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Review article

Covalent inhibitors design and discovery



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ABSTRACT

In the history of therapeutics, covalent drugs occupy a very distinct category. While representing a significant fraction of the drugs on the market, very few have been deliberately designed to interact covalently with their biological target. In this review, the prevalence of covalent drugs will first be briefly covered, followed by an introduction to their mechanisms of action and more detailed discussions of their discovery and the development of safe and efficient covalent enzyme inhibitors. All stages of a drug discovery program will be covered, from target considerations to lead optimization, strategies to tune reactivity and computational methods. The goal of this article is to provide an overview of the field and to outline good practices that are needed for the proper assessment and development of covalent inhibitors as well as a good understanding of the potential and limitations of current computational methods for the design of covalent drugs.

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1. Introduction

1.1. Covalent drugs

Non-covalent interactions, including electrostatic interactions, hydrogen bonds, van der Waals interactions, and hydrophobic contacts, all contribute to the affinity and specificity of ligands for receptors, substrates for enzymes, and binders for ion channels to name a few, but also to the activity of drugs whether receptor antagonists or agonist, enzyme inhibitors or ion channels blockers. Consequently, the development of drugs has primarily relied on the optimization of non-bonded interactions to improve potency for a given (or a few) target(s) and to increase selectivity (i.e., relative to off-targets). However, this approach, which has been adopted by many pharmaceutical companies and academic groups, neglects a whole class of drugs: those which can form a covalent bond with their targeted enzyme or receptor.

While specificity has always been perceived as a major focus for a successful medicinal chemistry program, statistics reported years ago revealed that marketed drugs bind to an average of six targets [1]. In this quest for specificity, drugs featuring reactive groups had often been red-flagged. Fear of toxicity caused by non-specific modifications of proteins, nucleic acids and other key biomolecules as well as conjugation with glutathione has, for a long time, restrained the development of covalent drugs. However, although intentionally discarded by most medicinal chemistry research programs, various covalent drugs have found their way to the market, including blockbuster drugs (Fig. 1). This apparent paradox results from their mode of action being unraveled after their therapeutic application has been discovered, a famous

example being aspirin, which irreversibly acetylates cyclooxygenase (COX) [2].

Recently, a boost in the development of covalent drugs has occurred (Fig. 2) [3,4]. It has been recognized that several properties of covalent drugs could be beneficial under certain circumstances compared to their non-covalent counterparts. For instance, the long-acting character of the covalent interaction opposes displacement of enzyme inhibitors by the natural substrates. This is particularly important when the biochemical pathway involves a build-up of the natural substrate upon inhibition, and when a covalent drug allows the same pharmacologic response to be achieved at a much lower concentration than a non-covalent drug for the same target.

In addition to toxicity concerns, inadequate characterization techniques for covalent inhibitors has impeded the development of covalent ligands. Indeed, with all the knowledge available in terms of enzymology, binding kinetics, pharmacokinetics and pharmacodynamics geared towards “conventional” non-covalent inhibitors, interpretation of data associated with covalent inhibition can be biased and erroneous. Often described as non-classical inhibitors, covalent inhibitors, when applied in a drug development project, also need non-classical treatments for their characterization.

1.2. This review

The objective of this review is to establish a framework for the development and optimization of covalent inhibitors and to illustrate how these concepts can contribute to the discovery of efficient

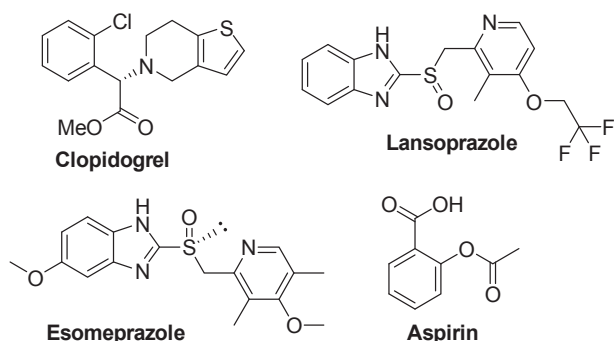


Fig. 1. Major drugs which act through covalent mechanisms.

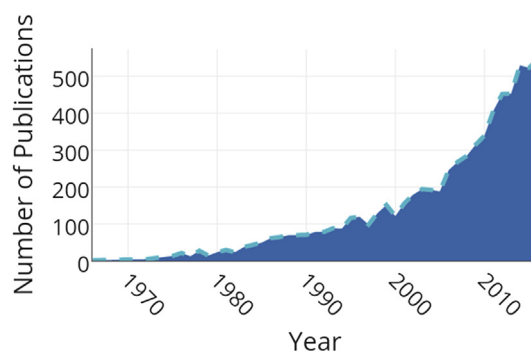


Fig. 2. Number of publications obtained from the search of the term “covalent drugs” in SciFinder®.

covalent drugs. We will discuss the various stages of covalent inhibitor design, from the choice of the biological target to the appropriate characterization of covalent inhibitor properties and optimization of those features through both experimental and computational methods. By no means is this review meant to be exhaustive but rather to be an overview of the field with illustrative examples. Our major focus will be on covalent enzyme inhibitors, although other classes of covalent drugs such as DNA alkylating agents exist.

