CHAPTER TWENTY-SEVEN

Targeted Covalent Enzyme Inhibitors

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Contents

1. INTRODUCTION

Small molecule drugs exert their pharmacological effects through binding to biomolecular targets, thereby modulating their activity (in the case of enzymes) or downstream signaling (in the case of receptors) with biological consequences that are relevant to the disease state being treated. The majority of small molecule drugs bind to their targets through noncovalent interactions (hydrogen bonds and hydrophobic interactions) or through reversible metal–ligand interactions. For these agents, PK/PD relationships are often understood in terms of equilibrium binding affinities (K_d) for their primary target(s) relative to steady-state free plasma concentrations. This seemingly uncomplicated scenario offers many advantages to the medicinal chemist: target potency and selectivity are determined through simple biological assays at equilibrium, reversibility of drug–target interactions allows tailoring the PD response

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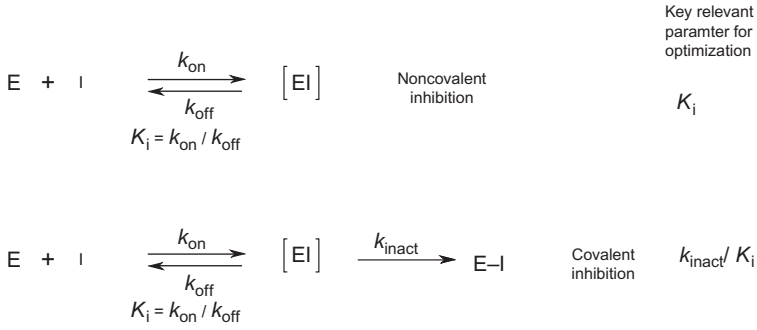
through modifying the PK profile, and the risk of idiosyncratic toxicities is reduced because the target protein is not modified by the drug (excepting, of course, cases of reactive metabolite formation). However, compounds that covalently modify their drug targets are well represented in the pharmacopeia. At present, 39 drugs approved by the FDA bind covalently to their targets, and approximately one-third of all enzyme drug targets have at least one example of an approved covalent drug.[1,2] Many of these compounds were discovered accidentally, noteworthy examples being aspirin, clopidogrel, and omeprazole.[1] Indeed, the formation of a covalent bond between a small molecule drug and its target protein has been largely avoided as a design strategy due to risks associated with immunogenic responses to covalently modified proteins.

Selective covalent modification of a biomolecular target can offer several important advantages in drug discovery—principally through establishing nonequilibrium binding kinetics.[3] For example, it is extremely difficult to target protein–protein interactions with noncovalent small molecule inhibitors due to the expansive binding surface over which the protein–protein interaction occurs.[4] Likewise, targeting the ATP binding site of a protein kinase can be problematic with a noncovalent inhibitor due to high intracellular ATP concentrations that often exceed the enzyme's K_m for ATP by several orders of magnitude. Each of these limitations can theoretically be overcome through the nonequilibrium condition established by targeted covalent modification. Indeed, for the case of kinases, this advantage has been taken further to address the epidermal growth factor receptor (EGFR) kinase T790M resistance mechanism in tumor cells, where this single mutation causes a significant enhancement in ATP affinity and is successfully surmounted with covalent EGFR inhibitors binding at the ATP site.[5–7] Likewise, targeting a uniquely positioned nucleophilic residue within a target's active site offers the opportunity to achieve selectivity for otherwise highly homologous proteins. The case of covalent kinase inhibitors has been extensively studied, and recently reported kinase cysteinome bioinformatics and chemical proteomic methods to characterize reactive cysteines within an enzyme offer useful tools for developing cysteine-targeting strategies for these enzymes.[7–9] Finally, the nonequilibrium condition established through targeted covalent binding can enable significantly lower therapeutic exposures because for protein targets that regenerate slowly, the pharmacodynamic half-life of the drug is much longer than its pharmacokinetic half-life.[10]

Equation (27.1) depicts the differences between covalent and noncovalent enzyme inhibition and establishes the need for unique approaches to study and optimize targeted covalent enzyme inhibitors.

In the case of reversible enzyme inhibition, the K_i for the enzyme (and occasionally k_{on} and k_{off} in the case of slow kinetics to reach equilibrium) is the relevant parameter for drug affinity optimization. For covalent inhibitors, it is the ratio of k_{inact} (the rate constant for covalent modification) to K_i (the equilibrium constant for prereaction association between enzyme and drug), or k_{inact}/K_i, that is important. This value is equivalent to the second order rate constant for bimolecular reaction of the enzyme and its inhibitor. The measurement of k_{inact}/K_i can be quite labor intensive and involves preincubating enzyme with inhibitor, followed by introducing substrate and measuring the percentage of remaining enzyme activity. Preincubation time is varied to produce k_{app} (the apparent rate constant for enzyme inactivation) for a given inhibitor concentration. Varying the inhibitor concentration enables generating linear plots according to the equation: $1/k_{app} = 1/k_{inact} + K_i/(k_{inact} \times [I])$. If $[I] \ll K_i$, then this equation simplifies to $k_{app}/[I] = k_{inact}/K_i$.[11] Therefore, plotting k_{app} as a function of [I] generates linear plots with a slope of k_{inact}/K_i. Because these experiments are very labor intensive, many programs attempt to compare compounds with IC_{50} values determined by varying inhibitor concentration at a single set preincubation time. This methodology is inadequate for generating SAR data because the IC_{50} values are dependent on preincubation time (which can vary between experiments), and information on the fundamental kinetic parameters for enzyme inactivation is not produced.[1] Newer methods for determining k_{inact} and K_i, such as using fluorescence spectroscopy to monitor changes in inhibitor fluorescence upon covalent binding to the target enzyme or deriving these parameters directly from time-dependent IC_{50} values, offer streamlined alternatives that provide high-quality experimental data.[12,13]



[27.1]

Kinetic and thermodynamic parameters describing covalent and noncovalent inhibition.

The question of broad target selectivity is an important consideration in programs utilizing covalent target modification strategies. This issue arises due to intrinsic reactivity of the drug molecule and the presence of surface nucleophiles on a number of proteins (such as albumin). The advent of mass spectrometry-enabled proteomics provides an important tool for determining selectivity of covalent target modification. Specially designed activity-based probes can be used to understand the functional activity of an entire enzyme family (for instance kinases or serine proteases, for which general probes have been developed).[14] When coupled to SILAC (stable isotope labeling with amino acids in cell culture) and click chemistry (to enable capture and enrichment of modified proteins, often using a biotinylated handle), these probes enable selectivity determination across relevant subsets of the cellular proteome. Likewise, it is possible to design clickable probes based on the covalent small molecule pharmacophore that enable capture and enrichment of all target proteins that covalently bind to the probe within the cell. Examples of programs where proteomic methods have been used to interrogate covalent enzyme inhibitor selectivity are presented in the case studies reviewed herein.

