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Strategies for discovering and derisking covalent, irreversible enzyme inhibitors

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Abstract

This article presents several covalent inhibitors, including examples of successful drugs, as well as highly selective, irreversible inhibitors of emerging therapeutic targets, such as fatty acid amide hydolase. Covalent inhibitors have many desirable features, including increased biochemical efficiency of target disruption, less sensitivity toward pharmacokinetic parameters and increased duration of action that outlasts the pharmacokinetics of the compound. Safety concerns that must be mitigated include lack of specificity and the potential immunogenicity of protein–inhibitor adduct(s). Particular attention will be given to recent technologies, such as activity-based protein profiling, which allow one to define the proteome-wide selectivity patterns for covalent inhibitors *in vitro* and *in vivo*. For instance, any covalent inhibitor can, in principle, be modified with a 'clickable' tag to generate an activity probe that is almost indistinguishable from the original agent. These probes can be applied to any living system across a broad dose range to fully inventory their on and off targets. The substantial number of drugs on the market today that act by a covalent mechanism belies historical prejudices against the development of irreversibly acting therapeutic small molecules. Emerging proteomic technologies offer a means to systematically discriminate safe (selective) versus deleterious (nonselective) covalent inhibitors and thus should inspire their future design and development.

Brief history & examples of covalent inhibitors

The design of selective covalent inhibitors is conceptually very attractive but in practice hard to achieve. That is because it is difficult to strike the right balance between reactivity and selectivity. In many cases, a highly electrophilic species (e.g., α-halo ketone, α,β-unsaturated ketone, fluorophosphonate (FP) or cyanamide) needs to be incorporated into the inhibitor to achieve covalent modification of a protein target [1]. Alkylation of other macromolecules can take place *in vivo,* leading to deleterious effects, or the reactive species may be scavenged by ubiquitous low-molecular-weight nucleophiles such as glutathione.

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Indeed many researchers avoid covalent inhibitors owing to the potential toxicity associated with the protein adduct(s), especially if the covalent modification is not selective. However, in cases where selectivity can be achieved and mechanism-based toxicity is not a concern, the increased biochemical efficiency associated with an irreversible mechanism can actually lead to heightened therapeutic margins, as lower drug concentrations are required for efficacy [2,3]. As a testament to the validity of this strategy, there are several examples of successful drugs incorporating tempered or masked electrophiles leading to covalent modification of their protein target (Figures 1 & 2). In fact, of the 74 enzymes that are inhibited by marketed drugs, 19 are irreversibly inhibited via covalent modification [4,5]. While this article will focus on covalent irreversible inhibitors, it should be noted that another important nonequilibrium binding mechanism involves slow dissociation binding kinetics, which leads to pseudo-irreversible or insurmountable inhibition. This mechanism is important to the drug action of the angiotensin II receptor antagonist candesartan, the muscarinic M3 receptor antagonist tiotropium, the histamine H1 receptor antagonist desloratadine, the CCR5 antagonist maraviroc and the HIV-1 protease inhibitor darunavir [2,3,6–8].

In the 1970s considerable effort was put into the design of mechanism-based enzyme inactivators or suicide substrates as an approach to develop highly selective enzyme inhibitors as drugs [9–11]. This approach avoids the direct use of a highly reactive species that can indiscriminately react with various macromolecules and instead aims to start with a relatively innocuous substrate analog, which is activated by the target enzyme to generate an electrophilic species that is attacked by a nucleophile in the active site, leading to irreversible inhibition of the enzyme. This approach is very challenging and some of the most notable successes were not originally designed as irreversible inhibitors; rather, their mechanism of action was discovered serendipitously. For example, omeprazole is a prodrug that covalently modifies gastric H^+/K^+-ATPase, the enzyme responsible for proton transport as the final step in gastric acid secretion [12]. It is converted under acidic conditions in the stomach to a tetracyclic sulfenamide intermediate that binds covalently to cysteine residues of the H^+/K^+-ATPase to form disulfide adduct(s) (Figure 1A) [13–15]. Clopidogrel is a prodrug that covalently binds to the adenosine 5′-diphosphate receptor $P2Y_{12}$ resulting in irreversible inhibition of platelet aggregation [16]. It undergoes hepatic metabolism to an active metabolite (Act-Met) containing a free thiol, which forms a covalent disulfide adduct with a cysteine of $P2Y_{12}$ (Figure 1B) [17–19].

There are several examples of covalent inhibitors that are successful drugs, and representative examples are shown in Figure 2 [2,3,6–8]. These examples should encourage medicinal chemists to consider this strategy when the biochemical mechanism supports such an approach. Aspirin is a NSAID that irreversibly acetylates an active site serine residue of the cyclooxygenases COX-1 (Ser-529) and COX-2 (Ser-516) (Figure 2) [20,21]. The covalent adduct results in a distortion of the arachidonic acid docking site, thereby blocking the approach of the substrate to the active site and leading to inhibition of COX-1 and COX-2 [22]. Tetrahydrolipstatin is a semisynthetic derivative of lipstatin that inhibits fat absorption [23]. It is a covalent inhibitor of gastric and pancreatic lipases, resulting from β-lactone reaction with the serine nucleophiles of the lipases to form stable ester bonds [24]. β-lactam antibiotics acylate the active site serine of penicillin-binding proteins (PBPs) and kill bacteria by inhibiting the final step of cell wall biosynthesis [25,26]. Class A and B PBPs are transpeptidases that catalyze the formation of peptide crosslinks between adjacent peptidoglycan strands and class C PBPs are D-Ala carboxypeptidases that may modulate the degree of crosslinking by removing the terminal D-Ala of the peptide. Bacteria acquire resistance to β-lactam antibiotics by producing highly mutated PBPs or by producing β-lactamases that catalyze the hydrolysis of the β-lactam ring, preventing their interaction with PBPs [27–29]. Clavulanate is a naturally occurring β-lactam [30] that forms a kinetically

stable acyl-enzyme intermediate and inactivates β-lactamase [31,32]. Nucleophilic attack by the active-site Ser-70 opens the β-lactam ring of clavulanate and the resulting oxazolidine ring opens to generate an imine adduct that is rapidly decarboxylated, revealing a covalently bound trans-α,β-eneamine adduct [32]. Therefore, clavulanate is used in combination with approved β-lactam antibiotics to overcome resistance in bacteria that secrete β-lactamase.

