Target Identification of Bioactive Covalently Acting Natural Products

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Contents

1 Introduction.......................................................................................................................
2 Covalently Acting Natural Products................................................................................
   2.1 Strained Ring-Containing Electrophiles....................................................................
   2.2 Unsaturated Ketones, Esters, and Amides.............................................................
   2.3 Aldehydes, Hemiaceetals, and Aminals.................................................................
3 Chemoproteomic Approaches to Target Identification of Covalently Acting Natural Products
   3.1 Reversible Versus Irreversible Natural Products and Methods for Target Identification
   3.2 Target Identification Using Natural Product-Based Probes....................................
   3.3 Activity-Based Protein Profiling (ABPP) for Identifying Proteome-Wide Druggable Hotspots Targeted by Covalently Acting Natural Products
   3.4 Covalent Ligand Discovery Against Druggable Hotspots Targeted by Natural Products
4 Conclusions....................................................................................................................
References................................................................................................................................

Abstract There are countless natural products that have been isolated from microbes, plants, and other living organisms that have been shown to possess therapeutic activities such as antimicrobial, anticancer, or anti-inflammatory effects. However, developing these bioactive natural products into drugs has remained challenging in part because of their difficulty in isolation, synthesis, mechanistic understanding, and off-target effects. Among the large pool of bioactive natural products lies classes of compounds that contain potential reactive electrophilic centers that can covalently react with nucleophilic amino acid hotspots on proteins and other biological molecules to modulate their biological action. Covalently...
acting natural products are more amenable to rapid target identification and mapping of specific druggable hotspots within proteins using activity-based protein profiling (ABPP)-based chemoproteomic strategies. In addition, the granular biochemical insights afforded by knowing specific sites of protein modifications of covalently acting natural products enable the pharmacological interrogation of these sites with more synthetically tractable covalently acting small molecules whose structures are more easily tuned. Both discovering binding pockets and targets hit by natural products and exploiting druggable modalities targeted by natural products with simpler molecules may overcome some of the challenges faced with translating natural products into drugs.

1 Introduction

Natural products from microbes, plants, and other living organisms are a rich source of medicinal compounds that have been utilized by humankind for thousands of years. Their further characterization and exploitation of important biological pathways have led to many different types of modern medicines to treat pathologies such as bacterial and fungal infections, inflammation, diabetes, neurodegenerative diseases, and cancer (Koehn and Carter 2005; Kingston 2011; Harvey et al. 2015; Laraia et al. 2018). Between 25 and 50% of marketed drugs are thought to be derived from natural products, and while such compounds populate many therapeutic areas, they have been particularly important to the areas of oncology and infectious diseases (Kingston 2011). However, while many natural products have shown therapeutic potential, developing natural products into drugs has remained challenging and most US pharmaceutical companies have reduced or eliminated their natural product groups. This is in part due to the difficulty of synthesis and execution of global structure–activity relationship (SAR) studies, challenges in large-scale isolation, slower discovery, and development compared to target-based high-throughput screening campaigns involving simpler, often “flat” molecules, hurdles in understanding mode of action or direct targets, and complex polypharmacology (Beutler 2009; Romo and Liu 2016). Despite these challenges, natural products have not only yielded classical active-site inhibitors against druggable targets, but have also been able to uniquely access druggable modalities that would be considered undruggable or difficult to tackle with traditional drug discovery efforts. These include, for example, FK506 or tacrolimus, which reduces peptidylprolyl isomerase activity by binding to FKBP12, creating a new complex FKBP12–FK506 complex, leading to the interaction with and inhibition of calcineurin resulting in inhibition of T cell signaling (Liu et al. 1991). Another example comes from rapamycin or sirolimus, which also binds to FKBP12 to form a FKBP12–rapamycin complex which then interacts with and inhibits mTORC1 signaling (Saxton and Sabatini 2017). Even in the twenty-first century, these molecular functions are difficult to “design” from first principles. Thus, target identification of natural products remains a critically important endeavor not just for
translational development of natural products into drugs, but also to gain insight into new modalities that can be targeted for drug discovery and to gain insight into natural strategies for target engagement. Upon identifying particular binding pockets within proteins targeted by natural products, synthetically simpler libraries of small molecules can potentially be developed against these sites to overcome the synthetic and isolation challenges faced with natural products. In this review, we will highlight activity-based protein profiling (ABPP) chemoproteomic strategies for mapping targets of covalently acting natural products and covalent ligand discovery approaches that can be utilized to potentially develop more synthetically tractable compounds that mimic the actions of the more complex natural products. We will also discuss diverse examples of electrophilic natural product scaffolds and their targets.

