Targeted Covalent Inhibitors for Drug Design

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Keywords: covalent drugs · drug design · electrophiles · inhibitors · protein modifications
In contrast to the traditional mechanism of drug action that relies on the reversible, noncovalent interaction of a ligand with its biological target, a targeted covalent inhibitor (TCI) is designed such that the initial, reversible association is followed by the formation of a covalent bond between an electrophile on the ligand and a nucleophilic center in the protein. Although this approach offers a variety of potential benefits (high potency and extended duration of action), concerns over the possible toxicological consequences of protein haptenization have hindered the development of the TCI concept. Recently, approaches to mitigate the risk of serious adverse reactions to this new class of agent have emerged, thus stimulating interest in the field and leading to authorization of the first cadre of TCIs to be marketed. The covalent inhibitor approach is rapidly gaining acceptance as a valuable tool in drug discovery, and is poised to make a major impact on the design of enzyme inhibitors and receptor modulators.

1. Introduction

The term “targeted covalent inhibitor” (TCI) refers to a compound designed to bind covalently to a specific molecular target and thereby suppress its biological function. This approach to drug discovery, which typically requires extensive bioinformatic support, adopts the principles of structure-based drug design, and may be distinguished from other pharmacological mechanisms that involve formation of a covalent adduct with proteins, for example, mechanism-based (“suicide substrate”) inhibitors that require activation by the target enzyme.[1] The process by which TCIs achieve “protein silencing” relies upon two related, but discrete, steps, the first of which involves the reversible association of the inhibitor (a high-affinity ligand) with its biological target in such a manner that a weakly electrophilic functionality (“warhead”) on the ligand is brought into proximity to an appropriately positioned nucleophilic residue on the protein (usually, but not always, a cysteinyl -SH group). In the second step, spontaneous reaction occurs between the participating functional groups on the ligand and protein to yield the covalently modified, inactivated protein. The action of a TCI can be described by the generic mechanism shown in Equation (1), where \( K_i \) refers to the noncovalent binding constant (affinity of the ligand for the enzyme) and \( k_{\text{inact}} \) to the rate constant for enzyme inactivation.[1]

\[
E + I \rightleftharpoons E-I \rightarrow E-I_{\text{inact}} \rightarrow E + I
\]

Equation (1)

A variety of chemical reactions may be involved in the formation of an adduct, some of which give rise to products that would be anticipated to exhibit reversibility on the biological timescale (e.g. acylations, formation of thioimides, hemiketals, or disulfides), while others are more likely to inactivate proteins in an essentially irreversible fashion (e.g. alkylations, Michael addition reactions).[2,3] Thus, by careful design of the ligand structure and judicious choice of reacting functionalities on the ligand and protein, a high degree of selectivity can be built into the TCI approach, together with factors such as reversibility that contribute to the duration of action of the inhibitor. As will be discussed in Section 3, selectivity has emerged as a critical consideration in successful TCI design.[4]

A number of potential benefits accrue from the TCI approach, including high potency (derived from complete blockade of the target),[5] low dose (a consequence of high potency), extended duration of action (determined by the turnover time of the protein as opposed to the pharmacokinetic (PK) properties of the drug), and general applicability to enzymes, receptors, and targets with shallow binding sites not amenable to conventional approaches.[1–4,6,7] In addition, techniques based on the use of activity-based probes,[8–10] click chemistry,[11] and proteomics mass spectrometry[12] allow for the assessment of selectivity and of time- and dose-dependent target occupancy in vitro and in vivo, which in turn enables the construction of target occupancy/biological effect relationships.[3] The latter replace pharmacokinetic–pharmacodynamic (PK-PD) relationships for this class of drugs, and are critical in the translation of the preclinical effects of TCIs to the human situation. Finally, in the kinase field, “second generation” TCIs such as afatinib and neratinib (HKI-272) have demonstrated high activity against mutant forms (T790M, L858R) of the epidermal growth factor receptor (EGFR) that represent the major source of resistance to chemotherapy with the “first generation” reversible EGFR inhibitors gefitinib, erlotinib, and lapatinib.[13] The T790M mutation in EGFR kinase increases the ATP affinity of the oncogenic L858R mutant by more than an order of magnitude, thereby allowing more extended duration of action.

From the Contents

1. Introduction 13409
2. Reversibility and Duration of Action 13412
3. Selectivity and Potency 13414
4. Metabolism and Disposition 13416
5. Mitigating Toxicity Risks with Covalent Inhibitors 13417
6. Summary and Outlook 13418
magnitude, thus rendering competitive inhibitors ineffective, but covalent modification of Cys-797 in the ATP-binding pocket by the above TCIs overcomes this resistant phenotype.[14]

