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Covalent inhibitors: an opportunity for rational target selectivity

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Abstract

There is a resurging interest in compounds that engage their target through covalent interactions. Cysteine's thiol is endowed with enhanced reactivity, making it the nucleophile of choice for covalent engagement with a ligand aligning an electrophilic trap with a cysteine residue in a target of interest. The paucity of cysteine in the proteome coupled to the fact that closely related proteins do not necessarily share a given cysteine residue enable a level of unprecedented rational target selectivity. The recent demonstration that a lysine's amine can also be engaged covalently with a mild electrophile extends the potential of covalent inhibitors. The growing database of protein structures facilitates the discovery of covalent inhibitors while the advent of proteomic technologies enables a finer resolution in the selectivity of covalently engaged proteins. Here, we discuss recent examples of discovery and design of covalent inhibitors.

Introduction

Despite the historical success of some covalent inhibitors (aspirin, beta-lactams and omeprazole for instance), most libraries for HTS have been pruned of molecules with mildly reactive functionalities that can potentially engage a target covalently. This is largely due to concerns that covalent protein adducts can yield haptens resulting in idiosyncratic immune response, or alternatively cause liver toxicity through promiscuous reactivity. This notion was supported by known cases of allergies to β-lactams and liver toxicity incurred by large doses of acetaminophen, which is metabolized to a reactive N-acetyl benzoquinone imine by P450. Notwithstanding these examples, we are frequently exposed to chemicals containing mildly reactive functionalities from environmental sources, including food and metabolites. It is also noteworthy that covalent inhibition is used endogenously in mammals with prostaglandins and oxidized fatty acids as two examples of endogenous covalent ligands. At late stages of inflammatory episodes, COX2 directs the synthesis of anti-inflammatory cyclopentenone prostaglandins for the resolution of inflammation through covalent inhibition of the IKKβ, thereby resulting in a negative feedback loop of the NF-κB inflammatory pathway.[1] Another example is the activation of the nuclear receptor PPARγ by oxidized fatty acids. Crystal structures of PPARγ bound to oxidized fatty acids revealed a covalent bond between a cysteine and unsaturated oxo fatty acids.[2]

To be safe and effective, covalent inhibitors must have a tuned reactivity that will be engaged with the target at a significantly faster rate than promiscuous reactions or inactivation by metabolism. Why take the risk to develop covalent inhibitors? Efficacy and the ability to design selective target engagement. It is well recognized that the efficacy of small molecule inhibitors or ligands correlates well with residence time ($1/k_{off}$) of the small molecule in the binary complex with its target protein.[3] With covalent inhibitors, the initial binary complex positions a mildly reactive functionality in proximity to a nucleophilic residue in the protein (most often a cysteine) and the high effective concentration promotes the reaction (Figure 1). This is particularly important when an inhibitor is in competition with an endogenous ligand present at high concentration (such as for ATP-binding pockets) or in competition with a high affinity

interaction (protein-protein interaction or transcription factor-DNA interaction). It should be noted that the rate of covalent engagement will vary depending on the nucleophilicity of the residue stargeted (for instance, some cysteine are predominantly ionized to a more nucleophilic thiolate at physiological pH while other are not) and the type of electrophile (for instance, disulfides and cyanoacrylates are very reactive but lead to reversible covalent engagement)[4]. In terms of selectivity, the juxtaposition of an electrophile in the inhibitor with an unconserved nucleophile in proximity of a conserved binding site across a protein family offers the opportunity to discriminate beyond the supramolecular binary complex. These two points are particularly relevant for kinases as will be discussed. While the most robust approach to design irreversible inhibitors is to capitalize on the heightened nucleophilicity of a cysteine thiol group present in the target protein,[5] covalent inhibitors engaging lysine residues have also been reported. This is conceptually distinct from mechanism-based inhibitors targeting the nucleophilic residue of hydrolytic enzymes (protease or esterase) with reactive warheads in that a catalytically essential residue is engaged and the reaction likely benefits from transition-state stabilization.