As mentioned earlier, the primary motivation for avoiding covalent inhibition approaches is the issue of immunotoxicity resulting from covalent protein modification that produces a hapten recognized as foreign by the immune system.[15] Wholesale strategies for avoiding intrinsically reactive functional groups (or seemingly innocuous groups that form reactive metabolites), coupled with reducing lipophilicity, have emerged as essential medicinal chemistry design principles for producing safer drug candidates.[16,17] These principles are augmented by extensive reactivity– and clinical exposure–toxicity relationship studies for drugs that produce reactive metabolites, which have resulted in dosage guidelines for compounds producing electrophilic species and zone classification systems based on dose and covalent binding to assess idiosyncratic toxicity risk.[18,19] However, it is important not to confuse the issue of toxicity from highly reactive species (epoxides, highly electrophilic Michael acceptors, etc.) produced by oxidative metabolism of compounds present at high concentrations in the liver with the safety risk of targeted covalent modification from mildly electrophilic functionality that is only reactive in the context of its protein target binding pocket.[1] The development of predictive immunotoxicity assays and more extensive clinical studies with targeted covalent inhibitors will better inform medicinal chemistry strategies pursuing targeted covalent inhibition.

The topic of covalent enzyme inhibition as a medicinal chemistry strategy has been reviewed several times over the past decade—often focusing on specific enzyme families or covalent modalities.[1,20] The purpose of this report is to broadly detail targeted covalent inhibitor approaches across a variety of enzyme families which have been published over the past 5 years. Covalent modification of essential enzyme cofactors as a design strategy and the development of covalent probes to enable chemical biology studies are beyond the scope of this review. This review will be divided into several sections based on reactivity of the electrophilic species (producing functionally irreversible or reversible covalent association with the target protein), and type of nucleophile with which the electrophile is known to associate in its biological context (sulfur, oxygen, or nitrogen).

2. FUNCTIONALLY REVERSIBLE COVALENT ENZYME INHIBITORS

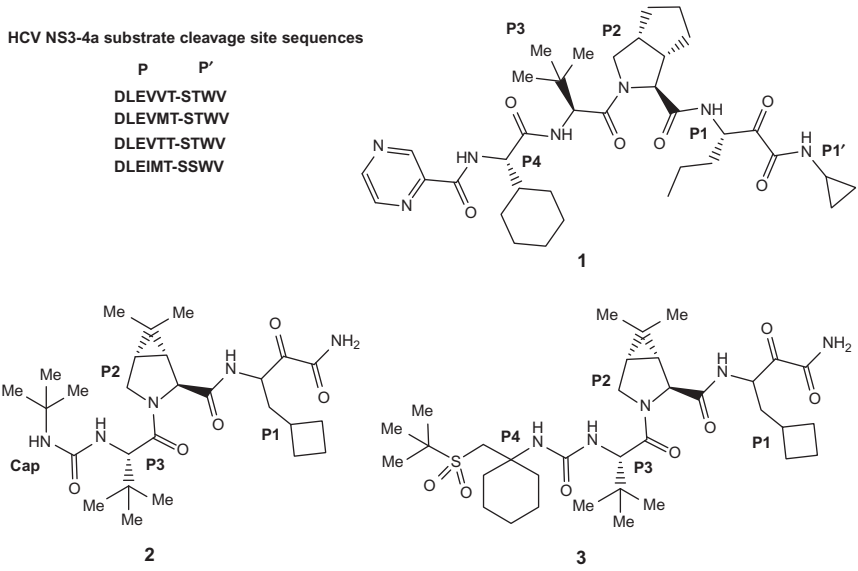
This concept refers to compounds that covalently bind their target and subsequently dissociate from it with a rate that is faster than the physiological degradation rate of the protein. In principle, this approach offers improved potency relative to noncovalent inhibitors while minimizing production of long-lived covalently modified proteins or protein fragments that could trigger immunological responses. It could, therefore, be a preferred strategy in cases where target resynthesis rate is rapid, necessitating higher sustained plasma concentrations of the covalent inhibitor to produce prolonged pharmacodynamic coverage of the target.

2.1. Oxygen-targeting electrophiles

Serine hydrolases and proteases represent a diverse set of targets for drug discovery because they are implicated in many different disease states, including thrombosis, infection, neurological signaling, cancer, and inflammation. While there are many different approaches to inhibiting these enzymes, covalent modification of the active site serine is attractive due to its role as a nucleophile in enzyme catalysis. This strategy offers the possibility of designing tight binding, reversible inhibitors, although significant challenges are associated with achieving enzyme selectivity due to the homology and breadth of this enzyme family.[21] This approach is precedented by several marketed inhibitors of DPP4, HCV protease, human neutrophil elastase,

gastric lipases, and acetylcholinesterase—the reader is referred to a comprehensive recent review for further information on these targets.[21]

Hepatitis C virus NS3/4a protease catalyzes the initial cleavage of the single HCV-encoded polypeptide into its component functional proteins and is essential for viral replication. Telaprevir, **1**, and boceprevir, **2**, are the only two marketed inhibitors of this protease and represent promising treatments for hepatitis C infection. These compounds were derived from peptidomimetic approaches studying peptide leads of varying length from P6′ to P5 of the natural substrate. Drug discovery efforts for **1** and **2** targeted the alpha keto amide serine trap based on its precedence in serine protease inhibition, knowledge of the NS5a/5b substrate, and product inhibition of the NS3/4a protease. The NS3a/4a cleavage sequences and structures of these inhibitors with each residue mapped onto the NS3a/4a binding site are shown below.



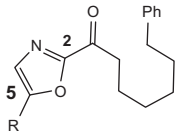
The potency and selectivity of these inhibitors are strongly influenced by structural features of their amino acid side chains, and iterative optimization at each site was required to achieve the proper balance of enzyme potency, activity in a cellular HCV replicon assay and suitable oral pharmacokinetics.[22–24] The S1 and S3 sites (binding regions for the P1 and P3 substituents) are shallow hydrophobic pockets accommodating small linear or branched aliphatic side chains. In the case of boceprevir, small cycloaliphatic substituents at P1 offered significantly enhanced selectivity

versus other serine proteases (human neutrophil elastase). The P2 amino acid has a strong influence on enzyme affinity and cellular replicon potency, with substituted proline derivatives providing substantial increases in activity. This increased activity is attributed to conformational constraints associated with tying back what would otherwise be the isoleucine side chain present at the P2 position of the substrate, but removal of the additional hydrogen bond donor of the backbone amide in acyclic derivatives could also have positive impact on cellular penetration. The P4 (**1**) or P3 capping group (**2**) projects into a small hydrophobic pocket that is important for influencing potency and selectivity.

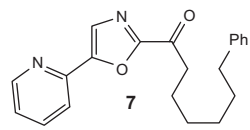
The binding mechanism for compound **1** involves an initial transient collision complex, followed by rearrangement within the binding site to produce the reversible covalent complex.[25] The dissociation half-life of this covalent complex is 58 min, with a steady-state inhibition constant ($K_i^{\star}$) of 7 nM.[24] Compound **1** demonstrated strong selectivity against other serine proteases, including kallikrein, thrombin, plasmin, and Factor Xa (no inhibition at 10 μM).[25] Compound **2** has a similar binding constant of 14 nM for NS3A, but a much longer dissociation half-life (20 h), and is 2200-fold selective versus human neutrophil elastase as a representative off-target serine protease.[23,26] Narlaprevir, **3**, is a structurally related compound that provides greater intrinsic activity in enzyme and replicon assays.[27,28] As with most anti-infective agents, daily dosage is high for these inhibitors (2.2–2.4 g/day for 12–44 weeks).

