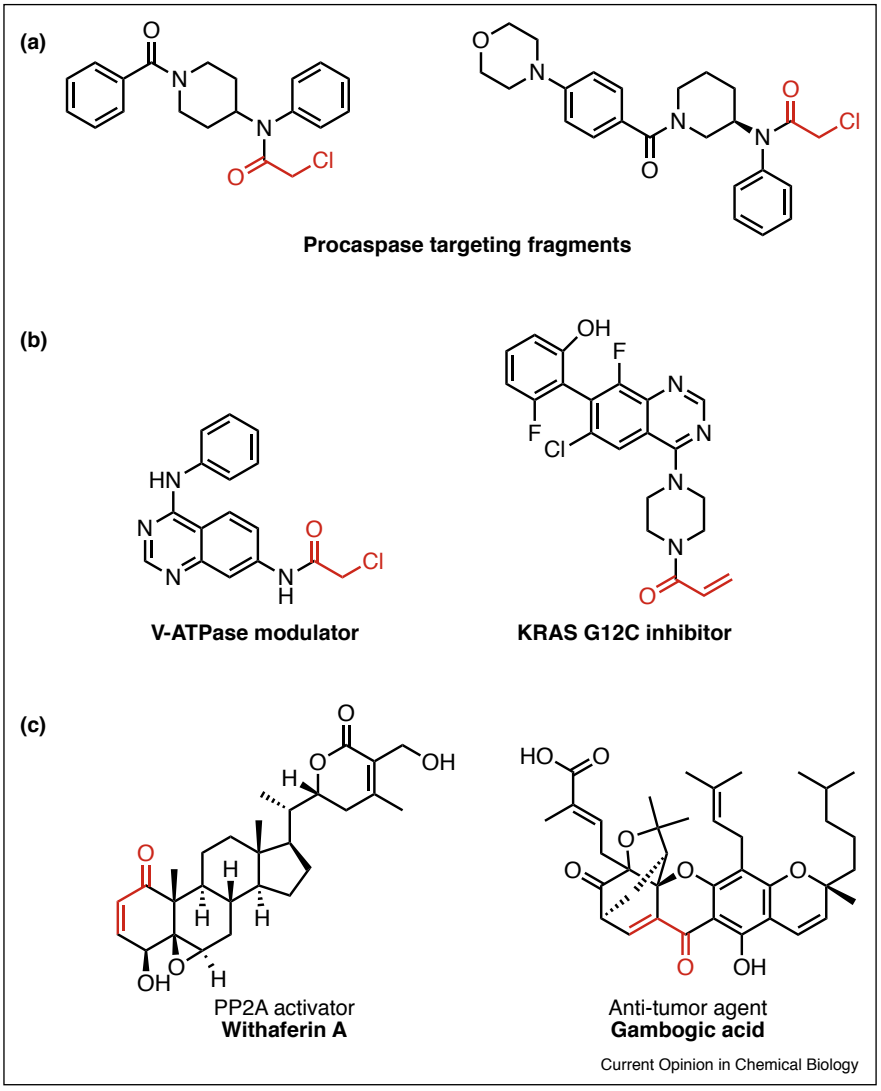
Drug-like small-molecule screening

Competitive isoTOP-ABPP has also been applied to drug-like electrophilic compounds. Dimethyl fumarate (DMF) is an electrophilic, immunomodulatory drug believed to function by covalently modifying cysteine residues. Blewett *et al.* found that DMF covalently modified conserved cysteines in the non-catalytic domain of protein kinase Cθ (PKCθ) and disrupted PKCθ-CD28 association during T-cell activation [12•]. T-cells expressing a cysteine mutant of PKCθ showed impaired activation; however, DMF treatment of these mutant-expressing cells showed a further reduction in activation, suggesting that DMF exhibits polypharmacology, and likely acts by concurrently targeting multiple cellular cysteines. Similarly, isoTOP-ABPP was used to demonstrate the high selectivity of a chloroacetamide-bearing quinazolinone for the vacuolar H^+ ATPase (V-ATPase) [53] (Figure 3b). In a variation of competitive isoTOP-ABPP, a desthiobiotin-linked IA probe was used to determine target engagement of a quinazoline-based KRAS G12C inhibitor [16] (Figure 3b). Lastly, Whitby *et al.* used isoTOP-ABPP to investigate proteome labeling by reactive metabolites generated *in vivo* upon treatment with the hepatotoxic drugs, acetaminophen, troglitazone, clozapine, and tienilic acid [54]. These studies demonstrate the utility of isoTOP-ABPP to investigate both target occupancy and promiscuity of drug-like small molecules.

Electrophilic natural-product screening

Natural products (NPs) exhibit structurally complex scaffolds that often demonstrate exquisite target selectivity [55], and often contain cysteine-targeting electrophilic motifs, including Michael acceptors and epoxides [43]. Typically, an alkyne variant of a covalent ligand can be used to assess target occupancy; however, the complexity of NP total synthesis and limited information of structure activity relationships, complicate the use of alkyne-tagged natural product analogs. Competitive isoTOP-ABPP,

Figure 3



Covalent ligand discoveries aided by isoTOP-ABPP **(a)** covalent fragments targeting procaspases **(b)** drug-like small-molecules targeting V-ATPase and KRAS G12C, and **(c)** electrophilic natural products. Electrophiles are highlighted in red.

whereby proteome treatment with the unmodified NP is subsequently followed by addition of IA-alkyne, has been applied to identify putative protein targets of several natural products including licochalcone A, celastrol, and curcumin [40,56,57]. Grossman *et al.* assessed the proteome reactivity of withaferin A, an electrophilic natural produce known to exhibit cancer anti-proliferative activity [58]. Withaferin A (Figure 3c) was shown to activate the tumor suppressor phosphatase PP2A, and a covalent fragment screen was used to identify a more synthetically tractable small molecule that recapitulates the anti-proliferative activity of withaferin A (Figure 3) [58]. Similarly, MTRP was used to map sites of labeling of several electrophilic natural products, including gambogic acid (Figure 3c), diverse α,β-unsaturated γ-lactones, and acetylbritannilactone [43].

Conclusions

The unique properties of cysteine, including nucleophilicity, redox susceptibility and polarizability, facilitates a central role for this amino acid in protein structure, function and regulation. Targeting disease-relevant cysteines can be a fruitful strategy to overcome some of the limitations of non-covalent drugs, including in the targeting of classically undruggable sites. IsoTOP-ABPP is one of many chemical-proteomic platforms that can aid in identifying novel ligandable and functional cysteines, revealing reactive cysteines upregulated in disease, and

elucidating the selectivity of electrophilic fragments, drug-like molecules, and natural products. Limitations in current isoTOP-ABPP approaches include the following: (1) only a subset of cellular cysteines are identified, thereby limiting the ability to assess compound selectivity across the entire cellular cysteinome; and, (2) typically cell lysates are treated under non-reducing conditions, which could result in oxidation or aggregation of a subset of proteins with highly reactive cysteines. Therefore, isoTOP-ABPP techniques need to continue to mature in order to provide important exploratory tools for the drug-development process.

Conflict of interest statement

Nothing declared.

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