Rivastigmine is a carbamate inhibitor of the serine hydrolase acetylcholinesterase, the principal enzyme that degrades acetylcholine at cholinergic synapses and is used for the symptomatic treatment of Alzheimer's disease. The crystal structure of rivastigmine with Torpedo acetylcholinesterase revealed that the catalytic serine nucleophile (Ser-200) was carbamylated, with the phenol leaving group being retained in the active site [33]. The decarbamylation of the adduct was found to be unusually slow, which may be explained by a movement of His-440 of the catalytic triad, such that nucleophilic attack of a water molecule is not permitted.

Neratinib is an irreversible inhibitor of the human EGF receptor tyrosine kinases EGFR and HER-2 and is in Phase III clinical trials for breast cancer [34–36]. It contains a 4-(dimethylamino)-crotonamide Michael acceptor that forms a covalent bond with a conserved cysteine residue, Cys-773 in EGFR and Cys-805 in HER-2. It is proposed that the Michael addition of the cysteine is accelerated owing to intramolecular general base catalysis by the dimethylamino group. Importantly, neratinib retains activity against tumors that have developed resistance to the noncovalent EGFR inhibitors gefitinib or erlotinib [35,37]. For instance, the T790M mutation in EGFR causes resistance to gefitinib. It has been shown that the T790M mutants retain low nanomolar affinity for gefitinib, but have an affinity for ATP that is increased by more than an order of magnitude [38]. It is postulated that this increase in ATP-binding affinity is the reason the mutants become resistant to reversible binding drugs such as gefitinib, but not to neratinib, since irreversible inhibitors are noncompetitive with ATP.

Despite these successful examples, there still appears to be reluctance toward developing irreversible inhibitors in the pharmaceutical industry. This largely stems from the perception that covalent drugs lack selectivity and the resulting protein adducts lead to toxicity. Until recently, no method existed to closely monitor the selectivity of covalent drugs or differentiate which protein adducts cause toxic effects. However, modern **chemical proteomics** has begun to provide technologies to experimentally address these concerns in relevant model systems [39]. For example, as will be discussed later, covalent inhibitors can be readily modified with clickable tags resulting in activity-based probes that are almost indistinguishable from the original agent. These probes can be applied to any living system across a broad dose range to fully inventory their on and off targets.

Potential advantages of covalent inhibitors

In recent years, the pursuit of covalent inhibitors as a medicinal chemistry strategy [40,41] has been reinvigorated by an increased emphasis on the biochemical mechanism and efficiency of drug action required for success [2], importance of residence time [6,8,42] and the advent of techniques to probe the selectivity of covalent binders [43,44].

Selective covalent binding of a drug candidate to the desired target can be beneficial owing to the increased biochemical efficiency associated with the nonequilibrium-binding mechanism (Box 1). The nonequilibrium binding of irreversible inhibitors limits the competition with high endogenous ligand concentrations, allowing the desired pharmacological effect to be achieved at lower drug concentrations/doses. In fact, 80% of marketed drugs have to compete with an endogenous ligand [7], therefore perhaps it is not

surprising that approximately 30% of marketed drugs that act on an enzyme target are irreversible [4].

Box 1

Pros and cons of covalent inhibitors

Pros

- Increased biochemical efficiency [2,3]:
  - Nonequilibrium binding limits the competition with high endogenous substrate/ligand concentrations.
- Less sensitive to pharmacokinetic parameters (i.e., clearance and protein binding).
- Potential longer duration of action dependent on the synthesis of new enzyme:
  - Pharmacodynamic effect outlasts pharmacokinetics of inhibitor;
  - Long residence time [6];
  - Less frequent dosing.
- Most efficient strategy when complete inactivation of target is required.
- Potential for improved therapeutic index assuming no mechanism-based toxicity:
  - Lower drug concentrations required for efficacy (reduced dosages);
  - If the drug inactivates the target and is eliminated quickly, off-target toxicities and drug–drug interactions will decrease.
- Potential to avoid some resistance mechanisms [37,38].

Cons

- Potential immunogenicity of protein adduct leading to an allergic response or drug hypersensitivity reaction (idiosyncratic) [45–48].
- Higher risk if covalent inhibitor lacks specificity (nonspecific covalent binding should be avoided).
- Not optimal for targets when the mechanism of action requires short residence time, transient inhibition or partial inhibition [49,50].

Furthermore, less than desirable PK properties can often be tolerated as the pharmacodynamic action of covalent inhibitors usually outlasts measurable plasma drug levels. Once covalently inactivated, the target is neutralized and the activity can only be recovered by synthesis of new protein. Therefore, as long as resynthesis of the protein is not too fast, only enough drug exposure to inactivate the target is necessary and sustained systemic exposure of the drug (long half-life) may not be required. If the drug inactivates the target and is eliminated from the circulation quickly, the potential for off-target toxicities and drug–drug interactions will decrease, which can lead to increased therapeutic margins.

There are several instances where the use of irreversible inhibitors is particularly advantageous. For example, when the biochemical mechanism of target inhibition involves buildup of substrate, covalent inhibitors are attractive because they prevent the achievement of mass-action equilibrium between the inhibitor and substrate.

Covalent inhibitors may also be preferred when complete and sustained target inactivation is required. The target activity can only be recovered by synthesis of new protein.

Potential disadvantages of covalent inhibitors

Much of the negative connotations surrounding covalent protein adducts can be traced to literature on drug candidates that undergo bioactivation to form a chemically reactive metabolite, which can covalently bind to target proteins [45–59]. Protein covalent binding emerged as a mechanism of drug toxicity in the early 1970s and many marketed drugs that have been associated with idiosyncratic adverse drug reactions (ADRs) are known to form reactive metabolites that are capable of covalently modifying proteins [52–54]. Immune-mediated ADRs are thought to be caused by an abnormal immune reaction triggered by an **immunogenic** drug–protein adduct [46–48]. According to the hapten hypothesis, drugs are too small to stimulate an immune response; however, the drug or metabolite can act as a hapten upon covalent binding to a protein. These drug–protein adducts provide **antigenic** determinants for the immune response, but it remains unclear what factors determine individual susceptibility for immunological tolerance versus immune reaction [45,55]. In one of the early examples, it was discovered that acetaminophen can be metabolized to a reactive metabolite, which covalently binds to microsomal proteins that can cause hepatotoxicity [56]. Although a clear causal relationship between covalent binding and toxicity is lacking, most pharmaceutical companies have instituted screens during lead optimization to weed out compounds that form reactive metabolites in efforts to decrease attrition [52,53]. This is logical, since idiosyncratic ADRs cannot be predicted from preclinical toxicology assessments and we have a limited understanding of which protein adducts are immunogenic and which are not. For covalent inhibitors that target an intracellular or membrane-bound protein, one could speculate that the resulting protein adducts would have a lower risk of immunogenicity compared with an extracellular protein adduct (extracellular antigen), which would probably have more effective antigen presentation to major histocompatibility complex and/or T-cell receptors, as well as be more likely to produce an antibody response. More work will be necessary to determine whether the localization of the protein could be a factor in differentiating a toxic drug–protein adduct from a nontoxic one [57]. Chemical biology tools and analytical technologies now exist such that the relationship between specific protein adducts and toxicity can begin to be systematically characterized [44].