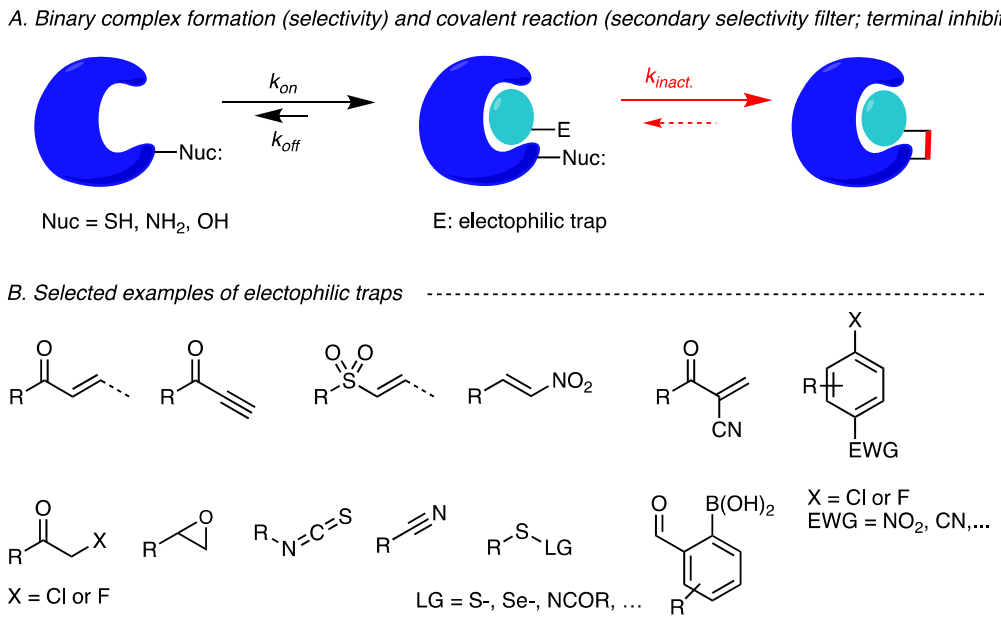

Figure 1. A. Covalent inhibitors bind to a target protein positioning an electrophilic trap (E) to engage a nucleophilic residue (Nuc:) in a covalent bond; B. Selected examples of electrophilic moieties used in covalent inhibitors. Some electrophilic traps form highly reversible covalent bonds (most prominently nitrile, disulfide or cyanoacrylate).

Discovery and design of covalent kinase inhibitors

Kinases have proven to be an attractive target class for the development of covalent inhibitors. There are approximately 530 protein kinases in the human genome, making this enzyme family one of the largest, and their regulatory role in important signaling cascades is known to be dysfunctional in a plethora of disease, most prominently in cancer. However, this enzyme class was originally perceived as undruggable due to the fact that while molecules with high affinity for the ATP-binding site were known (staurosporine for instance), this nucleotide-binding site is highly conserved across the family and achieving selective inhibition was deemed intractable. Furthermore, any drug targeting the nucleotide-binding site would be in competition with ATP, which is present at mM concentrations, making it difficult to achieve sustained

inhibition. The first inhibitors to achieve meaningful selectivity across the kinome were found to bind an inactive conformation adopted by some kinases. Prominent examples are imatinib and lapatinib that target the inactive “DFG-out” and “C-Helix out” conformations respectively. Although drug discovery efforts over the years have demonstrated the plasticity of the ATP-binding site of kinases,[6] achieving high kinase selectivity has proven extremely challenging.

Sequence alignment of kinases revealed unconserved cysteine residues in the vicinity of the nucleotide-binding site, which could provide a selectivity filter for moderately selective compounds targeting the nucleotide-binding site as only a specific subset of kinases would allow a suitable alignment of the reactive moiety (cysteine trap) and the targeted cysteine resulting in a covalent interaction. This was prominently illustrated with the design of design of RSK1, 2 and 4 inhibitor FMK (Figure 2A), which achieved selectivity by combining the presence of an aryl ring on the aniline enabling binding to a subset of kinases with small gatekeeper residues and a cysteine trap poised to engage Cys463 present in only 11 kinases; RSKs aside, the eight other kinases have a large gatekeeper residue precluding pharmacophore interaction.[7] Another early example includes PD168393 which was designed to interact with Cys797 in EGFR and eight other related kinases (Figure 2B).[8]

Natural products were also instrumental in establishing the selectivity of covalent kinase inhibitors. Hypothemycin and related resorcylic acid lactones (RALs, RALs, Figure 2C) were shown to selectively engage a subset of kinases bearing a cysteine preceding the DFG motif (Cys166 in ERK2).[9] The resorcinol ester recapitulates the key hydrogen bond networks of a purine and positions the *cis*-enone to act as a selective cysteine trap. Focused medicinal chemistry efforts to improve upon this natural product family yielded analogs with activities against VEGFRs, TAK1 and MEK-1.[10-12] It is interesting to note that hypothemycin analogs were shown to be efficacious in animal models of VEGFR-driven kidney cancer with dosing every other day despite the short half-life of the drug when exposed to physiological concentration of thiols,[13] thereby clearly highlighting the fact that terminal inhibition of the target can outlast a therapeutically effective concentration of the drug. The duration of effect is limited by target turnover and protein function is only restored following new protein expression.