2. Covalent drugs and natural products

2.1. Covalent inhibitors among natural products and synthetic drugs

A common strategy in drug discovery programs is to build insight into the chemical structures and binding modes of natural substrates (for enzymes) or ligands (for receptors) to design new drugs. The assumption is made that since substrates/ligands have co-evolved with their corresponding targets for optimal binding, mimicking their structural features should retain optimal biological effects. Peptidomimetism is, consequently, a widely used strategy in medicinal chemistry [5]. Another approach is to look for molecules from natural sources that are unrelated to the drug target under investigation. In fact, it has been reported that at least 60% of the drugs currently on the market were derived either directly from natural products or were inspired by natural products [6]. Natural products are often produced by living organisms to defend themselves or to confer them an advantage over other organisms. These properties have been exploited with natural products or analogues acting as antifungals [7], antibiotics [8] and anti-cancer agents [9]. As protein targets feature a number of nucleophilic residues, such as serine, lysine, cysteine and histidine, it is expected that biologically-active natural products may bear electrophilic moieties such as Michael acceptors [10], aziridines [11], epoxides [12] and β -lactams [13] to name a few (Fig. 3).

Studies of those bioactive natural products have also paved the way for the discovery of new biological targets.

2.2. Marketed covalent drugs

About 30% of marketed drugs that target enzymes act as covalent inhibitors [14]. While the abundance of covalent inhibitors is now established, the incorporation of reactive moieties was still disfavored in drug design until recently. Non-specific and/or off-target activity are usually cited as the main justifications to avoid the incorporation of reactive groups into new drug candidates [15].

However, a few examples of purposely designed covalent inhibitors that are currently on the market or in late stage clinical trials exist. Telaprevir [16] (Vertex pharmaceuticals) and Boceprevir [17] (Merck), two HCV protease inhibitors (Fig. 4), target the catalytic serine via the formation of a hemiacetal group, and were approved by the FDA in 2011. The development of Telaprevir illustrates the need for non-conventional analysis of activity data. Indeed, in the early stages, this active inhibitor was almost discarded due to poor performance in standard IC_{50} assays [16]. Kwong et al. noted that “Lessons learned from the development of telaprevir suggest that makers of innovative medicines cannot rely solely on traditional drug discovery metrics, but must develop innovative, scientifically guided pathways for success.”. Another example is Afatinib, an epidermal growth factor receptor (EGFR) inhibitor approved in 2013. The strong inhibition relies on an acrylamide moiety which reacts irreversibly with a non-catalytic cysteine present in the active site of EGFR. This covalent drug overcomes therapy-induced resistance [18,19]. Non-covalent inhibitors are

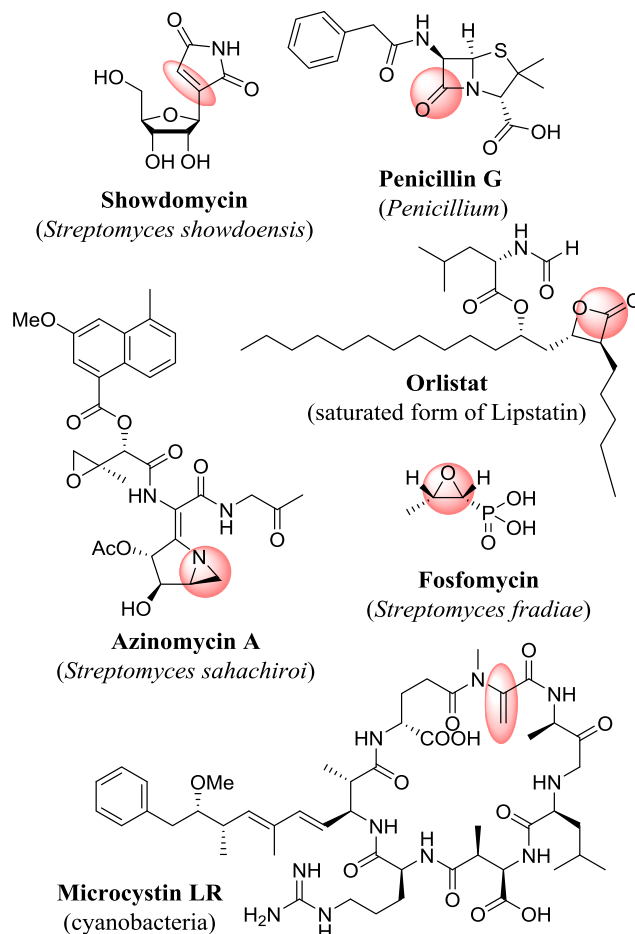


Fig. 3. Selection of electrophilic natural products or analogues.