2 Covalently Acting Natural Products

Hundreds of natural products have been shown, or proposed, to engage a wide array of biological targets in covalent bond formation including proteins, nucleic acids, and even small biological molecules themselves. Equally diverse are the chemical structures of the “reactive warhead” embedded into the parent natural product scaffold–motifs which in some cases would often be difficult to design from first principles (Potashman and Duggan 2009; Baillie 2016; Lagoutte et al. 2017), yet are continually influencing modern covalent drug design (Lagoutte and Winssinger 2017). Several excellent reviews have been written on covalently acting natural products, particularly with respect to their protein targeting abilities (Drahl et al. 2005; Leslie and Hergenrother 2008; Gersch et al. 2012; Wright and Sieber 2016; Pan et al. 2016; Jackson et al. 2017). Here, we briefly highlight various classes of electrophilic species presented by natural products that have been implicated in their mechanism of action.

2.1 Strained Ring-Containing Electrophiles

Nature extensively exploits ring strain in the creation of covalent warheads. Reactive carbonyl groups embedded in strained rings, as typified by the β-lactam and β-lactone functional group, are some of the oldest known and most intensely studied covalent pharmacophores found in natural products (Fig. 1a) (Böttcher and Sieber 2012). Indeed, the β-lactam antibiotics, typified by penicillin V (1), are perhaps the greatest success story in the use of covalently acting natural products to treat human disease. Their primary target (an active-site serine of bacterial transpeptidases) and other modes of action are discussed in Sect. 3. In the past three decades, β-lactones have garnished significant attention due to their protein targeting ability (Böttcher and Sieber 2012). Naturally occurring lipstatin (2) inspired
the antiobesity drug and lipase inhibitor orlistat (Bialecka-Florjanczyk et al. 2018). Omuralide (3) and salinosporamide A (4) are both potent cytotoxic agents which covalently modify an active-site threonine of the 20S proteasome subunit (Gulder and Moore 2010). This family of natural products is involved in various clinical trials for the treatment of myriad cancers (Potts et al. 2011). Despite the target specificity for 1–4, proteomic profiling studies of spongiolactone (5) revealed a complex portrait of targets and thus likely polypharmacological effects dictating its antiproliferative effects (Wright et al. 2017).

The epoxide functional group is another major class of reactive electrophile frequently exploited by natural products (Fig. 1b). The intrinsic affinity of the parent molecule to the target, prior to covalent engagement, is a common theme for promoting reactivity of otherwise unreactive substrates (Swinney 2009; Bauer 2015; Strelow 2017). Thus, many complex epoxide-containing molecules studied are not promiscuous, and in the case of multiple epoxidized molecules, only a single epoxide often reacts as discussed below. The bacterial-derived natural product epoxomicin (6) is a potent covalent proteasome inhibitor whose understanding of mechanism of action led to the discovery of the anticancer drug carfilzomib (Kim and Crews 2013). Fumagillin (7) targets the methionine aminopeptidase MetAP-2 wherein only the spiroepoxide is essential. Target identification for both 6 and 7 is discussed in Sect. 3. The diterpene triepoxide triptolide (8) has long been used in traditional Chinese medicine to treat a host of diseases including inflammation and cancer, and derivatives of 8 have entered clinical trials for the treatment of cancer. Triptolide induces apoptosis and cell growth arrest and is a potent inhibitor of
NF-κB- and NF-AT-mediated transcription (Meng et al. 2014). Many additional targets of 8 (both covalent and non-covalent) have been proposed (Leuenroth and Crews 2005). Triptolide covalently modifies a reactive cysteine on the XPB subunit of the transcription factor TFIH (Titov et al. 2011). Notably, the covalent locus of reactivity is believed to be the 12, 13-epoxide (He et al. 2015).

Cyclopropanes adjacent to electron-deficient π-systems (i.e., homo-Michael acceptors) have been frequently implicated in the alkylation of biological nucleophiles, particularly DNA bases (Fig. 1c). Not surprisingly, many natural products containing such motifs are potent cytotoxins including both acylfulvene (9) and duocarmycin SA (10), derivatives of which are being investigated for the treatment of cancer (Tanasova and Sturla 2012). DNA alkylation by 9 impairs transcription by RNA Pol II (Malvezzi et al. 2017), while 10 is a highly sequence-selective covalent alkylator of adenine-N3 in AT-rich sequences (Boger et al. 1994). The human gut microbe-derived family of colibactins, as typified by precolibactin A (10), alkylate and cross-link DNA and have been implicated in human colorectal cancers (Vizcaino and Crawford 2015).