Aldehydes and activated ketones are also well-precedented electrophiles for serine hydrolases. Alpha-ketoheterocycles were shown to be highly potent, competitive, reversible inhibitors of fatty acid amide hydrolase (FAAH), a potential target for pain which regulates signaling through degrading fatty acid amides such as anandamide and oleamide at their site of action. Their mechanism of inhibition involves attack of the active site serine on the heteroaryl ketone, producing a covalent hemiketal complex that is stabilized by an intramolecular hydrogen bond of the resulting hydroxyl group to the heterocycle. The importance of this hydrogen bond is illustrated by the significant potency difference between **7** (4.7 nM) and **8** (22 μM).[29] The potency of these inhibitors is also strongly influenced by the electron withdrawing character of the heterocycle C5 substituents, as demonstrated by a well-defined correlation between potency ($-\log K_i$) and Hammett σ_p constant for the substituent ($\rho = 3.01$; $R^2 = 0.91$).[30] This pronounced substituent effect is attributed to strengthening the covalent bond between the hemiketal carbon and the active site serine (Ser241) of

FAAH. Inhibitors **4**, **5**, and **6** demonstrate exquisite potency ($K_i = 400–800$ pM). The C2 substituent mimics the hydrocarbon side chain of the fatty acid amide substrate, and the optimum length of the linker alkyl group was demonstrated to be six carbons. Conformationally, constraining the C2 lipophilic side chain by replacing some of the linker group methylenes with a terminal biphenyl or biphenyl ether results in exquisitely potent and selective FAAH inhibitors.[31] Extensive SAR studies were conducted at the central heterocycle,[29] C2 side chain,[31,32] and C5 substituent.[33] Compound **7** is a potent ($IC_{50} = 4.7$ nM) FAAH inhibitor with $>300\times$ selectivity over any other serine hydrolase,[34] increases anandamide levels *in vivo*, and demonstrates potent analgesic activity in models of inflammatory and neuropathic pain.[35,36]



4: R = H
5: R = CO_2Me
6: R = CF_3



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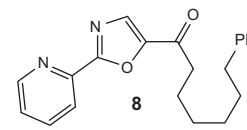
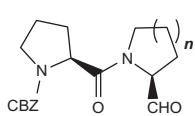
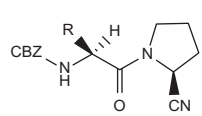


8

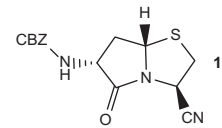
Peptidomimetic aldehydes and nitriles have also been used as electrophiles for prolyl oligopeptidase (POP), a cytosolic serine endopeptidase that has been pursued as a target for neurodegenerative and psychiatric diseases due to its role in neuropeptide maturation and degradation. Electrophiles are commonly appended to the alpha position of a pyrrolidine or piperidine, reflecting catalytic activity of the enzyme at the C-terminus of a proline peptide linkage. Compounds **9** and **10** are potent inhibitors of POP (Compound **10**, $K_i = 4.3$ nM), and crystallographic studies indicate that Ser554 forms a hemiacetal with the inhibitor aldehyde, the oxygen of which occupies the oxyanion hole formed by the active site Tyr473 and backbone NH of Asn555.[37] Pyrrolidine carbonitriles are also potent POP inhibitors, presumably forming a Pinner adduct with the active site serine. Compounds **11** ($IC_{50} = 20$ nM) and **12** ($IC_{50} = 3$ nM) are potent inhibitors of POP activity in cell extracts, whereas the conformationally constrained inhibitor **13** is considerably less active ($IC_{50} = 500$ nM).[38] These compounds demonstrated strong selectivity versus DPP IV activity in cells and cell extracts (Compound **13**, $IC_{50} > 100$ μM).



9: *n* = 1
10: *n* = 2

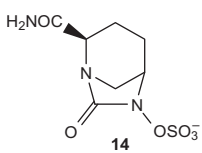


11: R = H
12: R = Me



13

The aforementioned examples of reversible covalent inhibitors focus on slow dissociation of the covalent complex to release the original inhibitor, effectively setting up an equilibrium between target and inhibitor. There are also several well-known examples of compounds that acylate their target enzyme, wherein enzyme function can be restored through hydrolysis of the acylated enzyme. Some of the best known examples are β-lactam inhibitors of serine β-lactamases. Because this topic has been recently reviewed, this section will focus on non-β-lactam inhibitors of these enzymes.[39] The most advanced non-β-lactam inhibitor of these enzymes is NXL-104 **14**, which contains an anti-Bredt urea that acylates the nucleophilic serine of β-lactamases, releasing the nitrogen atom bearing the sulfate moiety to produce a carbamate with considerably greater stability than the serine esters formed by reaction of the enzyme with β-lactam inhibitors.[40] Against a variety of Class A and Class C serine β-lactamases, compound **14** demonstrated an IC_{50} between 5 and 170 nM after a 5-min preincubation. This compound is presently in Phase III clinical trials in combination with ceftazidime or ceftaroline as a broad spectrum antibacterial therapy for nosocomial infections.



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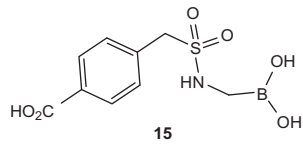

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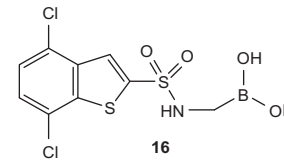
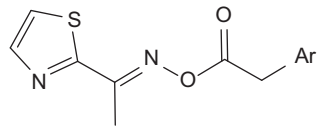


16

Boronic acid-based inhibitors of β-lactamases are transition-state mimics of the tetrahedral intermediate for the acylation reaction. A series of sulfonamide boronic acids represented by **15** demonstrated submicromolar K_i against AmpC β-lactamase, with **15** demonstrating an IC_{50} of 70 nM.[41] These compounds exhibit rapid binding kinetics, as evidenced by the lack of preincubation effects on enzyme potency. Crystallographic analysis of the complex between AmpC and these inhibitors reveals the covalent association of the active site serine Ser64 with the boronic acid to form the tetrahedral transition-state analogue with one oxygen of the boronate projecting into the oxyanion hole formed by backbone NH groups for Ser64 and Ala318. The other boronate oxygen interacts with conserved Tyr150 and an ordered water molecule known to participate in deacylation reactions of substrates. The related boronic acid **16** demonstrates activity across a broad array of β-lactamases from the TEM, AmpC, SHV, P99, and OXA classes, with IC_{50} values between 0.6 and 5.6 μM.[42]

Activated oxime esters were shown to be reversible covalent inhibitors of retinoblastoma binding protein 9, a serine hydrolase of unknown function that confers resistance to antiproliferative signaling of TGFβ and may be implicated in carcinogenesis.[43] Acylated thiazole oximes such as **17** were identified using fluorescent-labeled activity-based proteomic profile screening (fluopol-ABPP), wherein compounds are assessed for their ability to impede covalent labeling of the target protein by a fluorescent probe. These activated oxime esters acylate Ser75, forming a serine ester and releasing the oxime as a leaving group; enzyme function is regenerated upon hydrolysis of the serine ester. Compound **17** demonstrated an IC_{50} of 1.9 μM in this assay and showed no significant inhibition of other serine hydrolases across the 293T soluble proteome of transformed HEK cells.