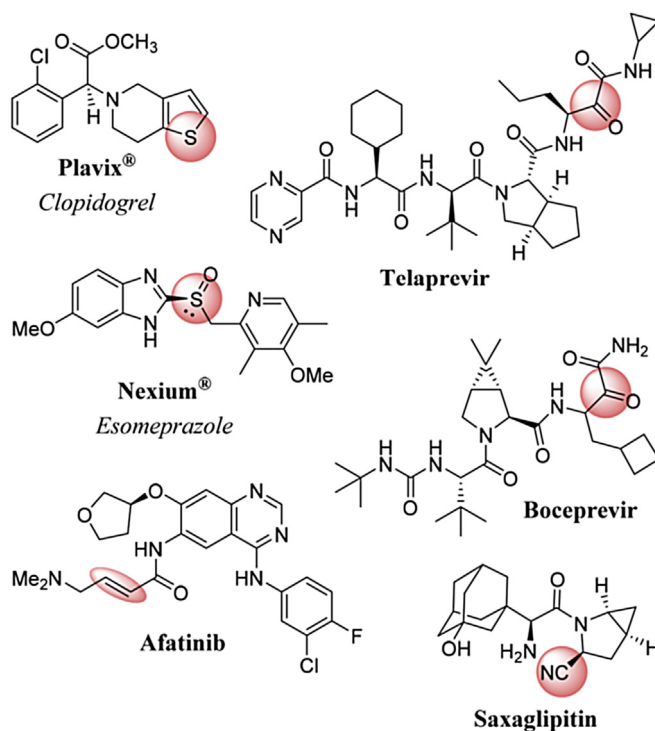


Fig. 4. Selection of covalent drugs on the market.

well documented for EGFR (e.g., gefitinib [20], erlotinib [21] and lapatinib [22]) and resistance towards those drugs has already appeared. In contrast, Afatinib proved to be effective against both EGFR and a resistant mutant, thanks to its increased efficiency and the permanent character of the complex formed with the conserved nucleophilic residue.

3. Mode of action and binding kinetics

3.1. Covalent bond formation mechanism

Despite the past reluctance of drug companies to develop covalent drugs, synthetic drugs with covalent modes of action have reached blockbuster status. In 2014, two of the ten top selling drugs in the United States were covalent, with Nexium® (Fig. 14), a proton pump inhibitor (PPI) used to relieve gastric reflux, being the most notable example. Despite this success, it is important to note that the covalent mode of action of those inhibitors was uncovered after the drug was discovered and that they were not designed with covalent interaction in mind. Examination of the chemical structures reveals no apparent covalent groups such as epoxides or Michael acceptors and indicates more complex modes of action are in play. Omeprazole (Prilosec® (racemate), Nexium® (single enantiomer)) was discovered in the 1970's by pharmacological screening. After several optimization and clinical studies, the drug was finally marketed in Europe as Losec in 1988 [23]. It was only two years before the release, in 1986, that Lindberg et al. described the covalent mechanism of action [24]. Interestingly, Lindberg found that Omeprazole was acting as a pro-drug. Its active form is its sulfenamide derivative which is formed by acid-mediated activation in close proximity of its target ($H^+, K^+ - ATPase$) (Fig. 5).

Clopidogrel is another example [25]. Clopidogrel interacts with the $P2Y_{12}$ receptor which is central to hemostasis and thrombosis. It has been found to be a pro-drug activated by P450 enzymes into the 2-oxo clopidogrel (Fig. 6). This metabolite then tautomerizes and ring-opens into a reactive thiol-containing compound. This thiol then forms disulfide bridges with two cysteine residues on the surface of the $P2Y_{12}$ receptor.

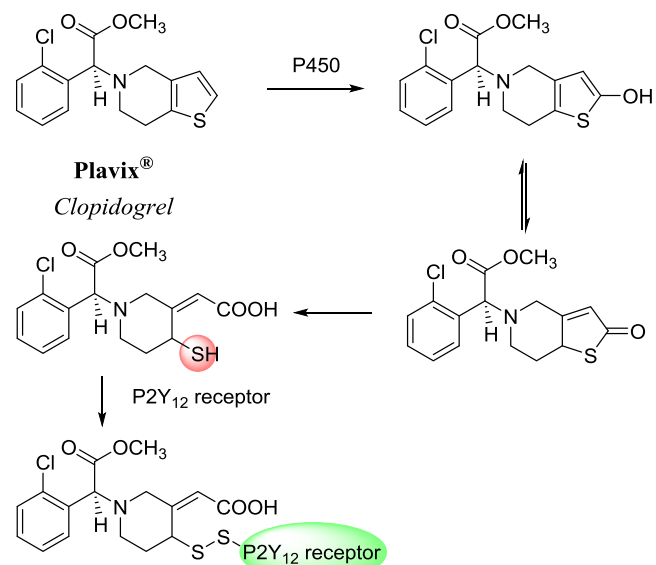


Fig. 6. Mechanism of action of Clopidogrel.