It is interesting to note that idiosyncratic ADRS (IADRs) are more frequently associated with compounds used at high daily doses. Therefore, the risk of IADRs from reactive metabolites can be mitigated if a low dose of the drug can be used. In fact, there are no examples of drugs that are dosed below 10 mg/day that cause IADRs [58,59]. In an analogous fashion, it could be inferred that the risk associated with developing a covalent inhibitor could be minimized if the dose was less than 10 mg/day. Fortunately, as pointed out in the previous section, covalent inhibitors often have increased biochemical efficiency and are less sensitive to pharmacokinetic parameters, which together, favor efficacy at lower doses.

In many cases, the potential benefits of a covalent inhibitor could outweigh the potential risk inherent in forming a protein adduct and this risk could in fact be minimal if a low dose (<10 mg/day) is achievable. However, covalent binding may not be optimal for targets when the mechanism of action requires short residence time, transient inhibition or partial inhibition [49,50]. For example, memantine is a NMDA receptor antagonist that has weak binding and short residence time [49]. These properties are desirable to achieve a clinically tolerated antagonist for this mechanism, because it is necessary to block excessive activation of the NMDA receptor, while leaving normal function relatively intact to avoid side effects. In this

case, an irreversible inhibitor would not be appropriate because excessive blockade of the NMDA receptor leads to clinically unacceptable side effects. Another example where an irreversible inhibitor with long residence time might lead to mechanism-based toxicity would be for treatments aimed at use-dependent ion channels [3,42]. In this case it is desirable to design compounds that block the channel in the open state and rapidly dissociate from the channel in the resting state. Lastly, some G-protein-coupled receptors that are subject to internalization may not be ideal candidates for covalent drugs [41,60].

Activity-based protein profiling to characterize the selectivity of covalent inhibitors

Activity-based protein profiling (ABPP) has emerged as a powerful chemoproteomic tool to characterize the selectivity of enzyme inhibitors on a global scale [43,61,62]. ABPP is a chemical strategy that utilizes active site-directed covalent probes to profile the functional state of enzymes in complex proteomes. **Activity-based probes** (ABPs) contain a reactive group to covalently modify the active site of enzymes in proteomes and a reporter group (typically a rhodamine or biotin) for detection and identification of protein targets (Figure 3A) [63]. Gel-based ABPP technologies enable visualization of labeling events using SDS-PAGE separation followed by either in-gel fluorescence (fluorescent reporter tag) or avidin blotting (biotin reporter tag). MS platforms, such as ABPP-MudPIT, enable the enrichment and identification of probe-labeled proteins from a complex proteome [64–66]. ABPs **(1–12)** have been developed for a number of enzyme classes [62], including serine hydrolases [67–69], cysteine proteases [70–72], serine/threonine [73] and tyrosine phosphatases [74], glycosidases [75,76], ubiquitin-conjugating/-hydrolyzing enzymes [77–79], proteasomes [80], oxidoreductases [81,82], ATP-binding enzymes (e.g., kinases) [83–85] and cytochrome P450s (Figure 4) [86,87].

Many potential targets do not possess a nucleophilic active-site residue (Ser, Cys or Lys) for covalent labeling by electrophilic ABPs. A possible solution to this limitation is to incorporate a photoaffinity group into an inhibitor scaffold to create a covalent adduct with the target upon exposure to UV light. This strategy has been successfully employed to create photoreactive ABPP probes **(13–19)** for metalloproteases [88–91], histone deacetylases [92,93], aspartyl proteases [94–96], Abl kinase [97] and the nicotinic acetylcholine receptor (Figure 5) [98].

Competitive ABPP

A competitive ABPP platform can be employed to identify protein targets and assess the selectivity of an enzyme inhibitor in native biological systems by measuring the ability of an inhibitor to slow the rate of reaction of the enzyme with a particular ABP [99–101]. Briefly, inhibitor-treated total tissue or cell extracts are subject to profiling with a relevant ABP, and IC_{50} values for inhibitor targets can be measured as a decrease in enzyme labeling by the ABP (Figure 3B). Competitive ABPP assays can be performed in complex proteome mixtures, enabling the simultaneous evaluation of inhibitor potency and selectivity within a relevant native proteome.

Click chemistry-ABPP

Original protocols for ABPP required the homogenization of cells and tissues prior to treatment with the ABPs, with the drawback of removing proteins from their native environment and disrupting specific activities. This limitation was circumvented by integration of **click chemistry** (CC) [102,103] and ABPP, resulting in the creation of smaller, more versatile probes using an alkyne or azide group as a latent reporter tag [104–107]. Replacing bulky reporter tags with alkyne groups enables the probe-labeling step to

occur *in vivo* within live cells and organisms. CC is applied to append an azide-functionalized reporter tag to the labeled proteins after cell lysis and homogenization (Figure 3c).

An important application of CC-ABPP is the evaluation of target selectivity of covalent inhibitors in whole cells and animals [108,109]. In many cases, an alkyne can be incorporated into a covalent inhibitor with minimal disruption to the cell permeability and binding interactions of the parent small molecule. The alkyne analog of the covalent inhibitor can be administered to mice, the tissue of interest can be harvested and subjected to CC conjugation with the desired reporter tag for identification of the target proteins. Several examples that utilize CC-ABPP to identify protein targets and compound selectivity will be discussed in the following sections.