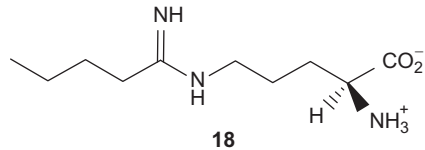


17: Ar=(4-Cl)Phenyl

2.2. Sulfur-targeting electrophiles

Several classes of reversible sulfur-targeting electrophiles have been reported, including the well-known peptidomimetic aldehydes and nitriles that are reversible covalent inhibitors of cysteine proteases, such as cathepsin S and cathepsin C, forming thiohemiacetals or thioimidates with their active site cysteine residue. Because these inhibitor classes have been recently reviewed, this section will focus on less exemplified electrophiles for reversible covalent modification of cysteine.[44,45]

Alkyl amidines form reversible covalent bonds with the active site cysteine residue of dimethylarginine dimethylaminohydrolase-1 (DDAH-1), which mediates the hydrolysis of N^{ω},N^{ω}-dimethyl-L-arginine, an endogenous inhibitor of nitric oxide synthetase.[46] Upon covalent bond formation with Cys274, the amidine nitrogen atoms participate in hydrogen bonding and ion pair interactions with the side chains of Asp79 and Glu78. Isothermal titration calorimetry was used to determine the potency of **18** against the wild-type enzyme (7 μM) and the C274S mutant (28 μM), which was shown crystallographically to be incapable of forming a covalent bond to the inhibitor's amidine group. The modest potency difference for these two orthologues suggests that the covalent bond is weak and does not contribute much to the overall binding affinity of the inhibitor.



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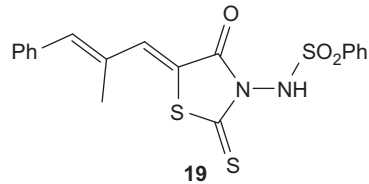
Benzylidene rhodanine derivatives were shown to be reversible covalent inhibitors of Hepatitis C NS5b RNA polymerase, an essential enzyme involved in transcription of viral RNA. These compounds, exemplified by **19**, covalently modify Cys366 through Michael reaction at the benzylidene group of the inhibitor.[47] This Michael reaction gets assistance from activation of the rhodanine ketone, which accepts a hydrogen bond from Ser367. Enzyme kinetic studies demonstrate noncompetitive behavior with nucleotide substrates, consistent with location of Cys366 outside the active site of the enzyme. These inhibitors impede growth of the RNA daughter strand by blocking the channel that the RNA uses to exit the enzyme. Reversibility was demonstrated through preincubation and dilution experiments, and compound **19** was the most potent inhibitor identified ($IC_{50} = 200$ nM). This compound demonstrated strong selectivity for NS5b RNA polymerase over cathepsin B; calpain; caspases 1, 3, 6, 7, and 8, and aldose reductase. This compound demonstrates low clearance (0.13 mL/min/kg) and volume of distribution (0.09 L/kg) in rat with a half-life of 9.2 h.[47]



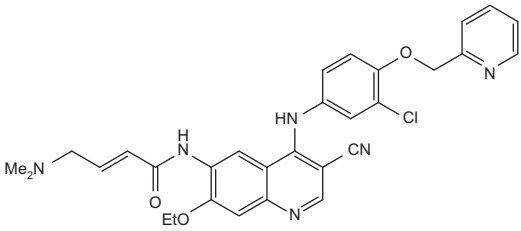
19

3. FUNCTIONALLY IRREVERSIBLE COVALENT ENZYME INHIBITORS

This concept refers to compounds that covalently bind their target and dissociate from it with a rate that is slower than the physiological degradation rate of the target. In cases where the target resynthesis rate is slow relative to the pharmacokinetic half-life of the compound, it offers the possibility for significantly prolonged pharmacodynamic effects.[20] However, the potential to produce immunogenic fragments of covalently modified proteins requires increased attention to selectivity and minimizing dose relative to functionally reversible targeted covalent inhibitors.

3.1. Sulfur-targeting electrophiles

A number of designed covalent inhibitors take advantage of the facile reaction between a cysteine residue in the target active site and a Michael acceptor. Covalent epidermal growth factor inhibitors are among the best-studied cases. The EGFRs (Her-1 and Her-2) are members of ErbB family of receptor tyrosine kinases. Uncontrolled activation of EGF receptors by overexpression of the receptors or of the ligands that activate EGFRs is implicated in cancers of the head, lung, breast, bladder, ovary, and kidneys. A number of first-line, efficacious, reversible EGFR inhibitors have been approved: Iressa, Tarceva, and lapatinib; however, most patients develop acquired resistance to these drugs. Covalent inhibitors with greater biochemical efficiency were developed to overcome this resistance. One of the first agents which showed clinical efficacy was HKI-272 or neratinib **20**. This compound shows good potency against EGFR (IC_{50}: 92 nM) and Her-2 (IC_{50}: 59 nM) and improved efficacy compared to EKB-569, a previously developed covalent EGFR inhibitor in Her-2-dependent tumor models.[48] The dimethylaminomethylene moiety attached to the acrylamide tail not only gives improved solubility but also presumably acts as an intramolecular base catalyzing the addition of Cys773 (EGFR) or Cys805 (Her-2) to the β-carbon of the acrylamide (structure **21**).[7] Phase I[49] and Phase II advanced non-small-cell lung cancer[50] and Erb-2-positive breast cancer[51] clinical studies have been published, with excellent efficacy seen in the breast cancer studies. In both trials, doses of 240 mg/day were used over several months with ~30% patients experiencing grades 3–4 diarrhea as the most common adverse event.



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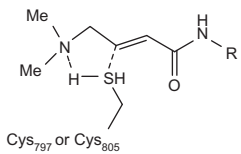
EGFR IC_{50}: 92 nM
Her-2 IC_{50}: 59 nM



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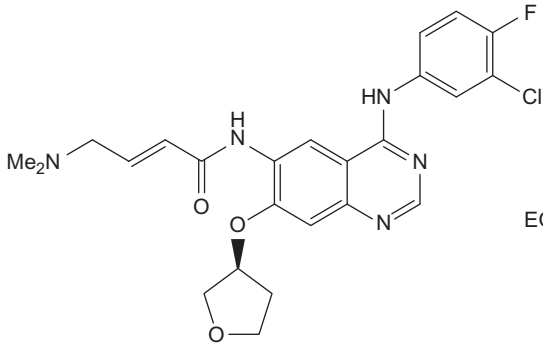
BIBW 2992 (afatinib **22**) is also an irreversible covalent EGFR inhibitor that is currently undergoing clinical assessment. Compound **22** shows excellent potency against EGFR (IC_{50}: 0.5 nM) and Her-2 (IC_{50}: 14 nM). It also shows excellent potency against two EGFR mutants, $EGFR^{L858R}$

(IC_{50}: 0.4 nM) and $EGFR^{L858R/T790M}$ (IC_{50}: 10 nM), the double mutant that is the cause for more than 50% of all acquired resistance to first-line reversible EGFR inhibitors.[52] The double L858R and T790M gatekeeper mutation provides resistance by producing a more active kinase (L858R) and by increasing ATP affinity (T790M).[5] Thus, covalent EGFR inhibitors can potentially overcome T790M resistance since, once covalently bound, they are no longer in a competitive, reversible equilibrium with ATP. Phase I trials of **22** showed good tolerability with maximum tolerated doses (MTDs) of 40–50 mg/day.[53] Slightly higher MTDs can be obtained using a 2–3 weeks on/1–2 weeks off dosing schedule. Two Phase II trials of **22** in NSCLC show positive results in EGFR inhibitor-pretreated patients with positive effects shown on progression-free survival and objective response rates, although these results show no advantage over currently marketed tyrosine kinase inhibitors. Currently, several Phase III trials are being conducted exploring **22** in EGFR-mutated NSCLC in comparison to cisplatin–pemetrexed therapy.