Another interesting example is Vigabatrin [26]. The level of γ -butyric acid (GABA) is balanced through the action of a decarboxylase which converts glutamate into GABA and a transaminase (GABA-T) which catabolizes GABA. The action of this GABA-T, which employs pyridoxal phosphate (PLP) as a co-factor, is shown in Fig. 7a. The reaction mechanism begins with the addition of GABA to the PLP-derived iminium to form the corresponding iminium derivative which is then hydrolyzed into the aldehyde. Vigabatrin, a suicide inhibitor of GABA-T, enters the same mechanism, however producing the iminium intermediate which, in contrast to GABA, is also a Michael acceptor (Fig. 7b). This reactive species is trapped by a lysine residue of the catalytic site forming a stable adduct and abolishing enzyme activity. In contrast to many other covalent inhibitors with complex mechanisms, Vigabatrin was rationally designed to act as described in Fig. 7.

3.2. Characterization

Characterization of inhibitors is essential for any successful drug discovery project. With the overwhelming number of parameters that needs to be screened, it is important to identify which ones are most important and to establish the optimal balance between accuracy and high throughput. It is a common practice in drug discovery programs to characterize potential inhibitors by the affinity or dissociation constants (K_i , K_d or the related IC_{50}). Lead optimization is then guided by the optimization of these values. They are usually determined *in vitro*, and are meaningful for rapidly reversible inhibitors (i.e., binding and unbinding are significantly faster than the measurement time of the experiment). However typical pre-incubation times can introduce a bias against covalent inhibitors that bind slowly and do not reach equilibrium before activity is measured [27]. As a result, covalent inhibitors may be overlooked if the pre-incubation time is not long enough to reach this equilibrium.

For a ligand forming a non-covalent complex with a target (Fig. 8a and Table 1), the dissociation constant (K_i) will be defined by the ratio of the two microscopic rate constants for the forward (k_1) and reverse (k_{-1}) binding event. In this common situation, the on-rate (k_{on}) is equal to the forward rate constant (k_1) while the off-rate (k_{off}) is equivalent to the reverse rate constant (k_{-1}).

If subsequent to the initial complex formation, a second

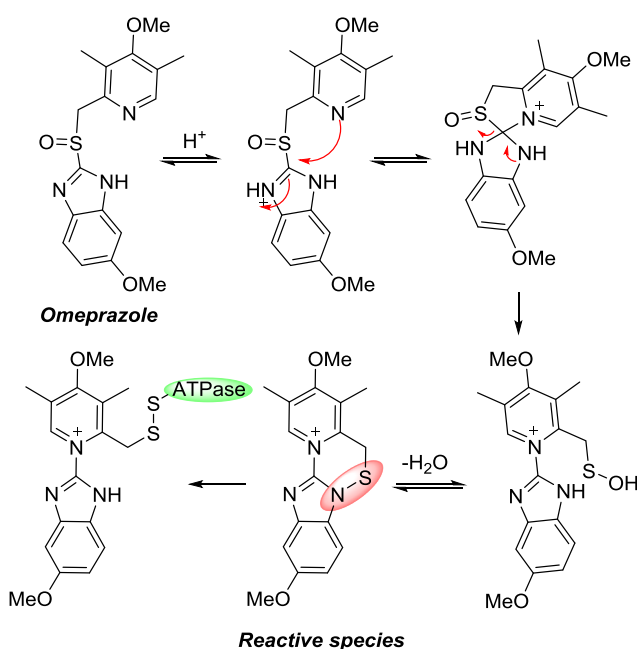


Fig. 5. Mechanism of action of Omeprazole [24].