Despite the anticipated benefits of the TCI approach in drug design, application of the concept to industrial drug discovery programs did not commence in earnest until the late 1990s,[15] in part due to the prior lack of the bioinformatics and proteomics tools, which now have become integral components of covalent inhibitor design. More importantly, perhaps, was the cautionary influence of research into the mechanistic basis of serious drug-mediated toxicities conducted during the preceding two decades that implicated chemically reactive drug metabolites as the causative agents in many cases.[16,17] Thus, it was demonstrated that the hepatotoxic effects of several approved drugs (e.g. acetaminophen, isoniazid, furosemide, halothane) and model hepatotoxins (e.g. bromobenzene, thioacetamide) in both animals and humans could be linked to the generation of electrophilic intermediates produced during the course of oxidation reactions catalyzed by cytochrome P-450 (CYP).[18] Radiolabeling studies showed that the reactive species generated from these agents became covalently bound to cellular proteins, and it was hypothesized that the resulting structural and functional damage served as the basis of the observed cellular toxicity.[19] In some situations, drug–protein adducts appeared to be immunogenic in susceptible individuals, triggering a response that was manifest as an idiosyncratic reaction, often weeks or months after initiation of therapy.[20] As a consequence, the pharmaceutical industry understandably became reluctant to embark on the development of compounds whose mechanism of action depended upon the covalent modification of cellular proteins. Molecules bearing reactive functional groups had long been viewed as unattractive drug candidates since they typically exhibited poor PK properties (notably high clearance),[21] while compounds that underwent metabolism to reactive electrophiles were now being screened out at an early stage of development in an effort to minimize the prospect of encountering drug-induced toxicities.[22–24]

Against the above backdrop, two factors emerged from research in the drug metabolism/toxicology fields that called into question the existing prejudice against covalent modifiers as therapeutic agents. Firstly, it was noted that not all drugs that underwent metabolic activation were toxic to the host, and although the underlying reasons for this were (and largely remain) unclear, the observation suggested that not all types of covalent modifications of proteins necessarily lead to toxicity. Support for this notion came from the discovery that a number of safe and effective marketed drugs exert their pharmacological effects through covalent modification of their respective protein targets; thus, aspirin and β-lactam-containing antibiotics (penicillins, cephalasporins, carbapenems) serve as acylating agents of cyclooxygenases (COX-1 and -2) and bacterial D-ala,D-ala-transpeptidase, respectively; omeprazole and related proton pump inhibitors are converted in gastric acid into reactive sulfenic acids that exist in equilibrium with sulfenamides and bind to the H+K+-ATPase in parietal cells in the stomach,[22–25] and clopidogrel and associated antplatelet drugs also undergo conversion into sulfenic acids (albeit in this case through oxidative metabolism) and covalently bind to the P2Y12 purinoreceptor on the surface of blood platelets[2,26] (Figure 1). It is worth noting that the types of adducts that are generated by these drugs involve either ester derivatives of serine residues (aspirin and β-lactams) or disulfide derivatives of cysteine residues—in both cases, linkages that can be reversed enzymatically in a biological environment (by hydrolysis and reduction, respectively), such that the lesions to target proteins would not be permanent in nature. Whether this feature contributes to the excellent safety record of these classes of drugs remains unknown, but the question is very relevant to the design of safe TCIs (see Section 2). Secondly, studies on the nature of the covalent protein adducts generated as a result of the metabolic activation of hepatotoxic drugs demonstrated that the reactive metabolites in question reacted with numerous liver proteins, and that the linkages to nucleophilic centers on proteins were not of the type that would be amenable to reversal through chemical or enzyme-mediated processes. For example, the reactive metabolite of acetaminophen, N-acetyl-p-benzoquinone imine (NAPQI), forms adducts to over 20 proteins in mouse liver,[2] thus indicating a marked lack of selectivity for binding to individual protein targets—an unsurprising finding in light of the high chemical reactivity of this electrophile.[28] Interestingly, NAPQI (and p-benzoquinone, which is formed from acetaminophen in parallel)[29] exhibits a strong preference for cysteinyl thiols as sites for alkylation on proteins, forming stable thioether conjugates at the C-3′ position on the aromatic ring (Figure 2).[30] These results, together with those from studies on the adducts derived from other electrophilic intermediates,[31] highlight an important feature of chemically reactive drug metabolites, namely that they tend to exhibit a high degree of electrophilicity and, as a result, bind in a relatively indiscriminate fashion to cellular proteins. These characteristics are in direct contrast to those of the ideal TCI, which bears a weakly electrophilic warhead that is delivered to a unique protein target.

Based on the emerging distinctions between chemically reactive metabolites and TCIs in terms of their intrinsic electrophilic properties and selectivity toward protein targets, it was argued that the time had come to reassess the merits of developing selective covalent modifiers as drug candidates.[2,3] Initial efforts focused on the design of acylating analogues of
naturally occurring β-lactams and β-lactones,[32,33] inspired by the diverse chemistry employed by protein-reactive natural products.[34] However, weakly reactive Michael acceptors (primarily acrylamide derivatives) soon became the favored warheads on TCIs aimed at protein kinases such as EGFR T790M, erbB2, Her2, and the Tec family kinase, Bruton’s tyrosine kinase (BTK), where alkylation of the active site Cys-481 by agents such as ibrutinib (PCI-32765) led to potent inhibition of the enzyme.[35–39]

Over the past decade, the TCI concept has quickly gained acceptance as a valuable tool in drug discovery, and a diverse array of proteins have been targeted by this approach, including a range of serine, threonine, and cysteine proteases[40] such as HCV NS3/4A[41] and NS5B[42] viral proteases, Dengue viral protease,[43] and methionine aminopeptidase 2.[44] Additional targets have included sickle cell hemoglobin,[45] fatty acid amidohydrolase,[46,47] Keap-1,[48] caspase-1,[49] glyceroldehyde-2-phosphate dehydrogenase,[49] 20S-proteasome,[50] and mutant Ras protein.[51] The most intense activity, however, has been in the field of protein kinases[52] where, in addition to BTK and the acquired drug resistant forms of EGFR, TCIs have been developed for Bmx,[53] FGFR,[54] GSK3β,[55] Itk,[56] JNK,[57] and mutant Ras protein.[51]

**Figure 1.** Examples of drugs whose pharmacological mechanism of action was found retrospectively to involve covalent modification of their respective biological targets. These compounds were not, however, designed a priori to act as covalent inhibitors, and so they are not classified as TCIs. Penicillin G (a) and aspirin (b) are inherently reactive acylating agents that form esters with serine residues of proteins, while omeprazole (c) and clopidogrel (d) require activation in vivo to generate electrophilic intermediates that form disulfide bridges to cysteine residues on their target proteins.