2.2 Unsaturated Ketones, Esters, and Amides

By far, the largest class of covalently acting natural product is those susceptible to hetero-Michael addition reactions—often by the nucleophilic amino acid side chains of proteins (Fig. 2) (Jackson et al. 2017). These addition reactions can be both reversible and irreversible. Notably, hetero-Michael acceptors are prevalent across much of natural product space (peptides, alkaloids, polyketides, and terpenes) and serve as the basis for much of modern covalent drug design (De Cesco et al. 2017). Nature employs both α,β-unsaturated amides (see 12), esters (see 13), and ketones (see 14) to achieve covalent target modification (Fig. 2a). Particularly important from a natural product’s perspective is the large class of exocyclic methylene lactones of which over 5000 members are believed to exist (see 15–18, Fig. 2b) (Kitson et al. 2009). Many of these compounds are reactive to thiols, both cysteine side chains and the pool of free intracellular thiols. The anti-inflammatory and anticancer sesquiterpene parthenolide (15) has been used for proteomics-based target identification as discussed in Sect. 3. The anticancer pseudoguaianolide helenalin (16) is also thiol-reactive (Widen et al. 2017). Nature also constructs various dimeric and higher-order structures featuring multiple electrophilic sites as typified by ainsliadimer A (17) and ainsliatrimer A (18). Mode of action studies by Lei et al. has found these compounds target IKKα/β and PPARγ (Li et al. 2014; Dong et al. 2015).

An important but less frequently encountered class of hetero-Michael acceptors is the family of quinone methides, which aromatize the following conjugate addition and thus providing an additional driving force for conjugation (Fig. 2c) (Bai et al. 2014). Celastrol (19), which possesses a myriad of biological activities including anticancer and anti-inflammatory effects, is cysteine-reactive (Klaić et al. 2014).
The antibacterial and cytotoxic natural product kendomycin (20) reacts with a proteasome histidine residue (Beck et al. 2014). Finally, it should be noted that few “stable” quinone methides exist; in many cases, the reactive electrophile is generated in situ (Bai et al. 2014). Pleurotin (21), for example, has been hypothesized to undergo in situ reduction to generate the reactive quinone methide 22 (Moore 1977).

2.3 Aldehydes, Hemiacetals, and Aminals

Nature also utilizes natural products containing electrophilic carbonyl groups at the aldehyde oxidation state, including free aldehydes, aminals, and hemiacetals, for covalent target engagement. The phytotoxic sesterterpene ophiobolin A (23) has been shown to condense with a lysine side chain of the calcium-binding protein calmodulin, presumably forming an extended enamine intermediate (Leung et al. 1988). Of note, 1, 4-dicarbonyl-containing natural products, including 23, have also been implicated in the formation of pyrroles via a Paal–Knorr-type process with intracellular amines (Chidley et al. 2016; Kornienko and La Clair 2017).
The bis-hemiacetal marine natural product manoalide (24) has also been proposed to react with lysines (Pettinger et al. 2017). Ecteinascidin 743 (25), an aminal-containing anticancer agent and approved drug (trabectedin), generates an iminium ion which covalently bonds to DNA bases (Aune et al. 2002) (Fig. 3).

In summary, nature has evolved a myriad of chemical warheads to covalently modify a range of biological nucleophiles and many historically successful natural product-based drugs have exploited these features. Given the enormous number of naturally occurring epoxide and $\alpha,\beta$-unsaturated carbonyl-containing metabolites, one can assume that we have only scratched the surface with respect to detailed target annotation as well as understanding the complex polypharmacology of bioactive natural products. Moreover, nature continues to impress the organic and medicinal chemist with its complex arsenal and sometimes bizarre mechanisms for generating reactive warheads (Fig. 4) (Kwan and Luesch 2010). One cannot help but stand in awe at processes such as the thiol-mediated conversion of leinamycin.

**Fig. 3** Natural products containing reactive functional groups capable of condensation reactions

**Fig. 4** Natural products containing exotic warheads: Unsaturated nitrones and episulfonium ions
(26) to DNA-reactive episulfonium ion 27 or the retro-dimerization of the alkaloid stephacidin B (28) into protein-reactive, \(\alpha,\beta\)-unsaturated nitrone-containing avrainvillamide (29) (Asai et al. 1996; Myers and Herzon 2003; Wulff et al. 2007). By studying these processes, searching for new ones, and distilling the essential features needed for efficient bond formation, new chemistry concepts relevant to small molecule drug discovery will no doubt be unearthed (Crane and Gademann 2016).