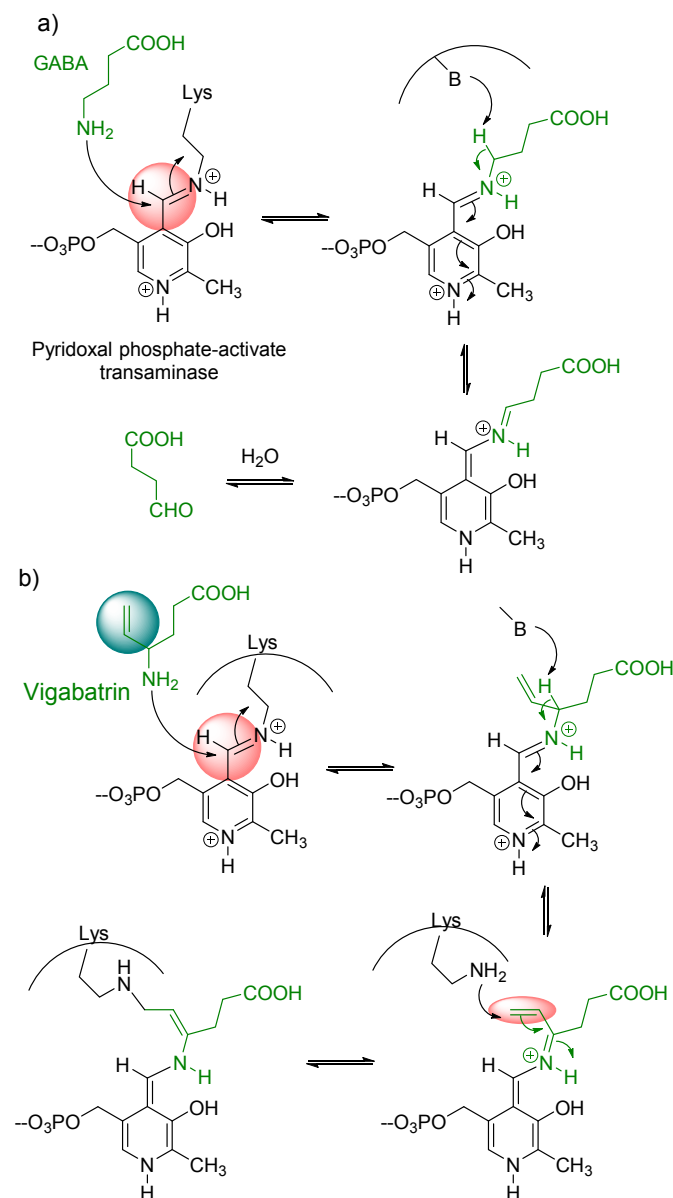


Fig. 7. Mechanism of action of Vigabatrin. a) deamination mechanism with natural substrate (GABA).

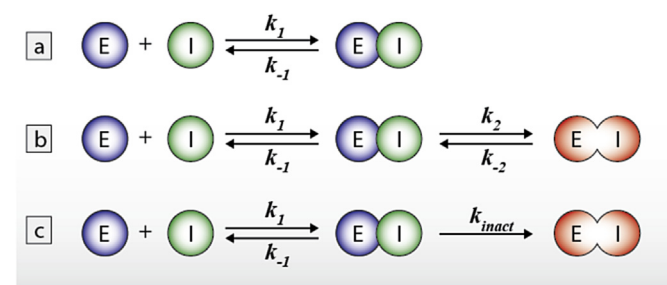


Fig. 8. Different modes of binding. a) One-step inhibition b) Induced-fit or reversible covalent inhibition c) Irreversible inhibition.

reversible step occurs, such as the formation of the covalent adduct, the binding process is more complicated. In addition to the initial complex dissociation constant (K_i), a global dissociation constant,

which takes the second equilibrium kinetics (k_2/k_{-2}) into account, is also defined (K_i^*). Moreover, this second step is usually slower than the first one and forms a tighter binary complex. The off-rate constant becomes proportional to not only the first reverse rate constant (k_{-1}) but also a combination of the covalent bond formation and dissociation rate (k_2 and k_{-2} respectively).

Finally, irreversible inhibition represents the extreme case where k_{-2} would be near or equal to zero. In this case, k_2 is referred to as k_{inact} and represents the only optimizable parameter, as K_i (or K_d) = 0 for all irreversible inhibitors, as summarized in Table 1.

Despite the different definitions for the off-rate, the rate that defines the formation of the initial complex is always defined by k_1 and the concentration of free ligand present at a given time. On one hand, while the association rate can have a significant impact on the desired properties of a drug, its utility as a metric in drug design is questionable as many parameters can influence the association rate. An association event is composed of a series of complex intertwined physicochemical factors (diffusion, solvation/desolvation, induced-fit ...) and it is therefore difficult to rationalize structural modifications that would alter its rate. In analogy of the catalytic efficiency of an enzyme capped by a diffusion limit, the rate of association of a ligand and its target cannot exceed this upper limit, which, under physiological conditions, is on the order of 10^8 – 10^9 M⁻¹ s⁻¹ [28]. On the other hand, the dissociation rate constant is less dependent of all physiological events since it only depends on the direct interactions made between the ligand and its target in the bound state and in the transition state for dissociation (e.g., hydrogen bonds, hydrophobic interactions, covalent bond). Unlike the on-rate, detailed structural information about protein-ligand interactions allows optimization of those interactions. This approach has in fact been a common practice in drug design. The off-rate of a specific ligand will determine the amount of time this ligand resides in the active site of its target. This concept is readily expressed as the residence time. As expected and nicely summarized by Copeland, the drug potency is often directly correlated with k_{off} while little correlation with k_{on} [29].

3.3. Residence time

Residence time has been the subject of extensive research over the past few years [30–32]. In contrast to the affinity constant, which is defined as the ratio of the association rate constant (k_{on}) and the dissociation rate constant (k_{off}), residence time (t_r) is defined as the reciprocal of the dissociation rate ($t_r = 1/k_{off}$) (Table 1), and corresponds to the average time a ligand remains bound in the binding site. In most structure-activity relationship (SAR) studies, the focus is on improving interactions between a ligand and its target in order to improve the affinity constant. Optimization of interactions will often lead to reduction of the dissociation rate and hence an improvement of the affinity constant.

