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Drug discovery considerations in the development of covalent inhibitors

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ABSTRACT

In recent years, the number of drug candidates with a covalent mechanism of action progressing through clinical trials or being approved by the FDA has increased significantly. And as interest in covalent inhibitors has increased, the technical challenges for characterizing and optimizing these inhibitors have become evident. A number of new tools have been developed to aid this process, but these have not gained wide-spread use. This review will highlight a number of methods and tools useful for prosecuting covalent inhibitor drug discovery programs.

Although nearly 30% of drugs on the market act via a covalent mechanism of action, the majority of those were not designed as covalent inhibitors and were discovered later to act via a covalent mechanism. Further, an increased understanding of organ toxicity from reactive metabolites in the 1970s and 80s led to a backlash against compounds with reactive functionality or compounds which were thought to have increased risk for forming highly reactive metabolites.[1,2] A recent analysis of 50 marketed drugs found not all drugs that form detectable thiol adducts in vitro exhibit drug induced toxicity.[3] The authors suggest that other parameters such as daily dose and reactive metabolite burden also contribute to the potential for adverse toxicological events. And while the formation of highly reactive metabolites can be minimized through the avoidance of toxicophoric moieties, reliably predicting idiosyncratic toxicity potential is next to impossible. For covalent inhibitors, it is thought that the risk of toxic events can be lessened through optimization of non-covalent interactions to improve target receptor recognition and modulation of electrophilic warhead reactivity to diminish undesired reaction with nucleophiles in vivo. These strategies have led to the design of increasingly selective covalent inhibitors.[4–7]

While the risks of covalent inhibitors are known, the sustained duration of inhibition of covalent inhibitors yields a number of potential advantages. These include: (1) improved biochemical efficiency as competition with endogenous substrates is reduced, (2) lower, less frequent dosing resulting in a lower overall patient burden, (3) a dissociation of pharmacokinetics from pharmacodynamics (PD) as PD is now dependent on protein resynthesis, making quickly cleared compounds more acceptable which would lead to a lower systemic drug exposure and (4) potential prevention of emergence of drug resistance due to continuous target suppression.[1,8] It has also been hypothesized that targets with shallow binding sites could be more efficiently inhibited with a covalent inhibitor, in effect making targets formerly thought of as 'undruggable', 'druggable'. Furthermore, covalent inhibitors from programs targeting EGFR,[9] BTK,[5] FAAH,[10,11] and MetAP2[12] have progressed to Phase II and III clinical studies with acceptable side effect profiles. In the case of the irreversible mutant EGFR inhibitors, similar side effect profiles to the reversible EGFR inhibitors were seen. In addition, in the past two years, a number of covalent inhibitors such as the 20S proteasome inhibitor carfilzomib for multiple myeloma,[13] the HCV NS3/4A protease inhibitor telaprevir,[14] the Cyp17 inhibitor abiraterone for metastatic prostate cancer[15] and most recently the EGFR inhibitor afatinib for metastatic NSCLC with EGFR

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mutations or deletions[16] have been approved by the FDA ushering in a new era for covalent modifiers.

As interest in the field has increased, technical challenges for prosecuting a covalent inhibitor program have become evident. In particular, covalent inhibitor programs face unique challenges including determining whether the nucleophilic residue of interest is reactive, whether covalent modification has in fact occurred, what the receptor occupancy of the inhibitor is, what percentage of receptor occupancy is required for a pharmacological effect to manifest, whether IC_{50} or k_{inact}/K_i[17] is the most appropriate measurement to aid in medicinal chemistry optimization and what the on- and off-target selectivity of the compound of interest is. In a recent review of targeted covalent inhibitors, it was noted that although a number of new tools exist for use in pursuing a covalent inhibitor strategy these methods are not yet being systematically applied.[18] This review will highlight a number of methodologies useful for developing and driving a covalent inhibitor program. To be noted is that while some techniques discussed are specific for targeted covalent inhibitors (TCIs), many of the methodologies can be applied to both TCIs and to irreversible mechanism-based inhibitors.

At the outset of a program for targeted covalent inhibitors, bioinformatic analysis is often used to identify appropriate nucleophiles (e.g. non-catalytic, poorly conserved, accessible, suitably positioned and oriented) near the binding pocket of the target protein. Confirmation that a molecule has reacted in the expected manner is then useful for subsequent optimization efforts. In the absence of a cocrystal structure which provides definitive proof, there are several methods to attain confidence in the proposed mechanism of action. Site-directed mutagenesis of the nucleophilic residue (e.g. C → S or K → R) and subsequent decrease/loss of the ligand's activity towards the still functional mutant provides support for the proposed covalent mode of action.[6,19,20] In addition, following incubation of a protein and a covalent inhibitor, enzymatic digestion combined with tandem mass spectrometry can also be employed in a peptide-mapping experiment to verify the site of covalent modification.[6,21,22] Furthermore for cysteine nucleophiles, the peptide-mapping findings can be compared with those following treatment with iodoacetamide which reacts with all accessible cysteines. Moreover, an important design strategy for the selectivity of targeted covalent inhibitors—in addition to tuning the warhead reactivity—is to optimize K_i. Thus, mutation of a residue influencing the shape/selectivity of the binding pocket (e.g. for EGFR, gatekeeper T → M) and/or affecting ATP affinity, and an ensuing loss in inhibitor potency/selectivity can lend further support for the proposed binding mechanism.[23] From a ligand perspective, an equivalent to a 'point mutation' entails preparation of the corresponding analog with an inactivated warhead isostere (e.g. acrylamide vs propionamide). Assuming that the binding mode of the two analogs is similar, the saturated analog should have greatly reduced activity.[24–28] By comparing directly with another, such an inactivated analog might also be useful in evaluating what aspects of a covalent compound's profile can be attributed to the reversible interaction with the target and which ones are due to the covalent interaction.