3 Chemoproteomic Approaches to Target Identification of Covalently Acting Natural Products

3.1 Reversible Versus Irreversible Natural Products and Methods for Target Identification

Many strategies have arisen to tackle the challenge of identifying direct protein targets of natural products. These include classical approaches wherein natural products can be immobilized onto solid supports for affinity-based isolation of protein targets (Wright and Sieber 2016). Approaches that enable target identification without chemical modification have also arisen, including cellular thermal shift assay (CETSA) or thermal proteome profiling (TPP) (Chang et al. 2016). Among various approaches, chemoproteomic strategies have been thus far been the dominant approach for natural product target identification, which relies on: (1) direct derivatization of natural products to incorporate photoaffinity cross-linkers (e.g., diazirines, benzophenones), biorthogonal handles (e.g., alkynes or azides), and/or biotin enrichment handles to enable covalent capture and enrichment; or (2) activity-based protein profiling (ABPP)-based competitive chemoproteomic profiling approaches pioneered by Benjamin Cravatt, wherein the parent natural product, usually covalently acting, is competed against a broadly reactive probe—for subsequent identification of the natural product protein targets by mass-spectrometry-based proteomics (Wright and Sieber 2016; Kanoh 2016; Pan et al. 2016). For reversibly acting natural products, these target identification approaches still remain quite challenging and laborious due to low abundance of the targets, high degree of non-specific binding, or synthetic difficulty in natural product derivatization without losing bioactivity. Furthermore, while these types of experiments can provide direct protein targets, identifying the specific site of binding within the protein target often requires additional structural elucidation.

As mentioned, many natural products possess electrophilic fragments capable of covalently reacting with nucleophilic amino acids within proteins to disrupt their biological functions (Drahl et al. 2005; Gersch et al. 2012). As seen in Sect. 2, \(\beta\)-lactones/lactams, exocyclic methylene lactones, spiroepoxides, enones, \(\alpha,\beta\)-unsaturated amides and esters, quinone methides, and aldehydes all take part in protein modification. These reactive groups have been shown to covalently modify
mostly serine, cysteine, threonine, and lysine, but also other amino acids such as histidine, arginine, and aspartic and glutamic acid on particular protein targets (Drahl et al. 2005; Gersch et al. 2012). The advantage of covalently acting natural products is their relative ease in target identification compared to reversibly acting molecules. This is in part because direct covalent attachment by the natural product itself enables harsher washing steps of the protein during the enrichment processes to reduce non-specific binding. It also abrogates non-specific reactivity issues that arise from photoaffinity tags and photo-crosslinking. It is also easier to identify the specific site of covalent modification or the druggable hotspots targeted by reactive natural products compared to reversibly acting natural products using competitive ABPP methods subsequently described. As previously mentioned, understanding the specific amino acid targeted by the natural product within a specific protein directly from complex proteomes or living systems provides significant benefit over just the identification of the protein target; the druggable hotspot(s) may represent a unique and functional modality that can be exploited for drug discovery without the laborious and sometimes impossible efforts of having to solve the three-dimensional structure of the natural product bound to the protein by either crystallographic or spectroscopic means. Furthermore, these sites of covalent natural product modification now represent ligandable hotspots that can be interrogated with far simpler and more synthetically tractable covalently acting small-molecule libraries using high-throughput competitive ABPP screening approaches to be subsequently discussed. We will discuss the two primary chemoproteomic methods for target identification of covalently acting natural products.