As the affinity is linked to the off-rate, one might assume that there is no need for specific characterization of ligand-protein binding kinetics. However very different values of k_{on} and k_{off} can produce the same affinity constant, and only considering the affinity does not give any information on the lifetime of the complex formed *a priori*. A good illustration of the major difference between kinetic and thermodynamic measurements was given by Thoma et al. [33]. In this study, a number of potential inhibitors were characterized in terms of their k_{on} , k_{off} and K_d . The data illustrated how two different classes of DPP-IV inhibitors displaying similar equilibrium dissociation constants may have very different kinetic profiles (Fig. 9). As we will see in the next section, these kinetic properties can greatly affect the pharmacodynamics of the potential drug candidate. In Fig. 9, two DPP-IV inhibitors exhibited the

Table 1
Different relation of the kinetic parameters.

	One-step Reversible covalent inhibition	Irreversible inhibition
k_{on}	k_1	$\frac{k_{on}}{K_i}$
K_i	k_{-1}/k_1	k_{-1}/k_1
K_i^*	—	—
k_{off}	k_{-1}	—
Residence time	$1/k_{-1}$	∞

same K_d but one of them dissociated 100-fold faster than the other one.

Biological systems are dynamic, and the ability to guide ligand design by assessment of kinetic rather than equilibrium parameters would likely increase transferability of *in vitro* results to *in vivo* efficacy. For example, kinetic parameters, such as residence times, have been directly linked to *in vivo* efficacy [34]. In this study, Lu and coworkers related the efficacy of Fcbl enoyl-reductase inhibitors, potential antibacterial agents, with their residence time. Interestingly, they found no correlation between K_i and survival rates *in vivo*. While 100% of the mice survived when administered the weakest compound of the series, only 20% survived when the most potent (lowest K_i) was used. Residence time is directly related to the duration of the binding rather than to the binding process. Longer residence times correspond to extended drug action and therefore to improved efficacy. As another example, Finasteride (Proscar®), an inhibitor of 5 α -reductase which bonds covalently with the co-substrate NADPH in the active site, exhibits a dissociation half-time of as long as 4.7–7.1 h, depending on the oral dose. This inhibitor blocks the production of dihydrotestosterone (DHT) via its precursor testosterone, and is used to treat benign prostate hyperplasia. With such a long residence time, Finasteride keeps the levels of DHT very low for more than 72 h after a single oral dose, and for more than 7 days after multiple dose administration [29,31].

The significance of residence time on different parameters such as efficacy, selectivity, toxicity and other related therapeutic effects has been reviewed elsewhere [30–32,35]. While those reviews describe residence time and kinetic parameters, they primarily focused on reversible inhibition. Hence, while most of the conclusions of those reviews are applicable to covalent inhibitors that act in a reversible manner (binding and unbinding at equilibrium),

some may not hold when irreversible inhibitors (no unbinding and virtually infinite residence time) are considered. It is worth mentioning that some non-covalent inhibitors may also exhibit long residence times, often resulting from an induced-fit mode of binding. This process, similar to covalent inhibition shown in Fig. 8, is characterized by a first step of complex formation, followed by a slower protein isomerization step that produces a final tight complex. Although these slow/tight binders share some mechanistic details in common with reversible covalent inhibitors, there exist some important differences from the drug design perspective. The covalent bond formation allows medicinal chemists to modulate binding kinetics by modulation of the steric and electronic properties of the inhibitor [36]. In contrast, modulation of a protein conformational change by small molecules is difficult to achieve. Optimization of covalent inhibitors will be discussed below.

3.4. Methods to measure kinetic constants of inhibition

In order to adequately characterize covalent inhibitors, we need to measure not only their affinities, but also their kinetic binding parameters. In this section, we will describe some of the main instrumental techniques that allow the kinetic characterization of covalent inhibitors.

Recording of *in vitro* biological activity remains the most widely used technique to measure inhibitor binding kinetics. This method is particularly used in the context of enzyme inhibition, as many approaches exist for quantifying the conversion of substrates to products, (e.g., cleavage of a substrate bearing a chromogenic or fluorogenic reporter molecule). For the determination of the off-rate (k_{off}) for instance, the target protein is pre-incubated with a given concentration of inhibitor until equilibrium is reached. The excess inhibitor is then washed away by rapid dialysis, chromatographic separation or rapid dilution. A reporter (e.g., a substrate) of protein activity is then added to the reaction media, and the recovery of the function over time is recorded. From the data obtained, a rate of dissociation can be derived and related to the off-rate of the covalent complex (k_{off}) (Fig. 10b). This experiment is also commonly used to evaluate the reversibility of the covalent inhibition. No activity recovery is observed in the case of irreversible inhibition.