**Figure 2.** Cytochrome P-450 mediated oxidation of acetaminophen to reactive quinone intermediates (NAPQI and benzoquinone) that covalently modify a wide range of proteins through reactions with cysteiny1sulfhydryl groups. For a detailed discussion of the underlying mechanisms of catalysis, the reader is referred to Ref. [29].
Kit/PDGFR,\cite{58} Nck2,\cite{59} PI3k\alpha,\cite{60} RSK,\cite{61} eEF-2K,\cite{62} and Src family kinases.\cite{63,64}

The object of this Review is to summarize the evolution of TCIs over the past decade, with an emphasis on the key findings from clinical candidates and recently approved covalent drugs. Representative examples of TCIs that have now received FDA marketing approval are shown in Figures 3 and 4. For a more comprehensive survey of electrophilic warheads, current TCI protein targets, and compounds in various stages of development, the reader is referred to several excellent reviews on the subject.\cite{1,3,6,13,24,65–70}

2. Reversibility and Duration of Action

Covalent inhibitors may be classified into two broad categories according to whether their adducts with the protein targets are functionally reversible on the biological timescale. Reversible TCIs bind covalently to their target and subsequently dissociate from it at a rate that is faster than the turnover rate of the protein.\cite{3} Examples of this mechanism may be found in the reaction between proteins and inhibitors bearing electrophilic warheads such as aldehydes, activated ketones, \(\alpha\)-ketoamides, \(\alpha\)-keto heterocycles, carbonitriles, and boronic acid derivatives, many of which serve as covalent transition-state inhibitors that act as reversible TCIs of the 20S- or 26S-proteasome,\cite{71} serine hydrolases,\cite{72} cathepsin cysteine proteases,\cite{73} and dipeptidyl peptidase IV\cite{74} (Figure 3). Irreversible TCIs, on the other hand, form adducts to their protein targets that either do not dissociate from the protein during its lifetime or do so with a kinetic half-life that is significantly longer than the resynthesis rate of the protein.\cite{3} Such compounds, many of which are kinase inhibitors (Figure 4), often contain warheads consisting of harder electrophiles, for example, epoxides, \(\alpha\)-haloketones, aclyoxymethyl ketones, aziridines, vinylsulfones, and activated acetyl enes, but most commonly employ Michael acceptors based on the acrylamide functionality, which is a relatively poor electrophile that requires proximity to the nucleophilic target for reaction.

In general, TCIs that act by a reversible mechanism will require sustained plasma concentrations to maintain efficacy at the target (just as noncovalent drugs do), whereas the duration of action of irreversible inhibitors can be quite prolonged, since the recovery of activity depends on the de novo synthesis of new protein. For example, it was
reported that, whereas interleukin-2 inducible T cell kinase (Itk) has a half-life of approximately 2 h in resting primary T cells in vitro, the covalent inhibition of Itk resulted in stabilization of the protein and functional silencing of the T cell receptor pathway for more than 24 h.\textsuperscript{[60]} Similar effects are observed in vivo, where the duration of the pharmacological effect of a TCI in animals and humans can greatly exceed its PK half-life. A case in point is the approved BTK inhibitor ibrutinib (IMBRUVICA), which has an apparent half-life in human subjects following oral dosing of 3 h,\textsuperscript{[75]} but affords >95% target occupancy 24 h after doses of 420 mg/day\textsuperscript{[74]} This effect has been referred to as an “uncoupling” of the PK properties of the inhibitor from its PD effects: although there is, of course, a relationship between the two, it is not the typical linear relationship that exists with noncovalent inhibitors, and it is, therefore, necessary to establish PK-target occupancy relationships to understand the temporal effects of TCIs. On the basis of the above considerations, it has been argued that the maximum benefit of the TCI approach is realized when the inhibitor acts irreversibly, and the protein target in question is turned over slowly, thus permitting less frequent administration and lower drug dose, thereby leading to a reduced propensity to cause off-target effects and drug–drug interactions. However, as discussed in Section 3, a high degree of selectivity for the protein target has emerged as an essential prerequisite for both reversible and irreversible covalent inhibitors.