### 3.2 Target Identification Using Natural Product-Based Probes

The chemoproteomics approach that has most commonly been used for covalently acting natural products is to derivatize or synthesize the natural product analog incorporating either a biotin enrichment handle or an alkyne to which a fluorophore-azide or biotin-azide handle can be appended by copper-catalyzed azide–alkyne cycloaddition reactions (CuAAC) (Fig. 5a) (Tornøe et al. 2002; Rostovtsev et al. 2002; Heeres and Hergenrother 2011; Gersch et al. 2012; Ursu and Waldmann 2015; Wright and Sieber 2016; Pan et al. 2016). This general strategy can also be coupled with competition studies with the parent natural product and quantitative proteomic approaches with stable isotopic labeling of cells (SILAC) or isotopic tagging of peptides (e.g., TMT labeling) to identify the direct targets of these natural products (Ong et al. 2002; Thompson et al. 2003; McAlister et al. 2014). With natural product probes bearing the full linker and biotin handles, the utility of these probes is often limited to in vitro experiments since the larger nature of the probe precludes cell penetrance. Nonetheless, this approach has been used repeatedly to identify the targets of many covalently acting natural products. Craig
Crews’ group has used this approach to gain invaluable insights into the mechanisms of action of multiple covalently acting natural products (Sin et al. 1997; Meng et al. 1999; Kwok et al. 2001). His group synthesized a biotinylated parthenolide analog and showed that this natural product derived from the feverfew plant exerts anti-inflammatory activity through targeting IKKβ and showed through mutagenesis studies that it reacts with cysteine C179 (Kwok et al. 2001). His team also discovered that epoxomicin (6), isolated from Actinomyces, covalently reacts with the LMP7, X, MECL1, and Z catalytic subunits of the proteasome using a biotinylated epoxomicin derivative (Sin et al. 1999; Meng et al. 1999). Subsequent studies by the Crews Laboratory showed that epoxomicin formed a morpholino ring...
between the amino-terminal threonine and the epoxomicin pharmacophore (Groll et al. 2000) and epoxomicin analogs were eventually developed into the multiple myeloma cancer drug carfilzomib or kyprolis (Kim and Crews 2013). Similarly, his group discovered that the antiangiogenic natural product fumagillin (7) was found to covalently react with methionine aminopeptidase, MetAP-2, using a biotinylated derivative of 7 (Sin et al. 1997). Through site-directed mutagenesis studies using a fluorescein-labeled fumagillin analog, Jun Liu’s group subsequently identified the site of covalent modification as His231 in MetAP-2 (Griffin et al. 1998). The anticancer natural product withaferin A, a steroidal lactone from the withanolide class of natural products found in Withania somnifera and other plant species, was shown to react with C328 of the intermediate filament protein vimentin using a biotinylated derivative of withaferin A (Bargagna-Mohan et al. 2007). Herbert Waldmann’s group also synthesized a biotinylated derivative of the tetramic acid natural product melophlin A and found that it targeted dynamin to modulate RAS signaling (Knoth et al. 2009).

Natural product probes bearing a biorthogonal alkyne handle can be used in complex proteomes, live cells, or in live animals, after which fluorescent or enrichment handles can be appended onto probe-labeled proteins for visualization or enrichment and identification of natural product targets. Cravatt et al., who have pioneered these types of chemoproteomic approaches, have utilized this approach successfully to map the on and off targets of countless pharmaceutical drugs, drug candidates, and tool compounds (Ahn et al. 2009, 2011; Bachovchin et al. 2011a; Lanning et al. 2014; Whitby et al. 2017; Niessen et al. 2017). In relation to natural products, Cravatt et al. synthesized a diverse alkyne-functionalized library of spiroepoxides, inspired by the many covalently acting natural products that bear
this scaffold including fumagillin and lumanicin D. They then screened this library for antiproliferative effects in breast cancer cells and identified a particular spiroepoxide MJE3 that covalently modified and inhibited the glycolytic enzyme PGAM1 (Evans et al. 2005). Edward Tate’s group has also made seminal contributions to chemoproteomic discoveries in natural product target identification. Their group showed, for example, that the reactive cyclic sesquiterpene zerumbone that has been shown to possess anti-inflammatory and anticancer activity reacts with numerous targets in cells involved in cell death, metabolism, cell cycle, and cytoskeletal pathways (Kalesh et al. 2015). Stephen Sieber’s group has also repeatedly used these approaches to successfully identify protein targets of covalently acting antibacterial natural products (Staub and Sieber 2008; Böttcher and Sieber 2010; Zeiler et al. 2011; Wirth et al. 2012; Nodwell et al. 2012; Battenberg et al. 2013; Wright and Sieber 2016). To highlight a few examples, the Sieber group functionalized multiple β-lactam antibiotics with alkyne handles for labeling diverse penicillin-binding proteins and subsequently introduced a series of new β-lactam probes that covalently labeled and inhibited a selection of additional bacterial protein targets, including the virulence-associated enzyme ClpP and resistance-associated β-lactamase (Staub and Sieber 2009). In another study, they functionalized a potent nucleoside antibiotic showdomycin with an alkyne handle and showed that it covalently binds to cysteines and inhibits multiple bacterial pathogenesis-associated enzymes, including MurA1 and MurA2 enzymes required for cell wall biosynthesis (Böttcher and Sieber 2010). They also defined multiple antibacterial targets of the dihydro-α-pyrone natural product rugulactone that bears two potentially cysteine-reactive Michael acceptors using an alkyne-functionalized rugulactone derivative, including covalent inhibition of 4-amino-5-hydroxymethyl-2-methylpyrimidine phosphate kinase (ThiD), involved in thiamine biosynthesis (Nodwell et al. 2012).