A remarkable example of selectivity with covalent kinase inhibitors was reported by Jänne, Gray and coworkers with the development of WZ4002 that selectively targets the T790M mutant of epidermal growth factor receptor (EGFR) (Figure 2D).[14] The clinical efficacy of EGFR inhibitors in non-small-cell lung cancer (NSCLC) is well established but limited by the development of drug-resistance mutations, particularly the gatekeeper T790M mutation. WZ4002 has a chloride substituent on the pyrimidine ring, which interacts with Met790 through a halogen bond and provides a mutant-specific binding enhancement. This compound was found to be 100-fold more potent against the T790M mutant over wild-type EGFR.

A detailed analysis of the position of the cysteine residue in the nucleotide-binding site of kinases taking into account the different the different kinase conformations has been reported highlighting the fact that over half of the kinome could be addressed with covalent inhibitors (Figure 2E).[15] To illustrate the predictive power of this analysis, a covalent inhibitor based on the imatinib pharmacophore was designed to target KIT/PDGFR (Figure 2F). This pharmacophore is known to bind reversibly to 20 different kinases, but within this set only KIT/PDGFR have the cysteine residue suitably positioned and were efficiently inhibited. on The imatinib scaffold has also been used to target another subset of kinases (JNK1 and 3, Cys154, JNK-IN-8, Figure 2F) with a different positioning of the electrophile.[16]

Extending beyond this cysteine-mapping analysis, Gray and coworkers recently identified a covalent inhibitor of CDK7 (THZ1, Figure 2G), which binds the nucleotide-binding site but extends an electrophilic warhead to reach a cysteine outside of the canonical kinase domain (Cys312).[17] Sequence alignment of

the 20 cyclin-dependent kinases (CDKs) indicates that Cys312 is unique to CDK7. A kinome-wide binding profile indicated that several other kinases are targeted by the pharmacophore, but only CDK7 showed a time-dependent inhibition, indicative of a covalent engagement. Extending on this study, the same group developed CDK12- and CDK13-selective covalent inhibitor THZ531. The absence of CDK12 and CDK13 inhibitors had hampered investigations into in the role of these CDKs in cancer. THZ531 caused a loss of gene expression with concurrent loss of elongating and hyperphosphorylated RNA polymerase II. In particular, THZ531 substantially decreases the expression of DNA damage response genes and key super-enhancer-associated transcription factor genes.[18] Collectively, these examples clearly illustrate the potential to design enhanced selectivity of kinase inhibitors by coupling the pharmacophore bias to the alignment of a cysteine trap with unconserved cysteines. To this end, kinases benefit from extensive structural information provided through the multitude of co-crystals of kinases with an inhibitor. Extensive medicinal chemistry efforts in the area have culminated in recently approved drugs that target kinases covalently.[19]

Covalent inhibitors of bromodomains and ATPase and GTPase

Bromodomains function as epigenetic readers and several non-covalent inhibitors have brought this target class into the drug discovery spot-light.[20] There are 61 bromodomains encoded in the human genome.[21] They share a conserved fold of four α-helices that form a binding cleft for theirinteraction with acetylated lysines residues on histones (Figure 3A). Sequence alignment revealed that 55 out of 61 human bromodomains contain a cysteine in the vicinity of the ligand-binding site and could be harnessed for covalent inhibition.[22] As an entry to discover a covalent ligand, As an entry to discover a covalent ligand, wwe screened a library using DNA display of PNA-encoded fragment pairs[23] against PCAF leading to the discovery of two compounds sharing a fragment from an FDA-approved drug containing a Michael acceptor (ethacrynic acid). Both of these compounds were evaluated against a panel of 32 bromodomains showing a unique covalent engagement profile consistent with pharmacophore biases in the initial binding complex for the two molecules. This profile established that at least five distinct cysteine positions in the human bromodomain family can be engaged covalently. Furthermore, these covalent bromodomain binders were competent in enriching low abundance bromodomains by pull-down for proteomic studies.