An interesting variation on the reversible TCI theme was reported by Taunton and co-workers,\textsuperscript{[77,78]} who described the inhibitory properties of a series of heteroaryl-substituted acrylamide derivatives bearing an electron-withdrawing group at the α-carbon atom. As predicted from model studies, kinase inhibitors bearing the α-cyanoacrylonitrile functionality readily formed β-thioether adducts with sulfur nucleophiles, but underwent facile retro-Michael reaction because of the decreased pKₐ value of the α-proton. Only when the complex between the inhibitor and the kinase was stabilized by hydrogen-bonding interactions did the compound behave as an irreversible TCI; when these interactions were lost, such as when the kinase unfolded or underwent proteolysis, the inhibitor dissociated from the enzyme. A variety of other electron-withdrawing substituents at the α-carbon atom produced similar effects, thereby allowing the reactivity of the Michael acceptor warhead to be “tuned” in a predictable manner.\textsuperscript{[79]} This novel approach, which led to the discovery of potent, selective, reversible covalent inhibitors of the p90 ribosomal protein S6 kinase (RSK2)\textsuperscript{[77]} (Figure 5), combines the advantages of reversible and irreversible TCI mechanisms so that protein silencing will persist until the kinase undergoes degradation, at which time the ligand dissociates, leaving no covalently modified peptide fragments for recognition by the immune system as “foreign.”\textsuperscript{[80]} Recently, this “chemically tuned electrophile” concept has been extended to the design of reversible covalent inhibitors of drug-resistant forms of EGFR,\textsuperscript{[81,82]} and it has been suggested that the favorable safety profile of the reactive α-cyano-α,β-unsaturated ketone bardoxolone methyl (CDDO-Me) reflects the formation of a transient covalent adduct with the thiol-rich electrophile sensing protein Keap1.\textsuperscript{[83]}

One aspect of the TCI approach that has received little attention thus far relates to the potential reversibility of covalent adducts that may be formed with off-target nucleophiles, for example, glutathione (GSH) and proteins with exposed nucleophilic centers such as serum albumin. In the case of TCIs that bear the acrylamide warhead, which currently represent the majority of compounds in development, adducts formed through 1,4-conjugate addition (the Michael reaction) can, in principle, revert to their original components through a retro-Michael process. As discussed by Johansson,\textsuperscript{[84]} the steric and electronic factors that control the forward reaction are relatively well understood through studies with model nucleophiles such as GSH, but the factors that affect the back reaction are often less clear and depend to a large extent on the local environment in the protein. Many acrylamide-based TCIs react spontaneously, albeit slowly, with GSH to form the corresponding thioether adducts. In contrast, the rate of reaction with a nucleophile on the protein can be relatively rapid, since the reaction partners are brought into close contact during the noncovalent binding step. Conjugation with GSH represents a common route of TCI metabolism in vivo, where the addition reaction may be accelerated through catalysis by hepatic and extrhepatic glutathione-S-transferase (GST) enzymes.\textsuperscript{[85]} It is interesting to note that agents such as afatinib (GILOTRIF) and the late-stage development candidate neratinib readily form GSH adducts in pH 7.4 buffer and in cytosolic fractions of human liver and kidney in vitro;\textsuperscript{[86]} however, the metabolites of these drugs present in human excreta do not reflect this pathway as a major clearance mechanism, with afatinib being eliminated in feces largely as the unchanged parent compound\textsuperscript{[86]} and neratinib being cleared primarily through CYP3A4-mediated oxidations.\textsuperscript{[87]} (Ibrutinib undergoes GSH-dependent metabolism, but in this case it is a reactive epoxide metabolite of the drug that is the major entity subject to conjugation.\textsuperscript{[88]} This apparent in vitro/in vivo discrepancy in terms of the importance of GSH conjugation in the metabolism of afatinib and neratinib raises the possibility that the addition of GSH (and possibly other nucleophiles) to the acrylamide warhead of these TCIs does occur in vivo, but that the process is reversible through a retro-Michael reaction. Support for this
hypothesis derives from the fact that these drugs form covalent adducts with plasma proteins, as evidenced by the presence of long-lived radioactive components in the systemic circulation of human volunteers given radiolabeled doses of the drugs.\textsuperscript{86,88,89} In the case of neratinib, this non-extractable radioactivity was demonstrated to be due to the formation of a covalent adduct with human serum albumin (HSA), in which the ε-NH$_2$ moiety of Lys-190 (in contrast to the more nucleophilic Cys-34 thiol) had become bound through a Michael reaction to the β-carbon atom of the acrylamide warhead (Figure 6).\textsuperscript{89} Subsequent in vitro studies showed that the neratinib–HSA adduct was formed in a reversible manner, such that free neratinib was released slowly from the protein conjugate upon incubation in pH 7.4 phosphate buffer at 37°C for 18 h.\textsuperscript{90} Interestingly, although the neratinib adduct with albumin was observed in the plasma of humans and monkeys, it was not seen in the plasma of dogs, rabbits, or rodents, thus demonstrating the species-dependent nature of this phenomenon.\textsuperscript{90} Given that most TCIs in development are relatively lipophilic molecules that exhibit high (non-covalent) binding to plasma proteins such as serum albumin, it is not surprising that some degree of covalent labeling of these proteins will ensue when the bound inhibitor adopts an orientation that places the warhead in proximity to a nucleophilic site on the protein. Therefore, the species-dependent formation of the neratinib–albumin conjugate probably stems from subtle structural differences in serum albumin across species.