### 3.3 Activity-Based Protein Profiling (ABPP) for Identifying Proteome-Wide Druggable Hotspots Targeted by Covalently Acting Natural Products

The aforementioned approaches have been very successful at identifying the targets of covalently acting natural products. However, many natural products are incredibly complex to synthesize or may not necessarily have appropriate sites for derivatization. Furthermore, identifying the specific site that has been covalently modified within the target can be challenging and laborious and requires considerable mutagenesis experiments upfront to nail down the specific site of modification. Identifying the specific sites of modification of large reactive natural products by mass spectrometry may also be challenging if the specific modified adduct on the protein is unstable, semi-reversible, or does not ionize well for mass spectrometry detection.
A chemoproteomic platform pioneered by the Cravatt group that greatly facilitates the identification of specific druggable hotspots targeted by covalently acting natural products is ABPP, which uses reactivity-based chemical probes to profile proteome-wide reactive, functional, and ligandable hotspots directly in complex proteomes (Fig. 5b, c). ABPP utilizes active-site-directed probes to profile the activities of entire enzyme classes. ABPP has now evolved to utilize broader reactivity-based probes to map proteome-wide ligandable amino acid hotspots. Pioneered by Benjamin Cravatt, Eranthie Weerapana, and Chu Wang, this modern version of ABPP termed isotopic tandem orthogonal proteolysis–ABPP (isoTOP-ABPP) enables the specific and quantitative mapping of proteome-wide sites of probe modifications (Weerapana et al. 2010). Reactivity-based probes consist of three features which enable this approach: (1) chemical probes that consist of an electrophilic warhead that can react with nucleophilic hotspots on proteins; (2) an alkyne handle for CuAAC conjugation of an enrichment handle for probe-labeled proteins and peptides; and (3) an azide-functionalized TEV protease recognition peptide linker bearing an isotopically light or heavy valine and a biotin group which can be appended onto probe-labeled proteins for subsequent avidin enrichment of probe-labeled proteins, digestion, isolation of probe-labeled tryptic peptides, and TEV release of probe-labeled peptides for subsequent quantitative proteomic analyses comparing isotopically light-to-heavy peptide ratios of probe-modified tryptic peptides. These probes react not only with catalytic sites within enzymes, but also solvent-accessible binding pockets, post-translational modification sites, cysteine oxidation sites, protein–protein interaction sites, and other types of regulatory or functional domains across the proteome. When coupled with isoTOP-ABPP to map reactivity of specific sites of probe modification, this overall approach enables a global method for mapping protein functionality and, more importantly, facilitates the identification of ligandable hotspots within protein targets that may have previously been undruggable (Weerapana et al. 2010).

When used in a competitive manner (competitive isoTOP-ABPP) (Fig. 5c), covalently acting small molecules, such as electrophilic natural products, can be competed against the binding of their corresponding reactivity-based probes to rapidly identify not only the targets, but also the specific sites of modifications, of these molecules (Weerapana et al. 2008; Wang et al. 2014; Backus et al. 2016; Hacker et al. 2017a). This isoTOP-ABPP approach has also evolved to employ chemically or UV-cleavable isotopic linkers, SILAC, multiplexed isotopic labeling strategies, isotopic incorporation into reactivity-based probes, and caged reactivity-based probes for in-cell use (Adibekian et al. 2011; Qian et al. 2013; Abo and Weerapana 2015; Abegg et al. 2015; Abo et al. 2017; Tian et al. 2017). There are multiple advantages of this method compared to the direct incorporation of analytical handles, conjugation to solid supports, or thermal stability assays. First, because the method involves not just the enrichment of proteins labeled with the broad reactivity-based probes, but also further enrichment of the specific probe-modified peptides, the proteome-wide coverage of probe-labeled sites is substantial. Second, this method allows for the use of the parent covalent natural product directly in vitro, in situ, or in vivo and does not require synthesis of
derivatives which may result in altered biological activity. Third and most importantly, this approach enables the identification of not only the direct target of the reactive compound, but also the specific amino acid druggable hotspot that is targeted by the natural product. There are also drawbacks to this approach though. First, this method is most amenable for covalently acting compounds as these entities are competed against other covalently acting reactivity-based probes. Second, this approach does not encompass all types of natural product reactivities. The chemical biology field has developed versatile reactivity-based probes that are compatible with proteomic profiling of probe-modified peptides for many amino acids, such as cysteines, lysines, serines, and methionines (Liu et al. 1999; Weerapana et al. 2008, 2010; Adibekian et al. 2011; Shannon et al. 2014; Lin et al. 2017; Ward et al. 2017; Hacker et al. 2017a). While there are many chemoselective bioconjugation reactions that have been reported and are being discovered for other amino acids, it is unclear whether these probes will be compatible with ABPP methods (Shannon and Weerapana 2015; Mix et al. 2016; deGruyter et al. 2017; Martín-Gago et al. 2017). Thus, if the covalently acting natural product (e.g., epoxomicin, fumagillin) reacts outside of the cysteine, lysine, serine, and methionine functional space, this approach currently does not work. Also, currently existing reactivity-based probes do not likely functionalize all possible ligandable sites within the amino acid scope of the probe and thus may still miss natural product targets. Third, there is no direct enrichment of natural product targets, so low abundance targets of natural products may be missed.