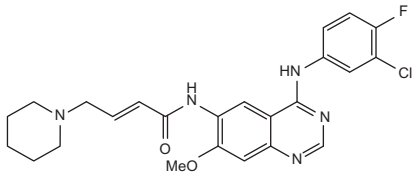


EGFR IC_{50}: 0.5 nM
Her-2 IC_{50}: 14 nM
$EGFR^{L858R}$ IC_{50}: 0.4 nM
$EGFR^{L858R/T790M}$ IC_{50}: 10 nM

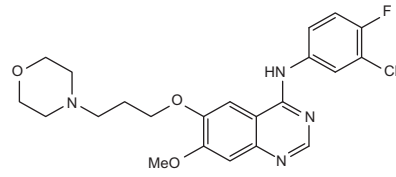
22

Another member of this class of covalent EGFR inhibitors is PF-00299804 or dacomitinib **23**. Compound **23** shows excellent potency across many EGFR family members: EGFR (IC_{50}: 6.0 nM), Her-2 or ERBB2 (IC_{50}: 46 nM), and Her-4 or ERBB4 (74 nM). Additionally, **23** shows efficacy in cell lines containing the L858R (H3255: IC_{50}: 0.007 μM) and the L858R/T790M (H3255 GR: IC_{50}: 0.119 μM) mutations which render the reversible EGFR inhibitor gefitinib **24** inactive.[54] Dacomitinib also shows excellent efficacy in SKOV3 and A431 human tumor xenograft models with MEDs of 15 and 11 mg/kg, excellent tumor drug exposure in these xenograft models, moderate clearance (24–49 mL/min/kg), and excellent oral bioavailability (56–80%) across

multiple species.[55] Phase I clinical studies with **23** showed a MTD of 45 mg and a comparable safety profile to other EGFR inhibitors.[56] Compound **23** demonstrated proof of concept in patients with NSCLC and is currently entering Phase III studies.

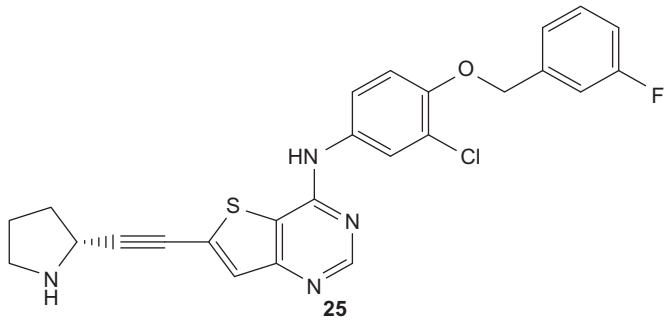


23
EGFR IC_{50}: 46 nM
Her-2 IC_{50}: 74 nM



24

In comparison to the acrylamide Michael acceptor used in the compounds described above, electron-deficient acetylenes have also been used to target the active site cysteine residues in EGFR enzymes. 6-Ethynylthieno[3,2-*d*]- and 6-ethynylthieno[2,3-*d*]pyrimidin-4-anilines such as **25** have been shown to have good EGFR, Her-2, and Her-4 potency and form covalent adducts with their target enzymes. The basic amine on the alkyne terminus is believed to act as a general base to deprotonate the active site cysteine, aiding nucleophilic addition to the alkyne. This compound has potent antitumor activity in BT474 tumor xenograft models at doses from 10 to 100 mg/kg and shows superior efficacy to the reversible EGFR inhibitor lapatinib.[57]

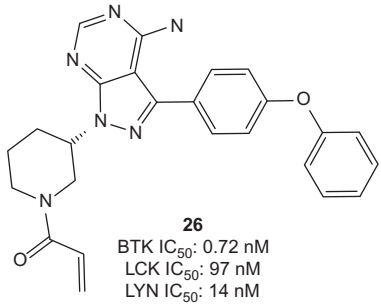


25
EGFR IC_{50}: 7 nM
Her-2 IC_{50}: 13 nM
Her-4 IC_{50}: 66 nM

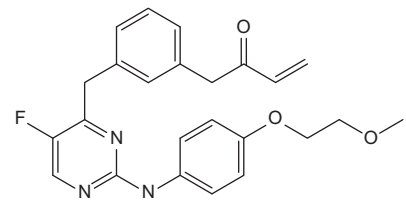
Bruton's tyrosine kinase (BTK) is a member of Tec family kinases and is a key component in B-cell receptor signaling. Genetic loss of function[58] and siRNA studies[59] have implicated BTK as a mediator of inflammatory signaling, making it a suitable target to treat rheumatoid arthritis. One successful strategy was to take a reversible BTK inhibitor with poor selectivity over LCK and LYN and incorporate a piperidine containing an electrophilic acrylamide to specifically target Cys481, thereby producing greater kinase

selectivity. The resulting compound, ibrutinib, **26** is a potent covalent irreversible BTK inhibitor (IC_{50}: 0.72 nM) with ~130-fold selectivity versus LCK and ~19-fold selectivity versus LYN.[60] Subsequent preclinical studies have shown **26** to be effective in diseases involving activation of the B-cell antigen receptor pathway,[61] especially chronic lymphocytic leukemia (CLL).[62] This compound has also been used as a tool to show that BTK is specifically required for IgE activation of human basophils compared to other basophil stimulators such as FMLP, C5a and IL-3.[63] A chemical probe was developed wherein **26** was linked to a BODIPY tag, enabling target occupancy to be determined by displacement of the probe with **26**.[64] This fluorescent probe demonstrates specific labeling of BTK in DOHH2 cells and mouse splenocytes, with no protein labeling detected in Jurkat cells, which do not express BTK protein. Compound **26** demonstrates complete target occupancy in mouse splenocytes for 12 h when given orally at 50 mg/kg. Compound **26** is currently in Phase II clinical trials for CLL.[65]

Another BTK inhibitor that has advanced to Phase II clinical studies is AVL-292.[66] This compound demonstrates strong potency against BTK in enzyme ($IC_{50} = 0.5$ nM) and Ramos cell-based ($EC_{50} = 8$ nM) assays, with excellent selectivity over Src-family kinases and B-cell signaling components ($IC_{50} > 700$ nM) across a family of 9 kinases. A chemical probe using a biotin tag for protein captured demonstrated 50% target occupancy in cell lysates at 5.9 nM concentration, in agreement with the Ramos cell assay results. In Phase I clinical studies, a single 2 mg/kg oral dose produced nearly complete target occupancy after 2 h that was sustained through the 8-h measurement period. In multidose studies, complete and sustained target occupancy was attained at the 250 mg QD dose level with good toleration. While the structure of AVL-292 has not been specifically disclosed, a single compound patent from Avila reports advanced physical form characterization for compound **27**, which is likely an advanced candidate compound, if not AVL-292 itself.[67,68]