Reversibility in GSH conjugation and formation of the protein adduct has a number of implications for TCI efficacy and toxicity. As proposed by the Pfizer group who studied neratinib, “It is reasonable to speculate that the release of neratinib from human serum albumin provides a transport system leading to release of neratinib in the more acidic environment of the tumor”,\textsuperscript{89} thereby providing a depot of latent inhibitor that is released slowly from its protein carrier. With regard to safety considerations, it has been shown, by using model electrophiles, that those covalent protein adducts that exhibit reversibility tend to be less cytotoxic in vitro than corresponding stable conjugates, presumably since the transient nature of protein adduct formation fails to trigger damage-signaling pathways.\textsuperscript{91} Similar considerations apply to the electrophilic intermediates now known to mediate the pharmacological activity of certain approved drugs, for example, aspirin, penicillin, omeprazole, and clopidogrel (see Section 1), all of which form covalent adducts with proteins that can be cleaved by metabolic processes to regenerate the native protein. The acrylonitrile functionality has also been reported to react in a reversible fashion with protein nucleophiles and GSH, as exemplified by the reaction of a metabolite of the veterinary drug furazolidone with thiol groups on microsomal protein (Figure 6).\textsuperscript{92} The net result of such reversible Michael additions is that the thiol serves as a vehicle to transport the electrophile within and outside the cell.\textsuperscript{93} Clearly, much remains to be learned about the biological consequences of reversibility in the reaction between TCIs and their target proteins, as well as with GSH and off-target macromolecules, and it is anticipated that a better understanding of this issue will emerge from ongoing development efforts with the current suite of TCI drug candidates.

### 3. Selectivity and Potency

As the TCI approach to drug discovery continues to mature, it has become generally accepted that high selectivity for the intended protein target is critical, and probably represents the single most important feature in the design of safe and effective covalent inhibitors.\textsuperscript{4,67,68,80} Safety concerns related to the potential immunogenicity of proteins forming a covalent adduct (and the modified peptides to which they ultimately are degraded) dictate that the range of proteins subject to forming an adduct by a TCI should be kept to a minimum to decrease the risk of idiosyncratic toxicity—this would appear to be particularly true for TCIs that operate through an irreversible mechanism whereby the covalent modification accompanies the protein throughout its lifetime—and a number of experimental approaches have been adopted to guide the

![Figure 6. Reversible Michael addition reactions seen with a) neratinib, a TCI of the EGFR receptor, and b) an acrylonitrile-containing metabolite of the veterinary drug furazolidone.](image)
development of highly selective covalent inhibitors. The initial design of the scaffold to which the warhead is to be attached is often based on known high-affinity, reversible ligands for the target, and may be optimized through measurement of the binding constant $K_i$. The selectivity of the covalent bonding step, on the other hand, is governed by the choice of a suitably tempered electrophilic warhead, and appropriate juxtaposition of that functionality on the inhibitor and a “noncatalytic, poorly conserved, assumed, suitably positioned and oriented” nucleophile on the target.\textsuperscript{[83]} The latter step is characterized kinetically by the term $k_{\text{rate}}$, the rate constant for inhibition, while the overall potency of the TCI on the enzyme is expressed as the ratio of $k_{\text{rate}}/K_i$.\textsuperscript{[3,6,75]} As noted in Section 1, high potency is desirable in terms of achieving efficacy at low dose, with the attendant benefits that accrue from a reduced body burden of parent drug and metabolites.\textsuperscript{[94]} From a practical standpoint, IC\textsubscript{50} values may be useful in rank-ordering compounds for potency within a structural series, but since the measured IC\textsubscript{50} value is highly dependent upon experimental conditions, the $k_{\text{rate}}/K_i$ ratio is the appropriate indicator of TCI potency.\textsuperscript{[1,83]}

Additional opportunities to enhance selectivity are available in the “chemically tuned electrophile” approach to reversible TCIs discussed in Section 2.\textsuperscript{[76,79]} since the complementary noncovalent binding interactions that stabilize the otherwise labile covalent inhibitor–protein complex may be absent even in closely related members of the target protein family. Clearly, strong bioinformatics support plays a key role in such design efforts.\textsuperscript{[36,58,61]} Optimizing the reactivity of the warhead for TCI use requires that a balance be struck between achieving a sufficiently high rate of reaction with the selected nucleophile on the target protein, as well as a minimal rate of reaction with off-target nucleophiles. A common approach to the latter objective involves the use of in vitro benchmarking strategies with protein surrogates, such as GSH, that assess the intrinsic chemical reactivity of the warhead with the thiol by using either LC-MS/MS or NMR techniques.\textsuperscript{[48,83,95–97]} However, caution should be exercised in extrapolating half-life values for the reaction between candidate inhibitors and GSH in aqueous buffer from one structural series to another, or from one laboratory to another, since the results can be influenced significantly by experimental conditions.\textsuperscript{[95]} It should also be appreciated that the pK\textsubscript{a} value of protein thiols can vary greatly from that of GSH in aqueous buffer—depending on their local environment—such that absolute reaction rates with GSH in vitro are not a reliable predictor of off-target reactivity in vivo.\textsuperscript{[84]} For these reasons, it is more appropriate to establish measures of relative, as opposed to absolute, reaction rates with GSH, cysteine, or other model nucleophiles, with benchmarking against a standard tempered electrophile.\textsuperscript{[9]} The calcium channel antagonist nivaldipine, a marketed drug with a favorable safety profile, has been employed for this purpose because of its weakly electrophilic cyano function, and exhibits an in vitro half-life of 40 h for the reaction with GSH, and 2 h with cysteine.\textsuperscript{[90]} (Figure 7). With the recent approval of ibrutinib, afatinib, and osimertinib as marketed covalent kinase inhibitors, it is likely that these compounds will find a place in future benchmarking studies. Recently, a number of workflows based on proteomics mass spectrometry have been adapted for use in TCI discovery programs, wherein they serve to confirm directly the site of adduction of candidate covalent inhibitors to their respective protein targets, as well as to assess the extent of reaction with abundant off-target proteins such as serum albumin and hemoglobin.\textsuperscript{[99–102]} Analogues of TCIs in which the acrylamide warhead is replaced by the saturated (propionamide) counterpart have proven to be useful tools in assessing the relative contributions of reversible and irreversible components to target inhibition.\textsuperscript{[94]}