The use of quinazoline-based covalent inhibitors extends beyond kinases. As previously discussed, tThe presence of nucleotide-binding pockets in diverse ATPases suggests that libraries based on quinazoline motifs might be applicable beyond covalent inhibition of kinases beyond kinases. Zhang, Cravatt and coworkers reported the discovery of a highly selective covalent inhibitor that targets a cysteine in the nucleotide-binding site of the vacuolar H^+ ATPase (V-ATPase, Figure 3B).[24] The discovery of this V-ATPase was achieved by profiling a library prepared to target the kinase EphB3. Impressively, this molecule had remarkable proteomic selectivity for V-ATPase with essentially one band out of crude lysates being labeled by the inhibitor as revealed by fluorescence imaging and chemical proteomic activity-based profiling. The site of covalent modification was mapped to Cys138 located in the soluble region of V-ATPase subunit A, which is which is thought to regulate the dissociation of V-ATPase. While chloroacetamide warhead could have been anticipated to afford some level of promiscuous reaction, the exquisite proteomic selectivity attests to the potential of such functionality in covalent inhibitors. D. It is noteworthy that kinases were not identified as targets of this compound considering the quinazoline pharmacophore is an important motif in kinase inhibitors. While some kinase binding should be expected, it does not yield a covalent adduct.

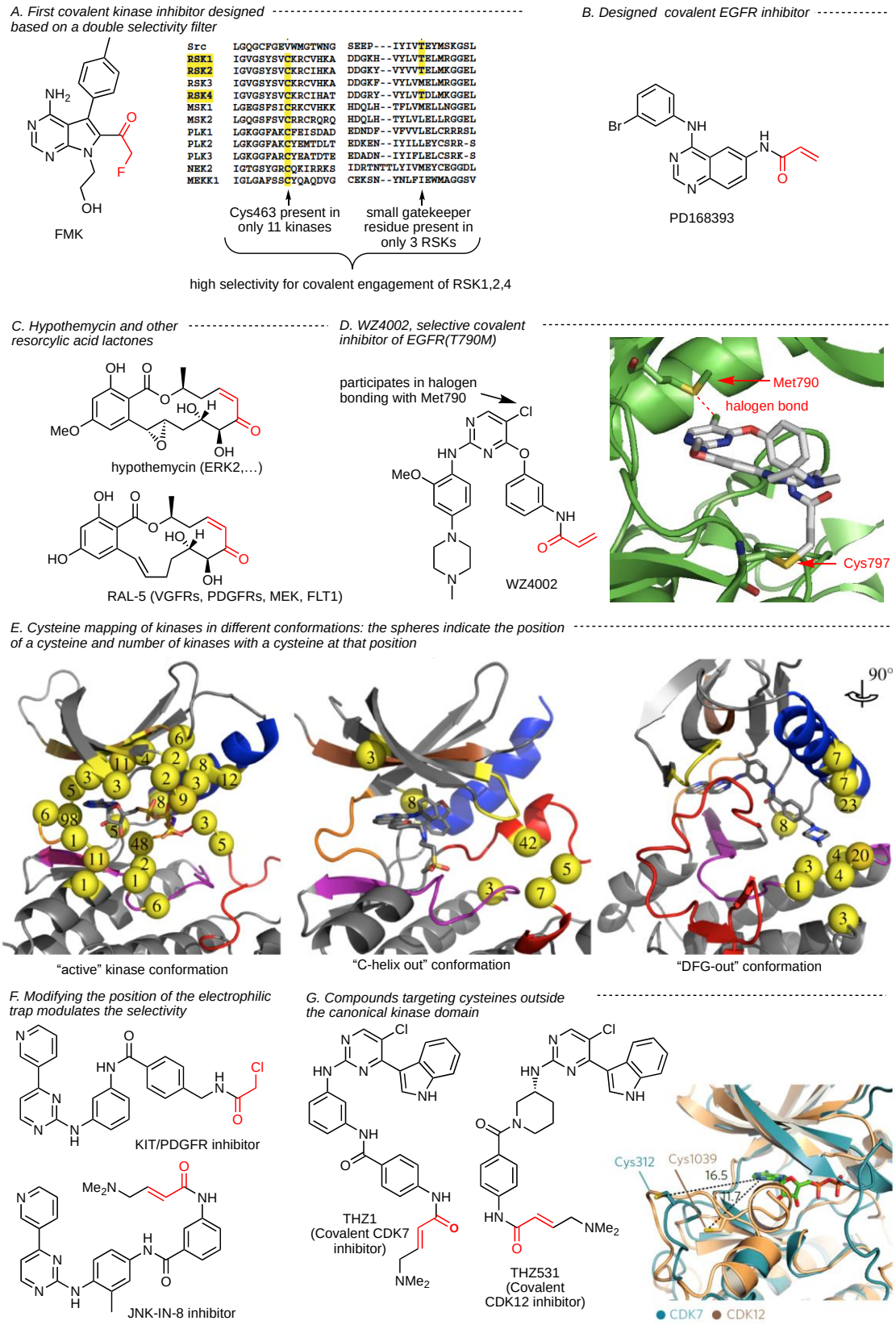

Figure 2. Designed covalent kinase inhibitors.