Highly selective covalent inhibitors for emerging therapeutic targets: fatty acid amide hydrolase as a case study

Fatty acid amide hydrolase (FAAH) is an integral membrane enzyme that degrades the fatty acid amide family of signaling lipids, including the endocannabinoid anandamide (AEA) [110,111]. Genetic or pharmacological inactivation of FAAH leads to analgesic and anti-inflammatory phenotypes in rodents without showing the undesirable side effects observed with direct cannabinoid receptor agonists. Selective pharmacological blockade of FAAH elevates the levels and prolongs the effects of anandamide (and other FAAs) only when and where it is synthesized and released on demand [112,113]. Therefore, there is much interest in developing selective FAAH inhibitors as a strategy to discern the endogenous functions of AEA-mediated endocannabinoid pathways and FAAH may represent an attractive therapeutic target for the treatment of inflammatory pain [114]. Several classes of FAAH inhibitors have been reported, including reversibly (e.g., tri-fluoromethyl ketones and α-ketoheterocycles; **20**) and irreversibly (e.g., FPs; **21**, carbamates; **22** and ureas; **23–27**) acting agents (Figure 6) [114,115]. Reversible inhibitors, such as the α-ketoheterocycle OL-135 **(20)**, have been found to display good *in vitro* potency and selectivity for FAAH relative to other serine hydrolases in mammalian proteomes [99,116,117], but produce only transient elevations in AEA *in vivo* [118]. The submaximal efficacy of reversible FAAH inhibitors may be due to their rapid metabolism, as well as the fact that near complete (>85%) blockade of FAAH activity is required to maintain elevated AEA levels *in vivo* [119]. For targets such as FAAH where inhibition leads to elevated levels of substrates, a further potential drawback of reversible inhibitors is that their efficiency and potency can be diminished by mass-action competition with endogenous substrates [2]. Irreversible inhibitors overcome this problem, but selectivity remains an important issue. Considering that FAAH is a serine hydrolase and that there are at least 200 members of this enzyme class in the human proteome, assessing and optimizing inhibitor selectivity represent major challenges. To help address this issue, ABPs against the serine hydrolase class of enzymes have been developed by linking a reactive FP group to a fluorophore or biotin reporter tag [67–69] and these probes have been utilized to profile the proteomic selectivity of FAAH inhibitors [99–101,109,120]. In the absence of an inhibitor, the FP probe labels all the serine hydrolases in the proteome. Serine hydrolases that show significant reductions in probe labeling intensity in the presence of inhibitor are scored as targets of the compound.

Ureas as selective covalent FAAH inhibitors

Recently, we [100,109,120] and others [121,122] have reported piperazine/piperidine aryl ureas as an emerging class of FAAH inhibitors. In 2007, we reported that the quinoline piperidine urea PF-750 **(26)** inhibited FAAH in a time-dependent manner (IC_{50}= 52 nM with 30 min preincubation) by covalently modifying the enzyme active site serine

nucleophile [100]. PF-750 was confirmed to be covalently attached to the Ser-241 of FAAH through a carbamate linkage by the PF-750-h/rFAAH crystal structure [123]. The irreversible covalent inhibition by PF-750 was rather surprising considering the stability of the urea functional group. Despite the covalent mechanism, PF-750 selectively inhibited FAAH relative to other mammalian serine hydrolases *in vitro* as determined by competitive ABPP (Figure 3B) [100]. Similarly, no off targets were observed for the benzothiophene piperazine urea PF-465 **(25)** [120]. By contrast, multiple serine hydrolase off-targets were observed for URB-597 **(22)** and OL-13 **(20)**, particularly amongst FP-labeled proteins migrating between 55 and 65 kDa [99–101,120]. To confirm that the different selectivity profiles of FAAH inhibitors determined *in vitro* were also observed *in vivo*, mice were treated with PF-750 or URB-597 for 1 h, then sacrificed and tissue was removed for competitive ABPP analysis with FP-rhodamine **(21)** [100]. Serine hydrolase targets of PF-750 and URB-597 were detected by SDS-PAGE and in-gel fluorescence scanning. At each dose tested, both URB-597 and PF-750 selectively targeted FAAH in the brain. PF-750 showed no detectable off-target activity in peripheral tissues (e.g., liver) either, however, URB-597 was found to block FP labeling of several liver serine hydrolases between the molecular masses of 55 and 65 kDa. A proposed mechanism that could explain this exquisite selectivity is a specific binding-induced activation of the urea in the FAAH active site, which renders the reactivity of urea similar to an amide.

Elucidation of the irreversible mode of action of the piperidine/piperazine ureas prompted us to modify the FAAH assay so that inhibitor potencies could be measured as $\mathbf{k_{inact}/K_i}$ values. Unlike IC_{50} values, k_{inact}/K_i values do not change with various preincubation times and have been described as the best measure of potencies for irreversible inhibitors [51]. Using this measure, PF-750 **(26)** was determined to have a moderate potency (k_{inact}/K_i = 791 $M^{-1}s^{-1}$) for FAAH. More recently, a series of biaryl ether urea analogs with improved potency has been reported [109]. PF-3845 **(27)** was the most potent inhibitor (k_{inact}/K_i = 14,310 $M^{-1}s^{-1}$) reported. Structural studies support that PF-3845 gains its potency from a more extended set of van der Waals interactions between the biaryl ether piperidine moiety and the hydrophobic acyl chain-binding pocket of FAAH based on a crystal structure of a PF-3845-h/rFAAH complex.

In vivo selectivity of the urea PF-3845yne & carbamate JP104 using CC-ABPP

Following confirmation that the carbamate URB597 **(22)** and the urea PF-3845 **(27)** covalently modified the serine nucleophile of FAAH, the alkynyl analogs JP104 **(28)** and PF-3845yne **(29)** were synthesized and their protein targets were directly analyzed *in vivo* by CC-ABPP [108,109]. Administration of these probes to $FAAH^{+/+}$ and $FAAH^{-/-}$ mice, followed by tissue homogenization and conjugation of a rhodamine reporter tag to probe-labeled proteins by CC, revealed their proteome-wide *in vivo* target selectivity (Figure 3c). The carbamate JP104 was selective for FAAH in the nervous system, but labeled several additional enzymes in peripheral tissues, including multiple carboxyesterases, whereas the urea PF-3845yne was completely selective for FAAH in both the nervous system and peripheral tissue. PF3845yne and JP104 selectively reacted with a single protein in mouse brain that was confirmed as FAAH based on its absence in $FAAH^{-/-}$ mice. In liver, however, PF3845yne and JP104 showed strikingly different profiles, with the former agent once again showing selective reactivity with FAAH and the latter inhibitor labeling a number of proteins that were found in both $FAAH^{+/+}$ and $FAAH^{-/-}$ mice.