A powerful approach to evaluate the proteome-wide selectivity of covalent kinase inhibitors was outlined by Lanning et al.,\textsuperscript{[4]} who employed activity-based protein profiling (ABPP) coupled with quantitative mass spectrometry to globally map the targets, both specific and non-specific, of covalent kinase inhibitors in human cell cultures. Probes for ibrutinib (BTK inhibitor) and afatinib (EGFR\textsuperscript{750M} inhibitor) containing a terminal acetylene moiety distant from the acrylamide warhead were incubated with intact cells or cell lysates for 1 h at 37°C, after which the reaction mixtures were treated with an azide–rhodamine reporter tag under copper-catalyzed azide–alkyne cycloaddition (click chemistry) conditions; proteins that formed an adduct with these probes were then isolated using streptavidin beads and visualized by SDS-PAGE with in-gel fluorescence scanning (Figure 8). By increasing the concentration of the probe over the range 1–1000 nM, it was shown that selectivity for the target (BTK or EGFR variant) decreased significantly. In the case of the ibrutinib probe, for example. MAP2K7, TEC, and MLTK all became labeled in addition to BTK, even at probe levels that corresponded to pharmacologically relevant concentrations of ibrutinib. Importantly, this loss in selectivity was paralleled by an increase in probe cytotoxicity, thus leading the authors to conclude that widespread nonspecific covalent binding (largely to cysteiny1 thiols in this case) was the probable cause of cell killing. Increasing the reactivity of the acrylamide warhead by incorporating an N,N-dimethylaminomethyl substituent at the β-carbon atom had a similar detrimental effect on the selectivity and a corresponding enhancement of cytotoxicity. Collectively, these findings underscore the need for a high degree of selectivity in the covalent modification of proteins by TCIs to mitigate the risk of off-target toxicities.

\textbf{Figure 7.} Reaction of nivaldipine with thiols (GSH or cysteine) in vitro to form thioimidated derivatives. Although the reaction is reversible, conditions have been established where the rate of the back-reaction is negligible.\textsuperscript{[88]} Nivaldipine has been used as a benchmark compound to rank-order the rates of reaction of candidate TCIs with GSH.
From a perspective of irreversible protein kinase inhibitors, Barf and Kaptein[67] summed up their view on the issue of selectivity as follows, “In a nutshell, the therapeutic applicability or the success of irreversible binding inhibitors is dependent on whether or not the covalent bond can be confined solely to the protein kinase of interest.” Thus, while absolute specificity in the action of TCIs probably is not achievable, efforts to optimize this property are of utmost importance in the design of covalent inhibitors. Finally, it should be noted that selectivity also can be achieved if exposure to the inhibitor is confined to its site of action. Omeprazole and its congeners are “masked” covalent drugs in that they are converted into reactive sulfenic acid/sulfenamide species only in the acidic environment of the stomach, which is the location of their target, the H+/K+-ATPase in parietal cells,[25] while tetrahydrolipstatin (orlistat) is poorly absorbed following oral administration and elicits its inhibitory effects on gastric lipases within the gastrointestinal tract.[103]

4. Metabolism and Disposition

The term “disposition” refers to the collective processes of drug absorption, distribution to/from the tissues, and elimination from the body. The fact that the pharmacodynamic response to a TCI may be “uncoupled” from its PK characteristics has led to the suggestion that a high metabolic clearance may be better tolerated with covalent inhibitors than with most other classes of drugs.[7,66] The underlying rationale is that if complete coverage of the target can be achieved in the initial period following dosing, then rapid metabolism of the inhibitor would serve to remove unchanged drug from the systemic circulation, thereby minimizing unwanted exposure in nontarget tissues. However, while such a scenario may confer a safety advantage, drugs that are rapidly cleared by metabolic processes usually exhibit a large first pass effect. “First pass effect” refers to the process whereby a portion of the drug being absorbed from the gastrointestinal tract is removed through metabolism by enzymes located in the gut wall and liver following oral dosing, thereby resulting in low oral bioavailability, a higher efficacious dose, and heightened intersubject PK variability. In practice, therefore, metabolic clearance cannot be too high for this class of drugs if the potential benefits of high potency and low dose are to be realized.[66]

A second consideration, which is not unique to the class of covalent inhibitors, relates to the phenomenon of metabolic activation, whereby an electrophilic center is introduced into the drug molecule during the course of its metabolism (usually oxidative metabolism catalyzed by cytochrome P-450 enzymes).[18] In the case of acrylamide-based TCIs, a potential opportunity for bioactivation is presented through the presence of the terminal double bond of the warhead, which can undergo CYP-mediated epoxidation (as occurs with ibrutinib).[86] Of course, bioactivation may occur elsewhere on the molecule, depending on the nature of its interaction with drug-metabolizing enzymes. In either case, the reactive intermediate formed will typically exhibit a much higher level of electrophilicity than the TCI warhead itself, the consequence of which is that the selective alkylating properties engineered into the covalent inhibitor are compromised, and the metabolite alkylates a broad spectrum of off-target proteins. Therefore, it is important that candidate inhibitors are screened at an early stage for their susceptibility towards metabolic activation, by using standard in vitro procedures that employ nucleophilic trapping techniques.[22] Where metabolic activation is detected and the responsible pathway elucidated, efforts can be directed to structural modifications that minimize, or eliminate, the offending reaction.[104] On a related issue, it is noteworthy that many TCIs have been reported to serve as time-dependant inhibitors of at least one human CYP enzyme[66]—a reflection of the formation of a reactive intermediate—and this property should also be assessed at an early stage in development for predicting potential drug–drug interaction liabilities.