Another recent example targeting the nucleotide-binding pocket of an ATPase (HSP72) was reported by Cheeseman and coworkers. HSP72 is a stress-induced chaperone implicated in the maturation and stabilization of a number of oncogenic targets. A challenge in achieving efficacy in HSP72 inhibition is the high affinity for the endogenous nucleotide substrate (K_D = 110 nM).[25] Based on the previously reported co-crystal structure of HSP72 with VER-155008 (Figure 3C), it was speculated that an alkyl chain terminating with an acrylate moiety could replace the aminobenzyl moiety and covalently engage Cys17. However, mass spectrometry analysis of the covalent adduct and a K56A HSP72 mutant confirmed that the covalent trapping proceeds on Lys56 rather than the anticipated Cys17.

Another study by Chiosis and coworkers reported the design of an inhibitor (YK5, Figure 3D) targeting an allosteric site of HSP72 (hHSP70), which is in proximity of Cys267.[26] This compound showed remarkable selectivity for HSP72. Using a biotinylated version of YK5 at five to ten times higher concentrations than those needed to inhibit cellular activity of HSP72 in cancer cells resulted in the selective formation of YK5-HSP72 adducts. Furthermore, at the physiologically relevant concentration of 10 µM (i.e. the concentration needed to maximally inhibit cancer-related HSP70 functions), YK5 was also inert when tested against a panel of 402 kinases. These findings point to the excellent selectivity of YK5 for its target.

Beyond the analysis of unconserved cysteines present in wild-type targets of interests, an analysis of mutations in human cancer revealed that a Beyond the analysis of unconserved cysteines present in wild-type targets of interests, an analysis of mutations in human cancer revealed that acquired cysteine is the most abundant somatic mutation.[27] The potential to harness one such cysteine for the design of covalent inhibitors was elegantly demonstrated in independent studies by the Shokat and Gray groups for the KRAS(G12C) mutant, one of the most common mutations in the KRAS proto-oncogene and present in half of the RAS-driven lung carcinoma (Figure 3E).[28] It should be noted that GTPases such as RAS have historically proven exceptionally difficult to inhibit due to the high affinity of the protein for GTP/GDP (pM). Shokat and coworkers screened a small library of fragments containing a disulfide electrophile and identified a chemical starting point for optimization of a covalent inhibitor with a Michael acceptor warhead. This led to the identification of a previously unrecognized allosteric binding site. Evaluation of the compound's cellular efficacy indicated a KRAS(G12C)-specific growth inhibition. The compound was shown to impair downstream signaling by allosterically biasing the inactive GTP-bound conformation and weakening its interaction with RAF. In parallel, Gray and coworkers designed a GDP analog (SML-8-73-1) containing a chloroacetamide functionality that was shown to engage Cys12 of KRAS(G12C).[29] While the presence of the phosphate curtailed cellular permeability of this compound, a caged version of the compound was shown to effectively disrupt RAS signaling in cells.

Covalent inhibitors extending beyond a given enzyme class: Targeting pathogens or PPI

Covalent quorum sensing inhibitor: Bacteria regulate growth through a mechanism known as quorum sensing. In *Pseudomonas aeruginosa*, this quorum sensing is initiated by a transcription factor, LasR that binds to the quorum sensing messenger 3-oxo-C_{12}-HSL. While high affinity ligands of LasR have been reported, an obstacle in achieving efficacy is the high affinity of LasR for its ligand 3-oxo-C_{12}-HSL. Meijler and coworkers reported the design of a covalent version of this ligand that harnesses the presence of cysteine residue (Cys79) in the vicinity of the binding site (Figure 4A). The covalent binding was anticipated to compete effectively with 3-oxo-C_{12}-HSL for binding to LasR and produce a slightly altered occupation of the binding pocket upon covalent engagement resulting in a less optimal conformational