Nonetheless, ABPP platforms have been successfully used to identify targets and druggable hotspots targeted by many covalently acting natural products. The isoTOP-ABPP platform (pioneered by the Cravatt, Weerapana, and Wang groups) has been used to map druggable hotspots targeted by a multitude of reactive metabolites, drugs, drug candidates, and tool compounds (Weerapana et al. 2010; Banerjee et al. 2013; Wang et al. 2014; Shannon et al. 2014; Lewallen et al. 2015; Blewett et al. 2016; Backus et al. 2016; Zhou et al. 2016b; Chen et al. 2017, 2018; Hacker et al. 2017a; Bar-Peled et al. 2017). Focusing on natural products, the Cravatt group has also used ABPP platforms and the serine hydrolase-directed fluorophosphonate activity-based probe to profile the targets of β-lactone natural product tetrahydrodilipstatin (THL) in mouse brain proteomes and showed that this compound inhibits multiple serine hydrolase enzymes, including Bat5, Abhd12, Pla2g7, and Tpp2 (Hoover et al. 2008). In another study, Porco et al. used ABPP platforms to discover that a novel rogaglate-derived β-lactone inhibits serine hydrolases ABHD10, ACOT1/2, CTSA, and SCPEP1 in prostate cancer cells (Laikiewicz et al. 2014). Adibekian et al. adapted isoTOP-ABPP platforms using a cysteine-reactive alkynyl benziodoxolone probe and a UV-cleavable linker bearing a biotin enrichment handle to discover the proteome-wide targets of curcumin, an anticancer diarylheptanoid natural product and showed that curcumin covalently modifies several key players of cell signaling and metabolism, including casein kinase I gamma (Abegg et al. 2015). Yang et al. used a similar multiplexed iTRAQ-ABPP strategy to profile the disparate proteome-wide cysteine reactivity of seven exocyclic methylene lactone-containing natural products (Tian et al. 2017).
A recent elegant collaborative study led by Michael Fischbach’s group showed that a family of non-ribosomal peptide synthetase gene clusters in gut bacteria encode for pyrazinone and dihydropyrazinone natural products. They showed in collaboration with the Cravatt group using isoTOP-ABPP platforms that the active form of these molecules is the initially released peptide aldehyde, which bears potent protease inhibitory activity and selectively targets the catalytic cysteines of a subset of human cathepsins (Guo et al. 2017). In another study, Nomura and colleagues used isoTOP-ABPP to map the proteome-wide cysteine reactivity of licochalcone A and found that this natural product impairs breast cancer cell viability through targeting C239 of prostaglandin reductase (Roberts et al. 2017).

3.4 Covalent Ligand Discovery Against Druggable Hotspots Targeted by Natural Products

The advantage of identifying the direct targets and druggable hotspots targeted by covalently acting anticancer natural products is that these targets can then be further pharmacologically interrogated with more synthetically accessible covalently acting chemical scaffolds for drug discovery efforts. Upon identifying the protein and druggable hotspot targeted by the covalently acting natural product that is responsible for its biological action, ABPP-based covalent ligand screening can potentially be used to pharmacologically interrogate this site in a target-based manner using moderate- to high-throughput screening approaches utilized by traditional target-based drug discovery efforts (Fig. 6a, b) (Bachovchin et al. 2009, 2010, 2011b). This method contrasts with having to perform medicinal chemistry efforts on natural product scaffolds that can be synthetically challenging, with readouts based on their bioactivity rather than affinity to specific protein targets. Cravatt and Backus showed the power of chemoproteomics-enabled covalent ligand discovery approaches in pharmacologically targeting classically undruggable or therapeutically intractable ligandable hotspots with cysteine-reactive covalent ligands. This work has been followed up by Cravatt, Weerapana, Nomura, and many others showing that cysteine- and lysine-reactive covalent ligands can be used to target classically intractable targets for potential therapy (Shannon et al. 2014; Bateman et al. 2017; Hacker et al. 2017a, b; Anderson et al. 2017; Grossman et al. 2017).