As noted in Section 2, GSH can add directly to the acrylamide warhead of a TCI (i.e. in the absence of metabolic activation) in a reaction that is catalyzed by one or more members of the GST family, and this process may occur in extrahepatic tissues as well as in the liver.[85] Certain GST enzymes, such as GST μ1, exhibit genetic polymorphism in the human population, thus raising the possibility that in cases where GSH addition to the TCI warhead represents a quantitatively important route of clearance and is mediated by this GST isoform, individuals may segregate into “slow” and “fast” metabolizer phenotypes. It has been suggested, therefore, that GSTM1 genotypes may need to be taken into consideration, which is not unique to the class of covalent inhibitors, relates to the phenomenon of metabolic activation, whereby an electrophilic center is introduced into the drug molecule during the course of its metabolism (usually oxidative metabolism catalyzed by cytochrome P-450 enzymes).[18] In the case of acrylamide-based TCIs, a potential opportunity for bioactivation is presented through the presence of the terminal double bond of the warhead, which can undergo CYP-mediated epoxidation (as occurs with ibrutinib).[86] Of course, bioactivation may occur elsewhere on the molecule, depending on the nature of its interaction with drug-metabolizing enzymes. In either case, the reactive intermediate formed will typically exhibit a much higher level of electrophilicity than the TCI warhead itself, the consequence of which is that the selective alkylating properties engineered into the covalent inhibitor are compromised, and the metabolite alkylates a broad spectrum of off-target proteins. Therefore, it is important that candidate inhibitors are screened at an early stage for their susceptibility towards metabolic activation, by using standard in vitro procedures that employ nucleophilic trapping techniques.[22] Where metabolic activation is detected and the responsible pathway elucidated, efforts can be directed to structural modifications that minimize, or eliminate, the offending reaction.[104] On a related issue, it is noteworthy that many TCIs have been reported to serve as time-dependant inhibitors of at least one human CYP enzyme[66]—a reflection of the formation of a reactive intermediate—and this property should also be assessed at an early stage in development for predicting potential drug–drug interaction liabilities.

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consideration during the development of covalent modifiers that fall into this category. The influence of potential reversibility in the formation of the GSH conjugate, discussed in Section 2, on the overall PK profile of TCIs remains to be determined, but the phenomenon may represent a further complication in dissecting the factors that govern the disposition of certain TCIs in vivo.

From the standpoint of drug discovery programs, it appears that a somewhat different suite of absorption, distribution, metabolism, and excretion (ADME) assays may be required when selecting lead TCIs for further study. Based on an analysis of the in vitro and in vivo preclinical ADME properties of 10 late-stage clinical development or approved covalent inhibitors, Moghaddam et al. concluded that the standard battery of assays was nondiscriminatory in the selection of promising compounds. Instead, nontraditional assays such as assessment of target mass modification, target confirmation by amino acid sequencing, the determination of cellular target occupancy, and target turnover rate data are the critical considerations for progressing candidates into development. Pharmacokinetics remain important to the extent that inhibitors intended for oral dosing need to exhibit acceptable oral bioavailability, although, as discussed above, less than ideal PK profiles (particularly in terms of clearance) may be acceptable for irreversible TCIs. Assessment of inhibitor selectivity becomes an important issue in lead optimization studies, where both the identities of off-target proteins and the levels of covalent modification associated with increasing concentrations of the inhibitor may be defined by using contemporary proteomics approaches. In vivo measurements of target occupancy as a function of time, initially in animals and subsequently in humans when the target is accessible, allow for the construction of target occupancy/PD relationships that replace PK/PD relationships in guiding the design of the clinical development program. Finally, TCIs need to have the requisite physicochemical properties (solubility, lipophilicity, pKₐ) and safety characteristics associated with drug-like molecules if they are to be developed successfully as therapeutic agents.

5. Mitigating Toxicity Risks with Covalent Inhibitors

The past decade has witnessed great strides in the development of targeted covalent inhibitors, several of which have now entered the market as approved drugs. The fact that these compounds are proving to be safe and effective chemotherapeutic agents has provided reassurance that selective covalent modification of proteins leading to target “silencing” does not necessarily incur a high risk of immune-mediated toxicity. However, concerns remain—particularly with irreversible covalent inhibitors—that this is a risky strategy in drug development. Two serious incidents, each resulting in deaths during clinical trials with irreversible TCIs, have occurred within the last year; the first of these involved the methionine aminopeptidase 2 inhibitor beloranib (ZGN-433), in late-stage development as an anti-obesity agent while the second involved the fatty acid amid hydrolase inhibitor BIA 10-2474 in a multiple dose Phase I trial for neuropathic pain indications (Figure 9). Although the cause of these tragic events remains under investigation and, at the time of writing, there is no evidence that the deaths were related to the covalent mechanism of action of the two agents in question, these incidents nevertheless sound a note of caution and highlight the risks inherent in the drug development enterprise. In light of the safety concerns, and in consideration of the various aspects of TCIs summarized in this Review, a number of approaches may be adopted to reduce the risk of encountering adverse reactions to this class of enzyme inhibitors. These include the following:

a) Maximizing selectivity of binding to the target protein. This goal is key in light of emerging data that suggest that off-target binding of TCIs leads to a greater risk of toxicity. Selectivity can be accomplished through: 1. optimization of the initial noncovalent association of the inhibitor with the target, and 2. application of bioinformatics techniques to optimize placement of an appropriately tempered warhead on the inhibitor in relation to an accessible, nonconserved nucleophile on the protein. Selectivity can be assessed experimentally by a combination of activity-based protein profiling and proteomics mass spectrometry techniques.