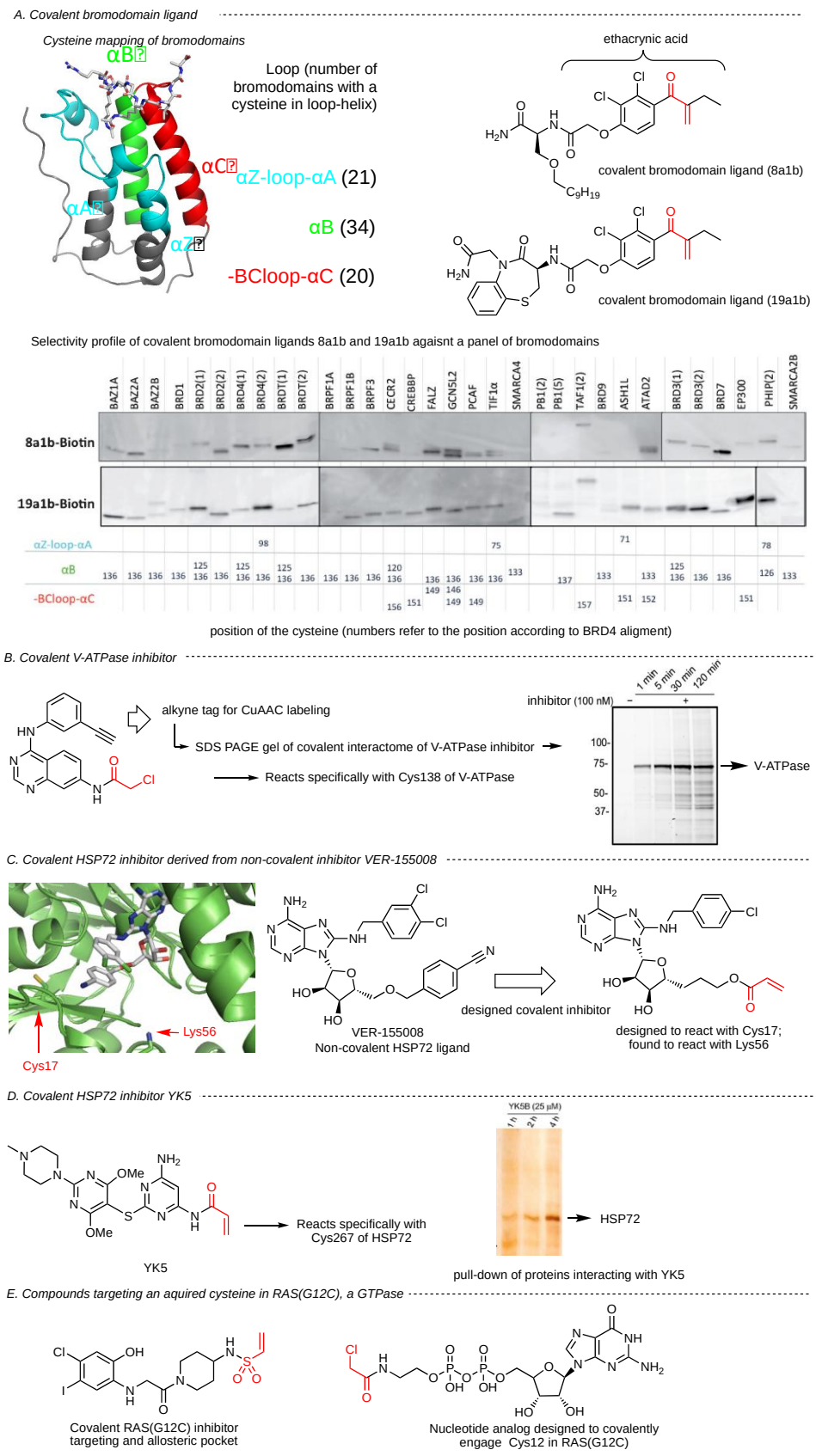

Figure 3. Covalent inhibitors of diverse Bromodomains, ATPases and a GTPase.

for transcriptional activation. Several functionalities were evaluated as cysteine traps (bromoacetamide, chloroacetamide and isothiocyanate) with varying alkyl chain lengths to identify ITC-12 as the most effective molecule. MS analysis confirmed covalent target engagement. Cellular assays confirmed the specific inhibition of gene expression regulated by quorum sensing, thus inhibiting the production of virulence factors and biofilm formation. This first example of covalent modification of a quorum sensing receptor provides a new avenue to combat antibiotic resistance among bacterial pathogens as well as a tool to further unravel the complicated quorum sensing regulation in *P. aeruginosa*.[30] A subsequent study by the same group reported several series of new inhibitors, one of which, fluoro-substituted ITC-12, displayed complete covalent modification of LasR, as well as promising *in vivo* results.[31]