In addition, since many covalent inhibitors exhibit relatively slow onset of inhibition, k_{on} can also be measured through *in vitro* assays. In the presence of a slowly inhibiting compound, the curve of inhibition (progress curve) will show a curvature that represents

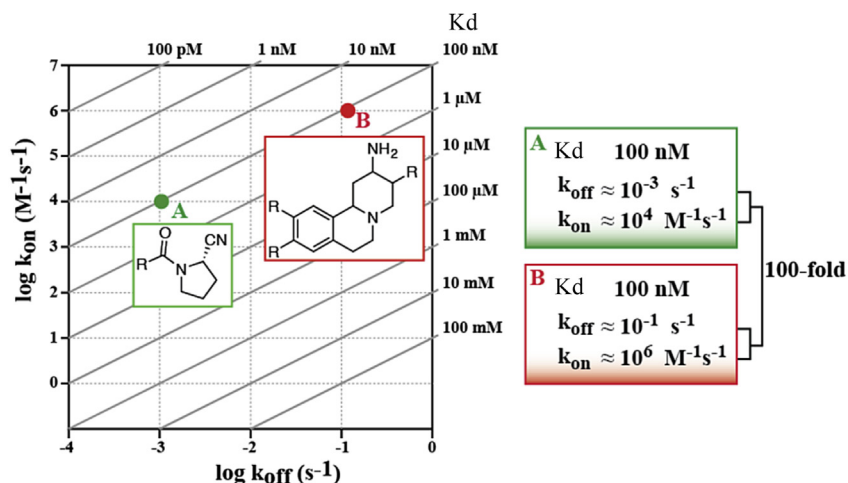


Fig. 9. $k_{on}/k_{off}/K_d$ -map of DPP-IV inhibitors, highlighting the difference in kinetic parameters observed for two compounds displaying similar affinities.

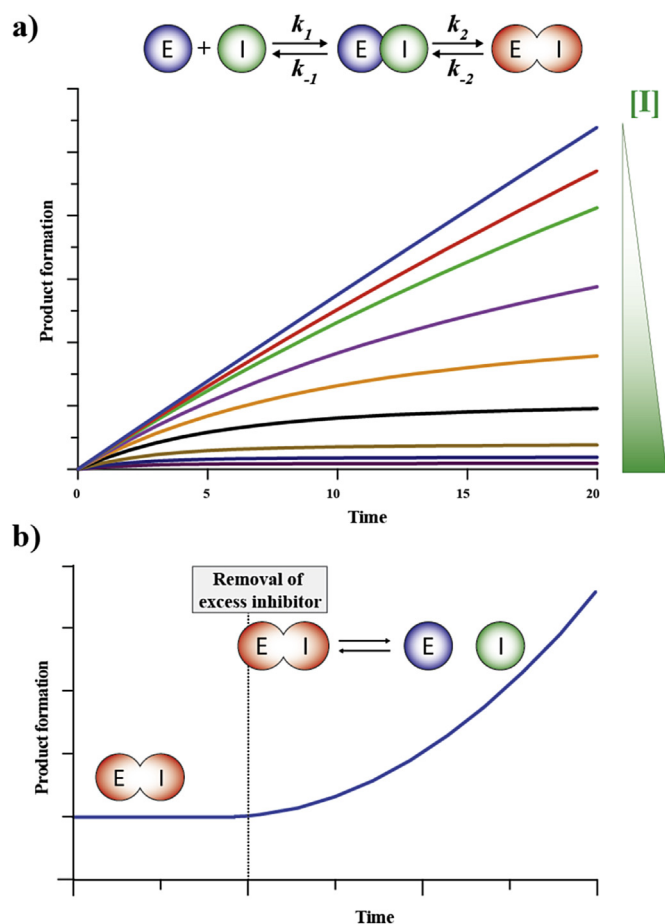


Fig. 10. a) Evolution of enzyme activity over time in the case of: covalent reversible inhibition. b) return of activity after washout of excess inhibitor.

the slow transition towards equilibrium, of the inhibition (Fig. 10a). Such enzyme assays have been used extensively for characterizing slow kinetics, whether covalent or not [37–40]. For example, the slow binding of Saxagliptin, a DPP-IV covalent inhibitor was characterized by Kim et al. via a steady-state absorbance assay of the cleavage of the Gly-Pro-pNA peptide [40]. The inhibitor as well the substrate were characterized in terms of their k_{on} , k_{off} and K_d . This allowed Kim et al. to propose a two-steps inhibition mechanism, from an initial encountering type of complex, to a covalent adduct with the serine in the active site (Fig. 11).

Another technique, surface plasmon resonance (SPR), has been gaining in popularity [41]. It offers the major advantage of being a fluorescent or radioactive probe-free method, which means it does not require any substrate or biological reporter molecule. In this method, the biological target (or the ligand) is immobilized on a surface. Then a buffer with a given concentration of ligand (or biological target) is flowed over the surface until equilibrium is reached. The complex is then slowly dissociated using a buffer with no ligand and the dissociation event is recorded (Fig. 12).

This extremely powerful technique has proven to be very useful in numerous binding kinetic studies, including in lead optimization stages towards the DPP-IV serine protease covalent inhibitors discussed above (Fig. 9) [33].