b) Maintaining a low dose. Reviews of the characteristics of drugs that have been withdrawn from the market because of an unacceptably high incidence of toxicity, or which have received “black box” warnings for toxicity, have stressed the role of daily dose. Thus, very few drugs given at ≤10 mg/day have been associated with serious toxicities, such as hepatic injury, while the incidence of serious adverse effects increases significantly in drugs administered at ≥50 mg/day. This appears to be due to the “body burden” of parent drug and metabolites, which should be kept as low as practical to minimize off-target effects. By capitalizing on the high potency inherent in targeted covalent drugs, and selecting candidates with good oral bioavailability, it should be
possible to develop safe, low-dose, once-a-day TCIs for a variety of therapeutic indications.
c) Minimal formation of reactive metabolites. There is a compelling body of information that implicates reactive metabolites as the mediators of some, but not all, drug-induced toxicities, and while it is not possible with current knowledge to predict which reactive intermediates will cause adverse reactions, it seems prudent to avoid those drug candidates that are found to be susceptible to metabolic activation.[22,104] Moreover, the introduction of a highly reactive electrophilic center through metabolism effectively overriders the controlled reactivity built into the TCI warhead, thereby leading to loss of alkylation selectivity and increased risk of off-target induced toxicity.

Although additional de-risking criteria could be added to the above list, such as a preference for reversible over irreversible covalent inhibitors, there is insufficient evidence at present to make a strong case for factors other than the three listed above. At this juncture, therefore, it seems reasonable to conclude that highly selective, low-dose TCIs that do not undergo metabolic activation should exhibit safety profiles comparable to those of conventional noncovalent drugs, while at the same time providing a number of therapeutic advantages associated with their non-equilibrium binding mode of action.

6. Summary and Outlook

Application of the targeted covalent inhibitor approach to drug discovery has increased rapidly in recent years, and based on the large number of covalent drug candidates presently in development, this trend is likely to continue for the foreseeable future. The major investment to date by the pharmaceutical industry has been in the area of covalent kinase inhibitors for the treatment of various forms of cancer, but with the growing clinical safety database for this class of drugs, it is likely that TCIs will be developed for indications in fields other than oncology. Proteins with slow turnover rates that possess accessible nucleophilic centers in a unique molecular environment are attractive candidates for the covalent inhibitor approach, which offers the promise of low-dose drug therapy with prolonged duration of action. While safety remains a concern, and will only be fully addressed after many years of clinical experience with marketed TCIs, initial results are encouraging.

As the field gains maturity, greater insight will be obtained through both experimental and computational approaches on the influence of warhead structure and reactivity on the selectivity, efficacy, and toxicity profiles of TCIs.[90,112-117] The biological consequences of off-target reactivity will become more clearly defined, and strategies to minimize broad proteome reactivity will emerge. Similarly, guidelines will be developed as to what constitutes the optimum PK characteristics for a covalent inhibitor and what might be the practical lower limit of target protein turnover time. Information on the potential for acquired resistance to TCIs in cancer chemotherapy is only beginning to be assembled, largely from clinical experience with EGFR inhibitors.[118,119] Novel protein targets will be identified, including those designed to perturb protein–protein interactions[120,121] and a new generation of highly selective, “third generation” kinase inhibitors will enter development. More challenging goals include the design of allosteric covalent enzyme inhibitors,[122] as well as covalent modulators of receptor function. Opportunities to broaden the scope of the TCI approach through targeting protein nucleophiles other than cysteinyl thiols will likely emerge; for example, some progress has been made in targeting the catalytic lysine residues in protein kinases, although achieving selectivity in the face of the numerous lysine residues frequently found on protein surfaces presents a significant design challenge.[123] These are exciting times for medicinal chemistry, and the coming decade will allow the promise of the targeted covalent inhibitor approach, as well as the attendant safety risks, to be more fully evaluated as a strategy for the design of novel therapeutic agents.

Abbreviations

ARPP activity-based protein profiling
ADME absorption, distribution, metabolism, and excretion
BTK Bruton’s tyrosine kinase
CYP cytochrome P-450
EGFR epidermal growth factor receptor
GSH glutathione
GST glutathione-S-transferase
HAS human serum albumin
NAPQI N-acetyl-p-benzoquinone imine
PK-PD pharmacokinetic–pharmacodynamic
TCI targeted covalent inhibitor.

Acknowledgements

I would like to thank Dr. Jus Singh, Dr. Wes Westlin, and Dr. Russ Petter, formerly of Avila Therapeutics, for introducing me to the fascinating subject of targeted covalent inhibitors, and for many interesting discussions on the topic.

How to cite: Angew. Chem. Int. Ed. 2016, 55, 13408–13421

Angew. Chem. 2016, 128, 13606–13619


Received: January 30, 2016
Published online: August 19, 2016