Covalent inhibitor of MEP: The methylerythritol phosphate (MEP) pathway is an essential metabolic pathway found in protozoa, including malaria parasites, and bacteria but absent in higher eukaryotes, making it a highly attractive target for the discovery of novel anti-infective agents. Notably, IspD, the third catalytic enzyme of the MEP pathway of different malaria parasites, has a cysteine in the vicinity of the catalytic site. Using high-throughput screening, Odom, John and coworkers identified a compound that inhibits IspD of *P. falciparum* and *P. vivax* through covalent interaction with Cys202 and prevents the growth of *P. falciparum* in culture, with EC_{50} values below 400 nM (Figure 4B). Enzymatic, genetic, crystallographic and modeling studies have established that covalent trapping proceeds through disulfide bond formation following attack of Cys202 on the isothiazolone core. The species-selective inhibitory activity of these small molecules suggests that they have potential as lead compounds in the pursuit of novel drugs to treat malaria.[32]

Beyond small molecule covalent inhibitors, Walensky and coworkers recently reported the design of a stapled peptide with an acrylamide functionality that can covalently engage an intracellular target to obstruct a protein-protein interaction taking place through a large surface groove interaction. This example is significant in light of the challenge to outcompete such interactions with small molecules. In their study, the authors identified a cysteine residue (Cys55) located at the tail of the surface groove of BFL-1, which interacts with the alpha-helical BH3 domains of NOXA (Figure 4C). Cancer often relies on the overexpression of anti-apoptotic BCL-2 family proteins that block programmed cell death by trapping pro-apoptotic proteins through protein-protein interactions. BFL-1 is a BCL-2 homolog implicated in melanoma, lymphoma and other cancers. A stapled version of the BH3 domain was derivatized with diverse acrylamides at the N-terminus in order to covalently engage the proximal Cys55. The authors found a range of candidates for covalent engagement with optimal alignment for the *R*-nipecotyl moiety (the opposite stereochemistry or a proline moiety only gave a fraction of covalent engagement). This stapled peptide was shown to selectively bind and react with BFL-1 in cells and restore apoptosis in treated cells. The compound showed striking selectivity for BFL-1 over its close homologs MCL-1 and BCL-X_L attesting to the engineered selectivity. Given the frequent proximity of native cysteines to regulatory binding surfaces, covalent stapled peptide inhibitors provide a new therapeutic strategy to inhibit key regulatory binding interfaces (protein-protein interactions) and provide a new dimension to stapled peptides.[33]

While targeting cysteine residues in proteins represent a rational choice based on the enhanced nucleophilicity of thiols, the paucity of this residue is both an asset for selectivity but also a limitation since many binding sites are deprived of surface-exposed cysteines. Thus, modifying solvent-exposed lysine residues would truly expand the range of proteins that can be targeted for covalent inhibition. The lysine side chain is well known to rapidly engage in imine formation with aldehydes under physiological conditions. The rapid equilibrium in this reaction however curtails the benefit of covalent reaction. A notable example is the discovery of a formyl coumarin which formed an imine with Lys907 of IRE1,