Further examples of selective, covalent inhibitors emerging from chemoproteomic endeavors

Carbamate inhibitors for the serine hydrolase family

Selective and potent covalent inhibitors for several members of the serine hydrolase family have recently emerged. These inhibitors are based on a carbamate scaffold that results in irreversible carbamylation of the active site serine nucleophile. The carbamate chemotype has emerged as a privileged scaffold for potent serine hydrolase inhibitors owing to its tempered electrophilicity and hydrolytic stability following carbamylation. The FP probe can be applied in a competitive ABPP platform to screen carbamate libraries against large numbers of serine hydrolases [124], circumventing the need for protein purification and substrate assays. These chemoproteomic endeavors have resulted in the development of inhibitors for both annotated and unannotated members of this enzyme family.

Recently, competitive ABPP screening of a carbamate library led to the development of a selective pharmacological agent against monoacylglycerol lipase (MAGL) [125,126]. MAGL is thought to be the primary enzyme responsible for hydrolyzing the endocannabinoid 2-arachidonoylglycerol. Using competitive ABPP, a potent and selective covalent inhibitor for MAGL, JZL184 **(30)**, was obtained (Figure 7). JZL184 is based on a piperidine–carbamate scaffold and demonstrates high *in vivo* potency resulting in near-complete blockage of MAGL activity at 4 mg/kg with minimal effects on other brain serine hydrolases, including FAAH. The structural similarity between the piperidine-carbamate MAGL inhibitors (i.e., **30**) and piperazine/piperidine–urea FAAH inhibitors **(24–27)** also inspired the use of competitive ABPP to develop dual FAAH–MAGL inhibitors such as JZL195 **(31)** [127]. The development of highly selective as well as polypharmacological probes, such as JZL184/195, provides researchers with valuable tools to dissect the roles of the endocannabinoids in a variety of biological systems.

Similar competitive ABPP methods were used to identify a carbamate inhibitor of the uncharacterized serine hydrolase, α/β-hydrolase 6 [124]. This inhibitor, WWL70 **(32)**, exhibited an IC_{50} value of 70 nM in brain membranes and was demonstrated to be highly selective for ABHD6 relative to 27 other serine hydrolase activities present in these proteomes. The development of potent covalent inhibitors of uncharacterized enzymes, facilitated by ABPP, generates valuable tools for annotating novel enzyme function.

Epoxide & vinyl-sulfone inhibitors for the cysteine protease family

One of the earliest applications of ABPP to inhibitor discovery was the use of cysteine protease probes to identify a selective inhibitor of cathepsin B. The cysteine protease-selective probe, DCG-04 **(2)**, was used in a competitive ABPP strategy to monitor the potency and selectivity of a library of epoxy-succinyl small molecules in rat liver extracts [128]. This study identified a selective covalent inhibitor **(33)** of cathepsin B, a protease that is implicated in tumor invasion. The synthesis of more elaborate epoxy-succinyl libraries introduced binding groups on either side of the epoxide and resulted in the discovery of covalent inhibitors for other papain fold cysteine proteases [129,130]. Detailed *in vivo* studies in mice, facilitated by ABPP tools, demonstrated that these compounds show overall rapid clearance in serum, which circumvented problems of nonspecificity induced by compound accumulation in tissues of interest.

Activity-based protein profiling and covalent inhibitors have also played a vital role in the identification of protein activities critical for the invasion and rupture of eukaryotic cells by the malaria parasite *Plasmodium falciparum*. A library of chloroisocoumarins and peptide vinyl sulfones were screened to identify compounds that block the release of the parasite

from host red blood cells [131]. These studies identified a compound JCP410 **(34)**, which contained a vinyl sulfone known to covalently modify cysteine nucleophiles on cysteine proteases. The protein target of JCP410 was identified as dipeptidyl peptidase 3 and a competitive ABPP platform using the broad spectrum cysteine protease probe, DCG-04 **(2)**, confirmed the target of the compound and additionally identified cross-reactivity with several members of the related falcipain family of proteases.

β-lactones & β-lactams as antibiotics

In addition to competitive ABPP platforms, CC-ABPP has found a similar niche in the area of small-molecule target discovery. CC-ABPP relies on alkyne functionalization of covalent inhibitors with minimal disruption to structure, binding affinity and cell permeability. Alkyne-functionalized inhibitors are administered to live cells or organisms and CC is used to tag inhibitor-modified proteins with a reporter group after cell lysis and homogenization (Figure 3c). A study by Sieber *et al.* highlights the use of click chemistry for protein target identification of covalent inhibitors [132,133]. Well-established antibiotics such as cephalosporin, ampicillin **(35)** and aztreonam are β-lactams that covalently modify their protein targets. Alkyne-functionalized versions of these antibiotics, for example AmpN **(36)**, were synthesized and their protein targets were investigated using CC-ABPP methods. These compounds were shown to target a diverse number of PBPs both *in vitro* and *in vivo*. These tools enabled the investigation of the protein targets of these common antibiotics in a variety of bacterial strains at different concentrations. Similar studies were carried out using a library of alkyne-functionalized β-lactones [134]. These studies identified selective inhibitors (e.g., **37**) for the bacterial caseinolytic protein protease, a serine protease that is crucial for virulence of many bacterial pathogens [135]. CC-ABPP facilitates the discovery of protein targets of bioactive small molecules and provides a tool to study potency and selectivity of these molecules in a variety of biological systems.

Inhibitors for protein kinase subfamilies

p90 ribosomal protein S6 kinases (RSKs) are members of the serine/threonine protein kinase family. Although ATP binding sites on protein kinases are highly conserved, the RSK family contains a threonine and a cysteine residue that act as selectivity filters to distinguish the RSK ATP binding sites from other kinases. With this information in hand, a fluoromethylketone inhibitor, fmk **(38)**, was developed that potently inactivates RSK1 and RSK2 in mammalian cells [83]. In order to assess the selectivity of **38**, the alkyne-functionalized variant fmk-pa **(39)** was synthesized [84]. Unlike the fluorophore or biotin tagged analogs of **38**, the alkyne-variant **39** demonstrated high cellular potency and, using CC, it was demonstrated that **39** achieves selective and saturable modification of endogenous RSK1 and RSK2 in mammalian cells. Furthermore, the fluorescent covalent probe **40** has been developed based on the irreversible EGFR inhibitor PD 168393 **(41)**. This probe was used to show that there is a linear correlation between inhibition of EGFR kinase activity and inhibition of downstream cellular signaling events [136].