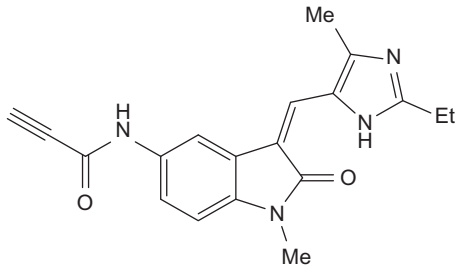


26
BTK IC_{50}: 0.72 nM
LCK IC_{50}: 97 nM
LYN IC_{50}: 14 nM



27
BTK IC_{50}: <10 nM
ITK IC_{50}: 10–100 nM

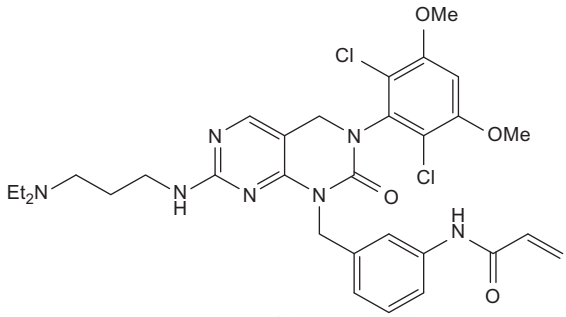
Nek2 is a serine/threonine kinase thought to play a role in bipolar spindle assembly driven by the microtubule motor protein Eg5 and is known to be overexpressed in breast tumor and diffuse large B-cell cancers. Nek2 possess a cysteine residue in its active site (Cys22) which is a suitable target for covalent inhibitors. Compound **28** (JH295) inhibits Nek2 (IC_{50}: 770 nM) and shows good selectivity over Cdk1, a key selectivity target, as Cdk1 inhibitors block mitotic entry and trigger mitotic exit. Incubation of 10 equivalents of **28** and 1 equivalent of Nek2 produces a single covalent adduct as seen by protein mass spectroscopy, and **28** was shown to inhibit cellular mitosis A549 cells after 95% of Nek2 activity was inhibited.[69]



28

Nek2 IC_{50}: 770 nM
Cdk1/CycB IC_{50}: >20,000 nM

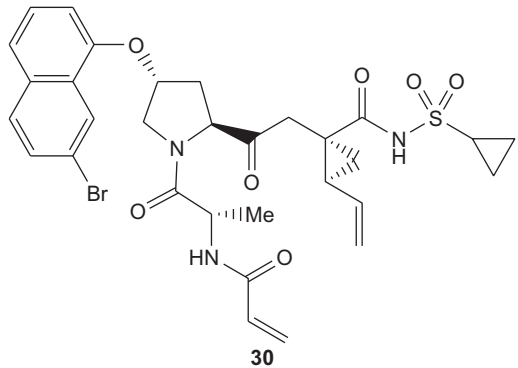
Fibroblast growth factor receptor (FGFR) kinases are a family of tyrosine kinases that play critical roles in normal development, wound healing, as well as tumor formation and progression. FGFR gain of function mutations and FGFR overexpression have been identified in bladder, gastric, colorectal, and additional cancers. Recent studies have identified **29** (FIIN-1) which effectively targets Cys486 of FGFR1 with an acrylamide electrophile. Compound **29** shows excellent potency for FGFR1, 2, and 3 (IC_{50}s: 9.2, 6.2, 11.9 nM) and only binds two other kinases with a K_d below 100 nM (Blk and Flt1) in a 402 kinase selectivity panel. This compound was confirmed to block activation of FGFR1 in MCF10A cells, and its binding to Cys486 was confirmed by incubating biotin labeled **29** in MCF10A cell lines expressing WT iFGFR1 and a C486S FGFR1 mutant, followed by immunoprecipitation, where binding to WT iFGFR1 was only seen. Compound **29**, however, shows weak potency against V561M FGFR1, a gatekeeper mutation that has previously been shown to reduce the FGFR1 potency of PD173074.[70]



29

FGFR1-3 IC_{50}s: 6.2–11.9 nM
FGFR4 IC_{50}: 189 nM

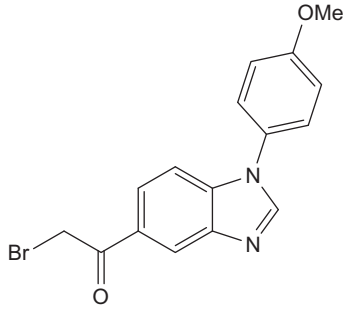
Cysteine trapping with acrylamides has also been used as an effective strategy to design HCV NS3/4A viral protease inhibitors. Compound **30** shows excellent HCVP inhibition (IC_{50}: 2 nM) but poor activity against a C159S HCVP mutant (IC_{50}: 1782 nM), indicating Cys159 to be the target of the acrylamide electrophile. The authors were also able to develop a biotinylated analog of **30** which was used as an occupancy biomarker in Huh-7 replicon cells to show that practically complete occupancy is needed to arrest replicon activity. An HCV NS3/4A turnover rate of 8–24 h could also be determined using this approach.[71]



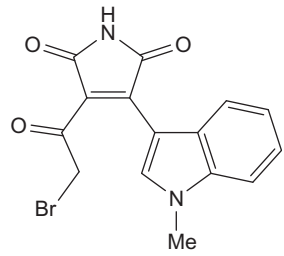
30

HCVP IC_{50}: 2 nM
C159S HCVP IC_{50}: 1782 nM

In addition to acrylamides, other electrophiles have been effectively used to target cysteine residues to produce covalent inhibitors. Glycogen synthase kinase 3 (GSK3) inhibitors can be switched from reversible to irreversible binders by changing the methyl ketone moiety that binds near Cys199 to the corresponding α-chloro or bromo ketones, which suffer nucleophilic attack. Compounds **31** and **32** are two representative inhibitors that show good to excellent GSK3 potency (**31** IC_{50}: 580 nM; **32** IC_{50}: 5 nM). Covalency was confirmed by dependence of GSK3 inhibition on preincubation time, and covalent binding of **32** was confirmed biophysically by MALDI–TOF analyses.[72]