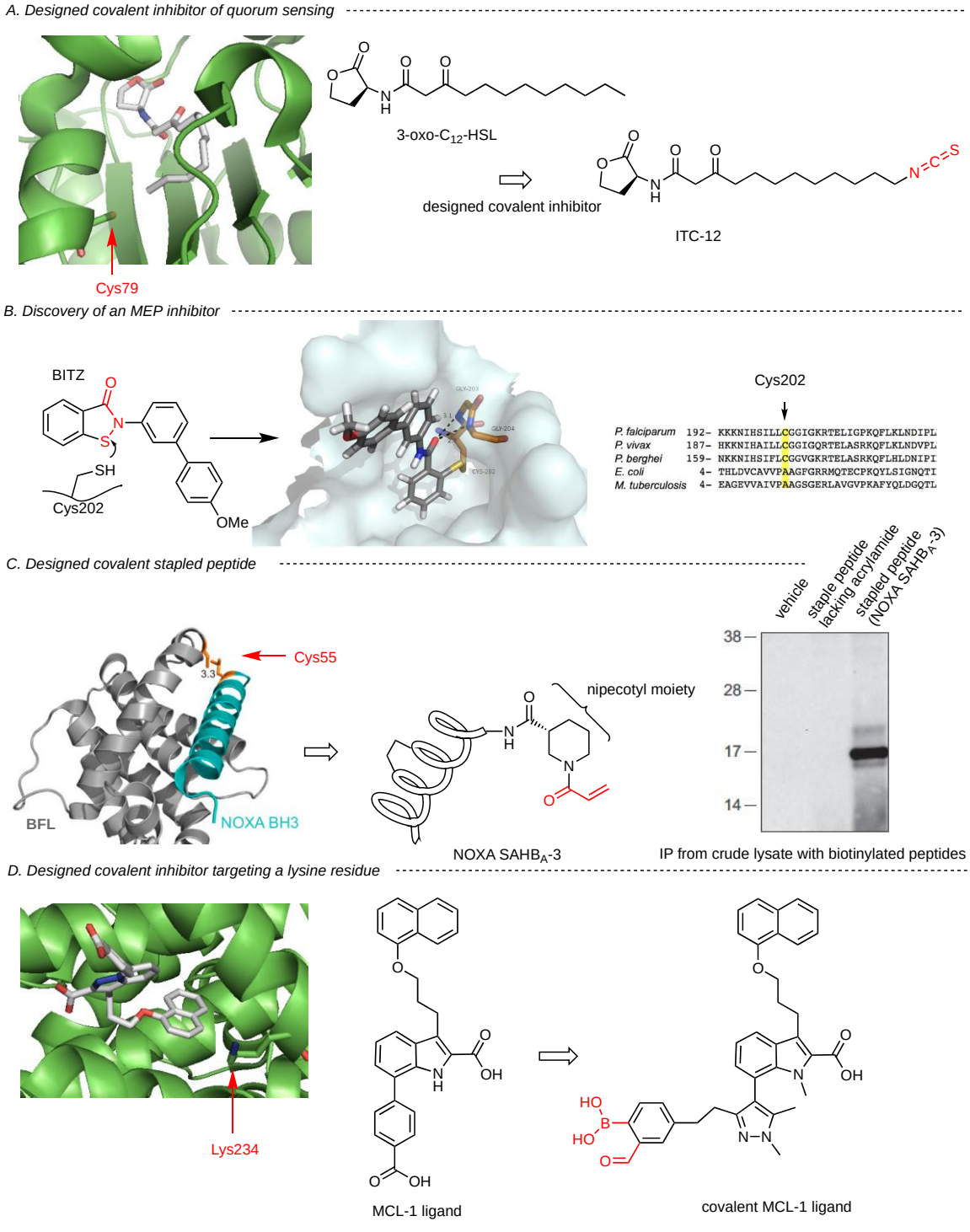

Figure 4. Selected examples of designed covalent inhibitors of other target classes.

however, washout experiments in cells restored enzymatic activity suggesting a reversible complex with the target protein.[34] The rate of equilibration can be dramatically slowed down with a boronic acid *ortho* to the aldehyde (such as 2-formylbenzeneboronic acid), which forms a stable iminoboronate.[35] This novel covalent trapping of lysines was explored by Su and coworkers to develop a protein-protein-inhibitor (PPI) of Myeloid cell leukemia 1 (MCL-1), a key survival factor in a wide range of human cancers, which binds and neutralizes the BH3 domains of pro-apoptotic proteins. Based on the X-ray crystal structure of MCL-1 with known inhibitors, it was anticipated that the compound could be extended to reach Lys234 with a 2-formylarylboronic acid (Figure 4D). The designed inhibitor containing the warhead had a 100-fold better IC_{50} than its non-covalent congeners (ligand lacking the formyl group and boronic acid). The covalent ligand proved to be highly efficacious in reactivating apoptosis in an MCL-1-dependent manner. Immunoprecipitation studies demonstrated that this covalent compound inhibited the MCL-1-BH3 interaction in cells while LC-MS showed clear evidence of covalent engagement.[36],

Conclusion

The examples presented highlight the benefit of covalent inhibitors in terms of designing additional target selectivity compared to traditional inhibitors relying solely on molecular recognition principles. Covalent target engagement offers the added advantage of terminally inactivating the target, which translates in high potency and extended duration of action. The covalent inhibitor approach is gaining acceptance in drug development and three designed covalent inhibitors have recently been approved for therapeutic intervention.[19,42,43] Genomic information coupled to the growing wealth of structural information offers a fruitful conjuncture to capitalize on the design of selective covalent inhibitors. While the majority of the work has focused on cysteines as the nucleophilic residue for covalent target engagement, it has been shown that the same principles can be extended to other amino acid side chains, notably lysine. Progress in the development of novel warheads will further extend our ability to engineer selective covalent inhibitors. The growing interest in covalent inhibitors will stimulate the development of libraries focused on covalent targeting.[44] Screening virtual libraries of electrophilic small molecules has proven a fruitful entry into covalent inhibitor leads.[45] The emergence of DNA-encoding as a rapid method to screen libraries of small molecules[46] is poised to make an impact in this area and novel screening methods to differentiate covalent from high affinity non-covalent inhibitor in a primary affinity screen have been reported.[47] In parallel, the development of novel reagents that broadly label cysteines will further enhance our ability to assess target selectivity without recourse to an inhibitor conjugated to a tag.[48] This is important because such conjugation can bias target engagement or be synthetically demanding

Acknowledgments

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