These examples highlight the utility of competitive ABPP platforms and CC-ABPP to identify novel covalent inhibitors, as well as to assess the selectivity of covalent inhibitors in complex proteomes *in vitro* and *in vivo*. As the repertoire of available ABPs expands to novel enzyme families, these chemical proteomic technologies will facilitate the development of highly selective covalent inhibitors for as yet untargeted proteins.

Future perspective

Almost 30% of the marketed drugs whose molecular targets are enzymes act by irreversible inhibition [4]. This high percentage is rather surprising considering the strong historical bias

against developing irreversible inhibitors as clinical candidates in the pharmaceutical industry. One of the main rationales for this bias is derived from the high inherent reactivity of functional groups generally associated with covalent modifications of proteins. Excessively reactive covalent modifiers can form covalent bonds with a large number of enzymes/proteins, often within (or even extending beyond) the same mechanistic class. Compounding these concerns, there has historically been no direct way to evaluate the selectivity of covalent inhibitors against a large number of proteins in native biological systems. Traditional approaches for testing selectivity have involved setting up individual substrate-based assays with a limited number of candidate 'off-target' enzymes. However, this approach excludes the analysis of uncharacterized enzymes, owing to the lack of substrate-based assays. Moreover, it does not take into account the often unpredictable relationship between compound efficacy and selectivity, which is affected by many variables, including target (and off-target) location, concentration and PK properties of the inhibitor and, therefore, must be empirically established *in vivo*. Functional proteomic methods, such as ABPP, have recently emerged that enable the selectivity of inhibitors to be evaluated against numerous enzymes in parallel directly in native cells and tissues. ABPP can also be combined with CC to create probes capable of fully surveying the direct targets of covalent inhibitors in living systems [104,105,108,109]. As discussed previously, these studies have already revealed covalent inhibitors that display a surprisingly high level of selectivity in the proteome [84,100,109,120,125].

This perspective is not advocating the broad application of covalent inhibitors for all types of targets. Rather, we argue that one should not rule out this approach, especially for enzyme targets where complete inactivation of the target is both desired and tolerated (i.e., no mechanism-based toxicity). Furthermore, the risk of developing a covalent inhibitor should be minimized if the compound is selective for the desired target. Selectivity can be achieved in several ways:

- Activation of a compound toward nucleophilic attack only within the target active site as proposed for the urea FAAH inhibitors [100,109,120] and β-lactam antibiotics [137];
- Selective distribution of a 'reactive' compound to the target tissue of interest, as is the case with orlistat, where the action of the drug is localized to the lumen with negligible concentrations in the plasma;
- Mechanism-based unveiling of a reactive group leading to covalent modification at the target site of action, as is the case with omeprazole;
- Combination of a tempered electrophile with a selective noncovalent binder.

The last approach sounds very attractive but in practice is quite challenging. Chemical biology methodology such as CC-ABPP should help advance this approach and successful examples have already emerged in the literature [84,109]. We must learn from our past successes and failures and, at the same time, be opportunistic in our application of new technologies to help guide the design of safe and efficacious drugs, whether they be covalent or noncovalent in mechanism.

Executive summary

- Selective covalent binding of a drug candidate to the desired target can be beneficial owing to the increased biochemical efficiency associated with the nonequilibrium-binding mechanism.
- Beyond their potential use as drugs, selective covalent inhibitors represent highly versatile pharmacological tools for assessing protein function *in vivo*.

- Chemical proteomic technologies, such as activity-based protein profiling, allow one to define the proteome-wide selectivity patterns for covalent inhibitors *in vitro* and *in vivo.*
- Covalent inhibitors can be readily modified with clickable tags resulting in activity-based probes that can be applied to any living system across a broad dose range to inventory their on and off targets.
- Ongoing clinical studies with an emerging cadre of highly selective covalent inhibitors should further clarify their therapeutic utility and possible risks.

Key term

Chemical proteomics	Involves the use of chemical tools for identifying small molecule protein interactions in complex biological systems and is generally based on activity-based protein profiling using covalent activity-based probes or compound-immobilized affinity chromatography
Immunogen	Molecule that can initiate an immune response. All immunogens are antigens, but not all antigens are immunogens
Antigen	Substance that can be bound by an antibody or surface receptor on T cells
Activity-based protein profiling	Applies chemical probes to profile the functional state of enzymes in complex proteomes. An activity-based probe can distinguish active enzyme from inactive zymogen or inhibitor-bound forms
Activity-based probe	Typical activity-based probes comprise a reactive group to covalently modify the active site of a particular enzyme class and a reporter group for detection and isolation of probe-labeled proteins
Click chemistry	Bioorthogonal reaction that applies the copper catalyzed, stepwise version of Huisgen's 1,3-dipolar cycloaddition reaction to form a stable triazole linkage between an azide and alkyne
k_{inact}/K_i	When characterizing covalent inhibitors, it is important to take both the equilibrium binding (K_i) and the rate of covalent bond formation (k_{inact}) into account. The best measure of inhibitory potency for an irreversible inhibitor is the second order rate constant obtained from the ratio k_{inact}/K_i. Unlike IC_{50}values, the ratio of k_{inact}/K_i is independent of preincubation time and enzyme and substrate concentrations

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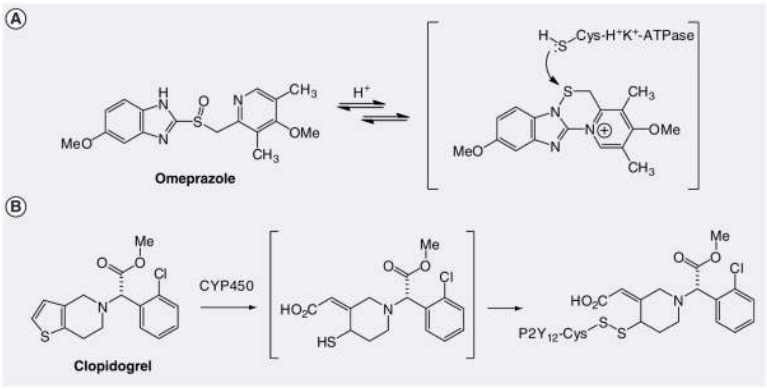

Figure 1.
Mechanism-based covalent inhibition via disulfide adduct formation.