In Cravatt and Backus’s study, they identified covalent ligands against >700 cysteines found in both druggable and more intractable proteins, including transcription factors, adaptor/scaffolding proteins, and uncharacterized proteins. Among the many validated examples of covalent ligands targeting characterized and previously unrecognized functional cysteines, one particular unique example they demonstrated was a cysteine-reactive covalent ligand that targeted the catalytic cysteine C360 of caspase 8, only in its inactive zymogen form, but not its active form, wherein they used these compounds to distinguish extrinsic apoptosis pathways in human cell lines versus primary human T cells (Backus et al. 2016). In another study, Nomura and Bateman performed a phenotypic screen with
From this screen, they identified a cysteine-reactive covalent ligand that targeted C1101 of reticulon 4 (RTN4), a protein involved in endoplasmic reticulum (ER) membrane curvature and tubule formation. The authors showed that targeting this C1101 of RTN4 impaired ER tubular formation and nuclear morphology during cell division leading to impaired colorectal cancer pathogenicity (Bateman et al. 2017).

The feasibility of simpler covalent ligands that can react with druggable hotspots targeted by covalently acting natural products was shown by Nomura, Grossman, and Ward recently (Grossman et al. 2017). The authors used isoTOP-ABPP...
platforms to map the proteome-wide cysteine reactivity of withaferin A and found that the primary target in breast cancer cells was an activating C377 on the protein phosphatase 2A (PP2A) regulatory subunit PPP2R1A, which led to the activation of PP2A activity, dephosphorylation and inactivation of AKT signaling, and impaired breast cancer pathogenicity (Grossman et al. 2017). They performed a parallel phenotypic screen with a simpler cysteine-reactive library and identified a simpler chloroacetamide that targeted this same C377 on PPP2R1A to recapitulate many of the properties observed with withaferin A. Further medicinal chemistry efforts led to the generation of JNS 1-40, a chloroacetamide that was in vitro, in situ, and in vivo selective for C377 on PPP2R1A, which activated PP2A activity, inactivated AKT signaling, and impaired breast cancer tumorigenesis in vivo (Grossman et al. 2017). While the simpler covalent ligand identified in this study was fortuitously identified to modify the same site as withaferin A, this study shows that gel-based or fluorescence polarization-based moderate- to high-throughput competitive ABPP assays can likely be performed against natural product targets to rapidly screen for simpler ligands that bind to the same sites and recapitulate the actions of their more complex natural product counterparts (Fig. 6b).

4 Conclusions

In conclusion, there are countless reactive natural products that likely target unique druggable hotspots across a diversity of therapeutic targets. Moreover, many more are likely yet to be discovered, some of which may exploit completely novel reaction chemistry and mechanisms of action. The translational potential of these natural products into drugs has been hampered in part by difficulty of synthesis, isolation, mechanistic understanding, and speed of translation compared to target-based drug discovery efforts. At least with covalently acting natural products, chemoproteomic platforms are becoming increasingly powerful and robust to enable target identification in a reasonable time frame. Covalent ligand discovery against druggable hotspots targeted by reactive natural products using target-based ABPP screening strategies may help to overcome some of the other challenges faced with natural product translation by developing more synthetically tractable small molecule leads that are inspired, not necessarily by the natural product scaffold itself, but rather the target and druggable hotspot. While this review focused on covalently acting natural products and covalent ligand discovery, these strategies can also be applied to reversibly acting natural products and chemoproteomics-enabled fragment screening using photoaffinity-labeling strategies. Recent studies by the Cravatt group showed how proteome-wide ligandable hotspots can be massively expanded through the use of quantitative chemoproteomic platforms and “fully functionalized” fragment probes bearing a variable small-molecule fragment conjugated to a constant tag bearing an alkyne and photoactivatable diazirine group (Parker et al. 2017). These types of strategies may be coupled with traditional photoaffinity-labeling-based natural product target
identification approaches to discover more synthetically tractable compounds that target druggable sites targeted by reversibly acting natural products. The future of this research area will no doubt greatly benefit small-molecule drug discovery.

References


Target Identification of Bioactive Covalently Acting …


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