$$E + I \underset{K_i}{\rightleftharpoons} E\bullet I \overset{k_2}{\underset{k_{-2}}{\dashrightarrow}} E-I \qquad (1)$$

Initial non–covalent complex — Final covalent complex

Eq. 1 Description of the general mechanism for the action of a covalent inhibitor consisting of (a) initial non-covalent interaction of the inhibitor with the target protein and positioning of the warhead close to the nucleophile of interest and (b) subsequent covalent bond formation to generate the inhibited complex. (Source: Reproduced with permission from Ref. 1. Copyright 2011 by Nature Publishing Group.)

As a consequence of the time-dependent nature of the interaction of covalent inhibitors with their target proteins and the fact that such a reaction should theoretically proceed to completion rather than establishing an equilibrium, a common recommendation is to utilize k_{inact}/K_i values in preference to IC_{50} values in order to evaluate the potency of covalent inhibitors[7] (Eq. 1, note: $k_2 \equiv k_{inact}$). An IC_{50} value is a poor indicator of a covalent inhibitor's potency as it is dependent on the incubation conditions (especially the incubation time) used. Despite this, a frequent practice, for reasons of either convenience or technical considerations, is to nevertheless measure IC_{50} values, albeit often after a pre-incubation time where maximum inhibition is observed.[29] Although a high correlation between IC_{50} and k_{inact}/K_i values can exist,[24,30] there are many instances where these diverge and the SAR of either a series of structurally closely related compounds (e.g. during optimization) or various scaffolds (e.g. during hit evaluation) might in some cases be better interpreted by dissecting the contributions of the reversible binding (K_i) from the irreversible binding (k_{inact}) components.[7,21,27] An illustrative example of this can be seen in a recently disclosed series of irreversible kynurenine aminotransferase (KAT) II inhibitors.[31] Comparison of k_{inact}/K_i values allowed for differentiation between various analogs possessing similar IC_{50} values (Fig. 1).

As an alternative to the time consuming determination of k_{inact}/K_i values or in those instances where the rate of covalent bond formation is extremely rapid (thus precluding accurate determination of k_{inact}/K_i values), a fluorescence-based assay has recently been introduced for kinase inhibitors. It is based on the rate of emission intensity upon addition of a thiol to a Michael-acceptor appended quinazoline/quinoline scaffold (also applicable to other fluorophore systems).[24] This allows direct measurement of the covalent bond-forming step independent of target enzyme activity and in the absence of ATP. The assay was validated with a series of EGFR inhibitors in a model system where site-directed mutagenesis of cSrc had been employed to produce a protein possessing a cysteine in a structurally equivalent environment as C797 of EGFR and/or a methionine analogous to the T790M mutation in drug-resistant EGFR. The results obtained, in combination with those from other activity-based assays, also contributed to a better understanding of the factors influencing drug resistance, e.g. reduced reactivity towards the mutant as evidenced by a decreased initial velocity of covalent bond formation (Fig. 2).

Another aspect to consider when pursuing a covalent approach is an assessment of the irreversibility of the inhibition. This can be accomplished using washout experiments and/or competitive binding experiments with a reference probe. In a washout experiment, a target protein is treated with an inhibitor, excess compound is removed by either washing out,[22,26] dialysis[7,23] or gel filtration,[32,33] and the residual activity and/or the time required for recovery of activity is measured. In contrast to non-covalent inhibitors, the inhibitory activity seen should not be lost or diminish immediately following washout/dialysis/filtration. This in vitro effect demonstrates one of the advantages expected in vivo with covalent inhibitors, namely an extended duration of action even when the drug has been cleared systemically (i.e. the PK/PD disconnect). The overall pharmacological effect in vivo is then largely dependent on the de novo protein resynthesis rate in response to protein inhibition, which can differ drastically from that of the resting/non-inhibited state, e.g. it has recently been reported that, whereas the half-life of Itk in the resting state is about one hour, this increased to 22 h upon treatment with a covalent inhibitor.[27]

Studies in the HIV protease field have shown that (i) resistance was found to correlate with an increase in the dissociation rate and