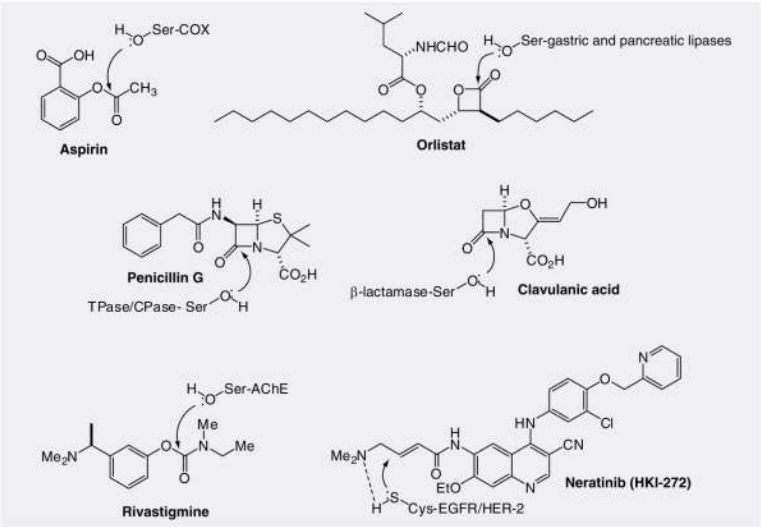

Figure 2.

Examples of covalent inhibitors including their protein target(s) with active-site nucleophile. The arrow indicates the position of attack by the nucleophile on the drug resulting in covalent modification of the target.

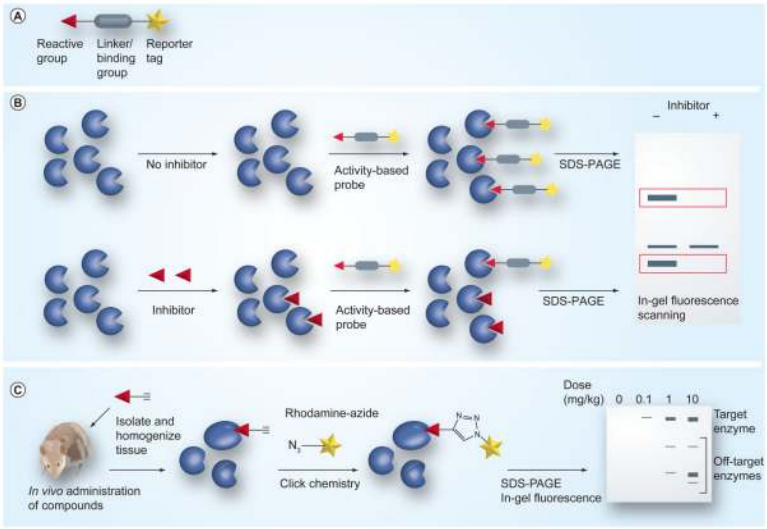

Figure 3. Assessment of global selectivity of covalent inhibitors by activity-based protein profiling (ABPP)

(A) Representative structure of an activity-based probe (ABP), which contains a reactive group, a linker or binding group and a reporter tag. **(B)** Competitive ABPP to determine the selectivity of an inhibitor against an enzyme family that is targeted by a particular ABP (with fluorescent reporter tag in this example). Probe-labeled proteins are analyzed by SDS-PAGE (in-gel fluorescence) and those that show significant reductions in fluorescent intensity in the presence of inhibitor are scored as targets of the inhibitor. **(C)** Click chemistry ABPP profiling to characterize the selectivity of covalent inhibitors *in vivo*. Covalent inhibitors are converted to activity-based probes via incorporation of an alkyne handle and these probes are administered to living systems (cells or animals). Probe-labeled proteins are conjugated to rhodamine-azide using click chemistry and analyzed by SDS-PAGE (in-gel fluorescence).

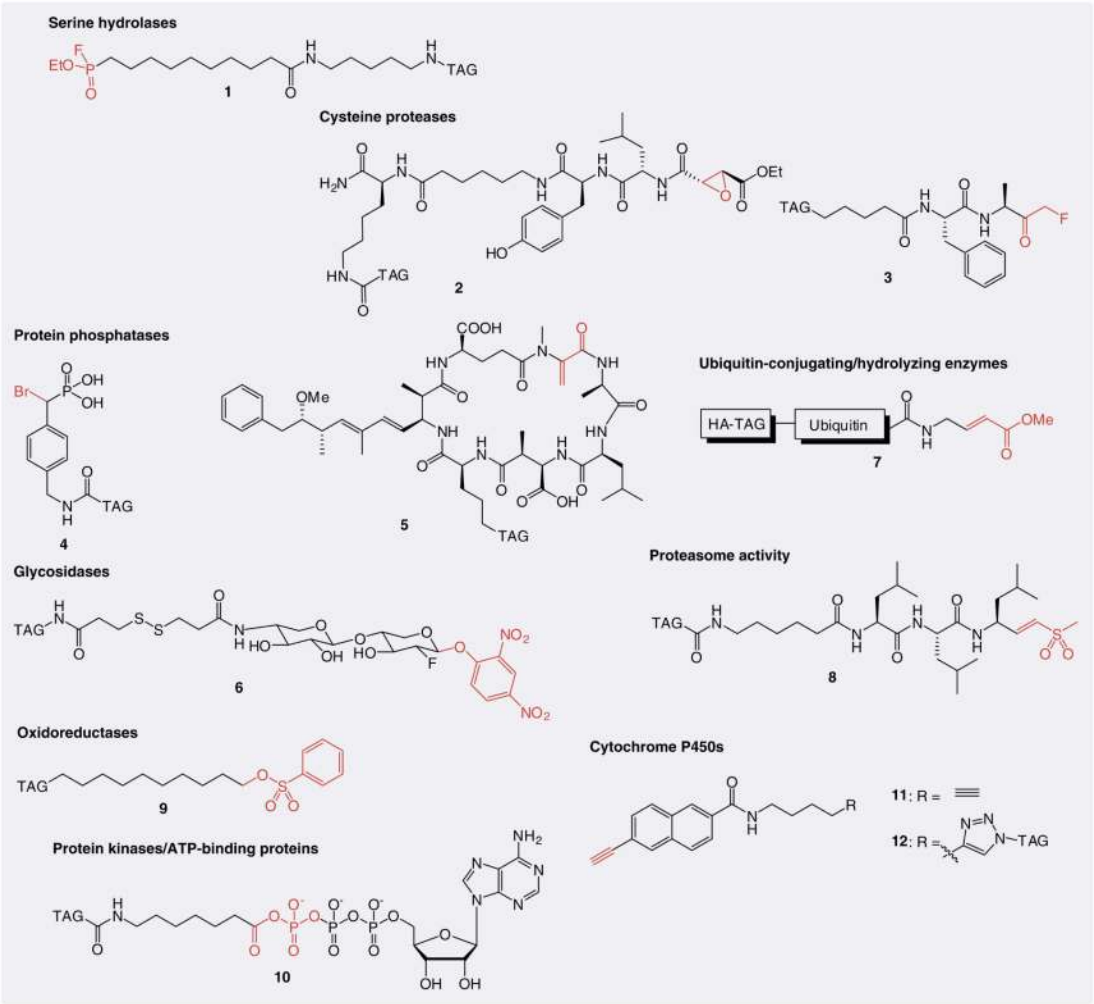

Figure 4. Representative activity-based probes for individual enzyme families or subfamilies
Reactive groups are highlighted. Tag: Biotin, rhodamine, TAMRA, BODIPY or HA.