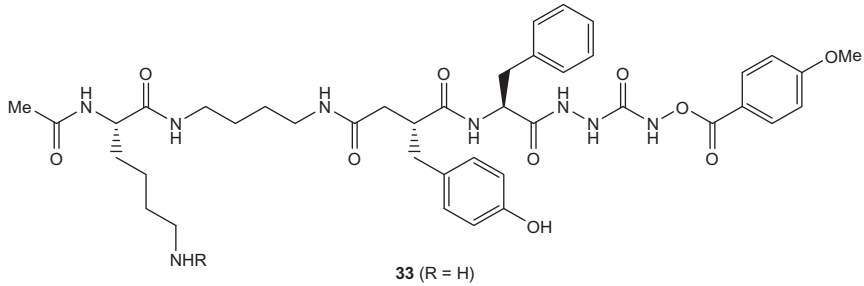


31
GSK3 IC_{50}: 580 nM

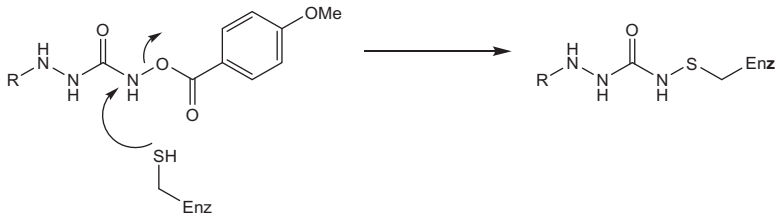


32
GSK3 IC_{50}: 5 nM

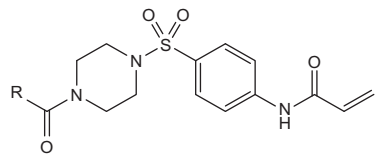
O-Acyl hydroxamates have been used to prepare aza-peptide covalent inhibitors of papain family cysteine proteases such as cathepsin B. Compound **33**, which contains an acyl-*N*-((4-methoxybenzoyl)oxy)hydrazinecarboxamide unit, shows potent inhibition of cathepsin B and cathepsin Z (cathepsin B IC_{50}: 13 nM, cathepsin Z IC_{50}: 170 nM) with very fast rates of enzyme association (cathepsin B k_{on}: 75,000 M^{-1} s^{-1}). The corresponding biotinylated analog of **34** showed strong binding to cathepsin B in labeling experiments and survived reducing conditions (pH 6.8, 150 mm DTT, 100 °C, 2 min) prior to SDS-PAGE gel loading.[73] The *p*-methoxybenzyl group creates an electron-deficient nitrogen on the hydrazinecarboxamide, facilitating attack of a nucleophilic cysteine leading to a *N*-mercaptohydrazinecarboxamide covalent structure.



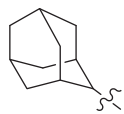
33 (R = H)
Cathepsin B IC_{50}: 13 nM; K_{ass}: 75,000 $M^{-1}s^{-1}$
Cathepsin L K_{ass}: 65,000 $M^{-1}s^{-1}$
Cathepsin Z IC_{50}: 170 nM
34 (R = biotin)



Tissue transglutaminase 2 (TG2), a Ca^{+2} enzyme responsible for making intermolecular peptide cross-links between the γ-carboxamide of a glutamine and an ε-amino group of a lysine, has been shown to be important in mitochondrial energy function.[74] Overactivity of TG2 has been closely associated with Celiac and Huntington's diseases. Covalent inhibitors of TG2 have been recently developed by targeting Cys277 using unsubstituted acrylamides **35** (TG2 IC_{50}: 10 nM) and **36** (TG2 IC_{50}: 120 nM). In addition to unsubstituted acrylamides, other electrophiles were incorporated that led to active compounds (substituted arcrylamides, oxirane-2-carboxamide, nitriles, ynamide, diazoketone). These analogs showed time-dependent TG2 inhibition and modest second order inhibition rate constants were determined (**36** k_{inact}/K_i: 1087 $\mu M^{-1}\,s^{-1}$). A log–log plot of k_{inact}/K_i versus IC_{50} (30 min incubation) shows a high correlation of these measures. Compound **35** shows excellent selectivity against TG1 and TG3 with modest selectivity shown over FXIIIa. *In vitro* stability/metabolism studies in mice shows moderate plasma stability ($T_{1/2}$: 248 min) and high mouse liver microsome intrinsic clearance (3203 mL/min/kg).[75]

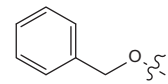


R =



35
TG2 IC_{50}: 10 nM
TG1 IC_{50}: 3400 nM
TG3 IC_{50}: 80000 nM
FXIIIa IC_{50}: 180 nM
mouseplasma $T_{1/2}$: 248 min
mLM Clint: 3203 ml/min/kg

R =

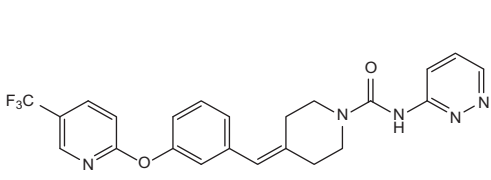


36
TG2 IC_{50}: 120 nM
TG2 k_{inact}/K_i: 1087 $\mu M^{-1}s^{-1}$
TG1 IC_{50}: 3700 nM
TG3 IC_{50}: >80,000 nM
FXIIIa IC_{50}: 420 nM

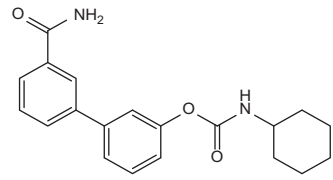
3.2. Oxygen-targeting electrophiles

Harder electrophiles (e.g., ureas, carbamates, epoxides) enable covalent modification of hydroxylated amino acids (e.g., serine) in a number of targets. FAAH, a serine hydrolase that hydrolyzes the endogenous endocannabinoid anandamide, can be inhibited with excellent potency (hFAAH k_{inact}/K_i: 40,300 $M^{-1}\,s^{-1}$: IC_{50}: 7.2 nM) by urea **37**, a covalent inhibitor that targets Ser241 in the active site of FAAH.[76] Compound **37** shows exquisite selectivity for FAAH as assessed by competitive activity-based protein profiling (ABPP) in human and mouse brain membranes and soluble liver proteomes using a rhodamine-tagged fluorophosphonate (FP) ABPP probe.[77] Even at 100 μM, **37** selectivity blocked labeling of only

FAAH in soluble liver lysates, whereas first-generation inhibitor URB597 (**38**) blocked labeling multiple proteins. Compound **37** shows excellent pharmacokinetic properties in both rat and dog and demonstrates statistically significant activity in a CFA model of inflammatory pain after oral administration at 0.1 mg/kg. Related covalent FAAH inhibitors have also been disclosed.[78,79] Compound **37** is currently in clinical trials for pain and other indications.

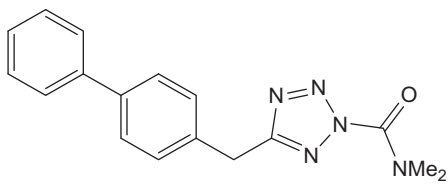


37
hFAAH k_{inact}/K_i: 40,300 $M^{-1}s^{-1}$
hFAAH IC_{50}: 7.2 nM



38

Monoacylglycerol lipase (MGL) is a serine hydrolase related to FAAH which hydrolyses 2-arachidonoylglycerol, an endocannabinoid-like anandamide. Compound **39** (LY 2183240) is an electrophilic urea that inhibits hMGL by targeting Ser129 with $IC_{50} = 20$ nM. Tryptic digest of the compound **39**-inhibited hMGL followed by MALDI-TOF MS analysis clearly shows the incorporation of a dimethylamido (-C(O)NMe_2) fragment as a result of serine acylation.[80] Compound **39** also shows potent hFAAH inhibition (IC_{50}: 37 nM)[81] and displays nociceptive effects in mouse models of inflammatory pain.[82]

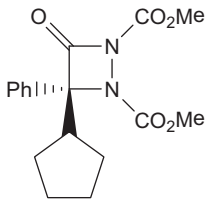


39

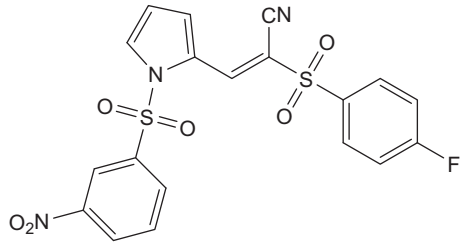
hMGL IC_{50}: 20, 54 nM
hFAAH IC_{50}: 37 nM

The serine hydrolase protein phosphatase methylesterase-1 (PME-1) demethylates protein phosphatase 2A, potentially leading to cancer and neurodegenerative disease. Potent covalent inhibitors of PME-1 have been identified, **40** (IC_{50}: 10 nM)[83] and **41** (IC_{50}: 600 nM),[84] using fluorescence polarization-activity-based protein profiling (fluopol-ABPP). Both **40** and **41** show excellent serine hydrolase selectivity via competitive ABPP using FP, chloroacetamide, and sulfonate ester probes. Compound **41** is believed to inhibit PME-1 via a covalent irreversible mechanism,

since recovery of enzyme activity was not observed after gel filtration chromatography. The PME-1 adduct with compound **40** was isolated, but not with compound **41**.

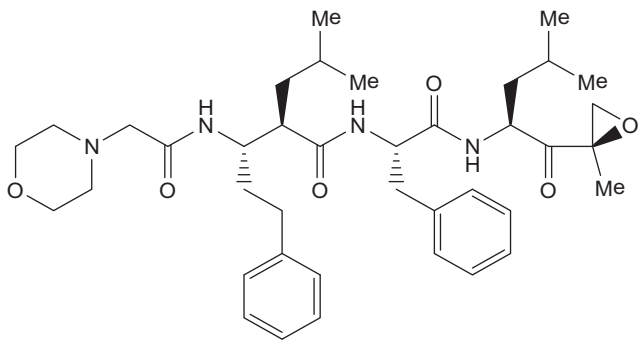


40
IC_{50}: 10 nM



41
IC_{50}: 600 nM

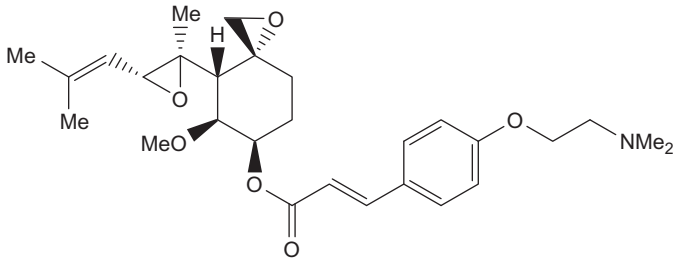
Carfilzomib (**42**) is a potent inhibitor of the human 20S proteasome (k_{inact}/K_i: 34,000 M^{-1} s^{-1}) and shows more than 300-fold selectivity over other proteasome catalytic activities.[85] Inhibition of the 20 S Proteasome is a clinically validated approach for the treatment of hematological cancers (leukemia, lymphoma, and myeloma) as evidenced by bortezomib (PS-341, Velcade™), a proteasome inhibitor that shows efficacy in multiple myeloma and non-Hodgkin's lymphoma. Compound **42** inhibits the 20S proteasome by irreversibly binding to an active site serine. The compound induces apoptosis and growth arrest in hematologic (RPMI 8226 and HS-Sultan) and solid tumor (HT-29) cell lines and shows efficacy in HT-29, RL, and HS-Sultan human tumor xenograft models.[86] Compound **42** has progressed rapidly through the clinic and showed positive effects in Phase II trials of multiple myeloma.



42
h20S proteosome k_{inact}/K_i: 34,000 $M^{-1}s^{-1}$
h20S proteosome 1 h IC_{50}: 6 nM

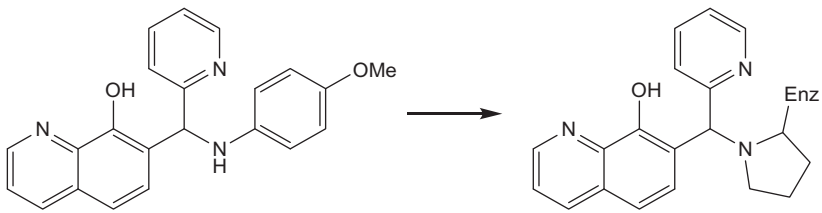
3.3. Nitrogen-targeting electrophiles

Beloranib (**43** ZGN-433) is a covalent inhibitor of methionine aminopeptidase 2 (METAP2). Originally developed as an angiogenesis inhibitor,[87] it was subsequently discovered to have antiobesity effects, and it is currently in early clinical trials. An active site histidine in METAP2 is believed to react with one of the epoxides of **43** leading to covalent enzyme inactivation.



43

Macrophage migration inhibitory factor (MIF) plays a key role in the pathology of cancer and inflammatory diseases. While the mechanism of how MIF exerts its biological effects is not clear, it has tautomerase activity that is likely linked to its cytokine activity. It was recently been disclosed that irreversible inhibition of MIF can be achieved using 7-((arylamino)(pyridin-2-yl)methyl)quinolin-8-ols such as **44**. Compound **44** reacts with MIF tautomerase by making a covalent bond with Pro-1 as shown by X-ray crystallographic analysis and time-dependent inhibitory potency profile.[88]



44

4. CONCLUSIONS

The past 5 years have witnessed a resurgence of interest in covalent enzyme inhibitors due to a renewed appreciation for their prevalence in the human pharmacopeia and the recognition that more challenging biological targets will require unconventional drug discovery strategies to produce clinical candidates. While covalent inhibitor approaches carry increased safety

risk due to the presence of a reactive moiety, these concerns can be mitigated by appropriately targeting the inhibitor through modulating its electrophilicity, emphasizing noncovalent binding interactions to improve selectivity and minimizing dose with careful attention to pharmacokinetic properties and potency. Recent advances in protein mass spectrometry and chemical biology offer the possibility of quantifying selectivity against entire protein families in a cellular context, providing additional information to gauge safety risks. Many of these methods have yet to be applied systematically to targeted covalent inhibitor programs, offering significant opportunity for the medicinal chemist to devise new strategies for monitoring target occupancy and off-target effects for irreversible or slowly dissociating compounds. Indeed, the number of irreversible covalent inhibitor programs cited in this review where primary pharmacologic activity was judged based only on IC_{50} values demonstrates the broader need for more rigor in designing even basic screening strategies for covalent inhibitor approaches. The diversity of electrophilic moieties utilized for reversible and irreversible enzyme inhibition for programs exemplified herein illustrates the breadth of opportunities already available for generating targeted covalent inhibitors. However, there is significant room for elevating design sophistication through understanding electrophile reactivity under physiological conditions and in the context of their target protein binding pocket properties, which would enable selecting the optimum electrophile for targeted covalent inhibition. Further advances in this field promise to make covalent inhibition a more prominent tool for the medicinal chemist to approach contemporary targets that pose significant challenges for traditional noncovalent strategies.

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