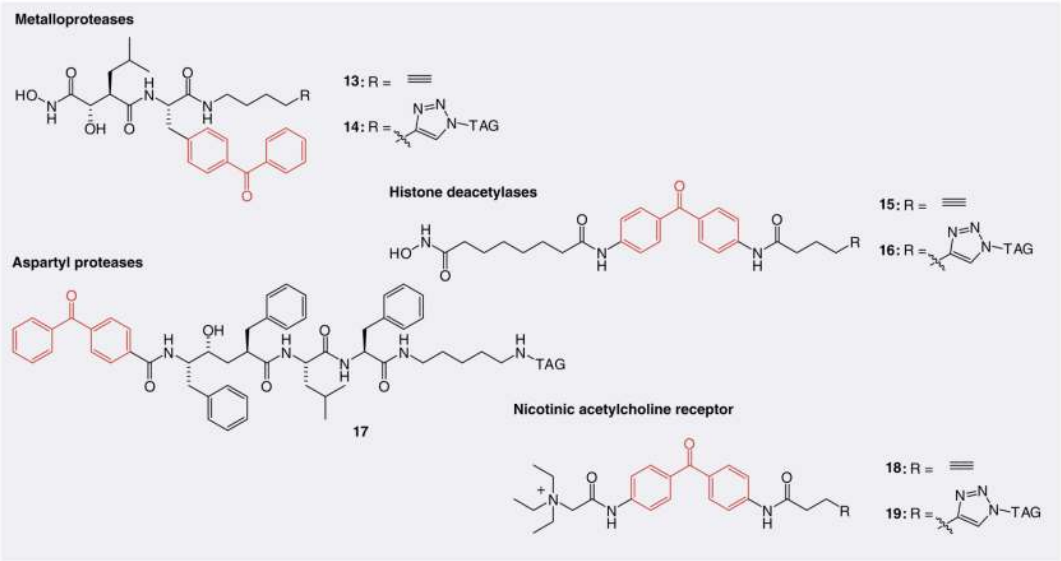

Figure 5. Representative photoreactive activity-based probes that achieve target selectivity through binding affinity and covalent labeling is accomplished by exposure to UV light
Tag: Biotin, rhodamine or TAMRA.

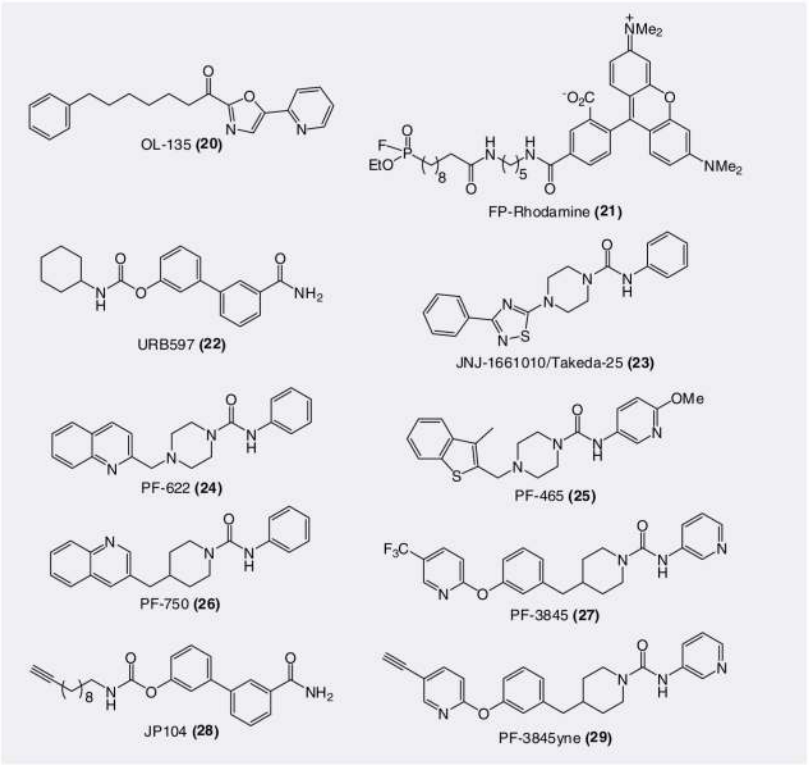

Figure 6.
Covalent fatty acid amide hydrolase inhibitors; OL-135 is reversible.

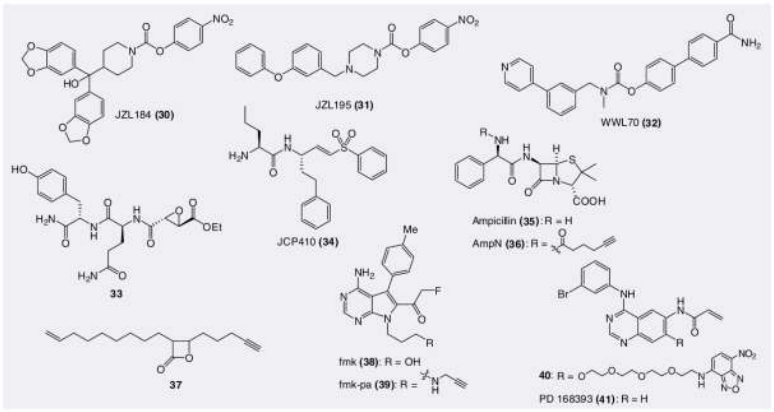

Figure 7.
Covalent inhibitors and clickable covalent probes emerging from chemoproteomic endeavors.