Lastly, recent developments in kinetic methods for isothermal titration calorimetry (ITC) [42], along with improvements in hardware will likely make it a major player in the future years. The advantage of ITC over SPR is the ability to work in solution with no need for immobilization of one of the two binding partners. ITC is

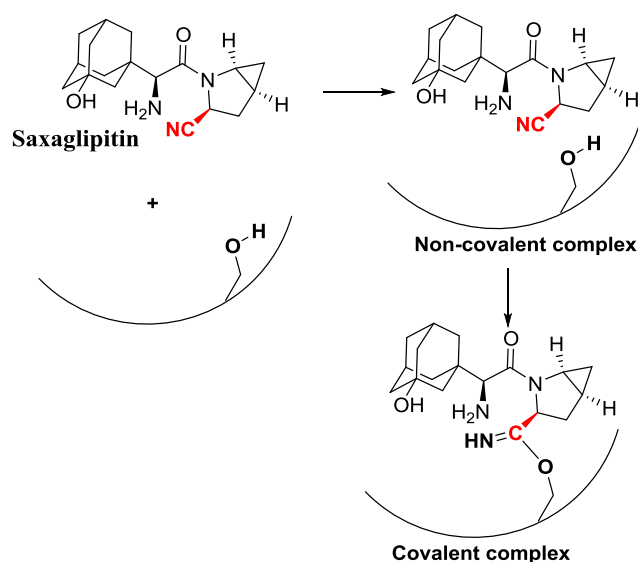


Fig. 11. Saxagliptin two-step covalent binding.

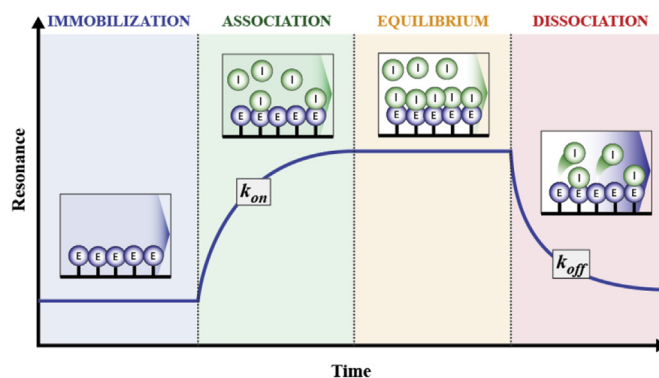


Fig. 12. Basic principle of surface plasmon resonance. For further details, please refer to review [41].

commonly used to quantify solution equilibrium constants and stoichiometries as well as thermodynamics quantities such as binding enthalpies, entropies and free energies. This technique has already been applied to a variety of biological systems [43,44]. For example, Lluís et al. investigated dual inhibitors of dimethylarginine dimethylaminohydrolase-1 (DDAH-1) and nitric oxide (NO) synthase [45]. ITC was used to characterize continuously the interaction of their inhibitor of wild-type DDAH-1 as well as the mutation of the cysteine in the active site (C274S) mutant in a label-free manner which is complementary to stopped-flow IC_{50} assays. They managed to match previous results and to gain more information on the binding of the inhibitor; the binding of the inhibitor to the wild type enzyme was found to be entropy-driven, likely due to water displacement, with binding to mutant was less entropy-driven since water is retained in the active site.

3.5. Integration of binding kinetic with pharmacokinetic and pharmacodynamic

The binding kinetics of covalent drugs have major implications for their efficacy. Specifically, the long residence times of covalent drugs can lead to favorable pharmacokinetics (PK) and more effective inhibition of the target. The pharmacodynamic (PD) profile is important when optimizing a lead compound (Fig. 13). In fact,

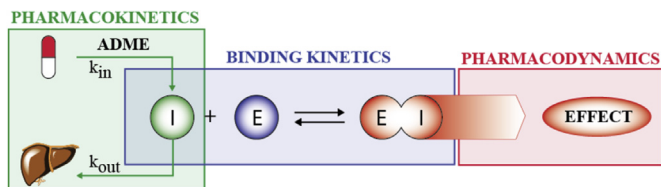


Fig. 13. Links between pharmacokinetics (PK), binding kinetics (BK) and pharmacodynamic (PD).

this profile will determine the percentage of inhibition and duration required to have a noticeable effect on the pharmacological properties *in vivo*. Not all targets require the same levels of inhibition to exert a specific effect. Thus, during classical (i.e., non-covalent) lead optimization stages, PK and PD profiles are usually linked together. If a drug requires 50% of the target to be occupied to have a measurable effect, the PK profile of this drug will need to be optimized in order to retain 50% of the target occupied. Typically, the desired properties involve a proper balance between metabolism/excretion and an exposure long enough to the target such that once or twice a day dosage is sufficient to maintain the desired effect.

As shown in a PD simulation (Fig. 14), the concentration of a non-covalent inhibitor in rapid equilibrium quickly decays and the inhibition level drops below 50% while the drug is being cleared. In contrast, the concentration of a covalent reversible drug, with a residence time of just 20 min, decays slowly with a much longer clearance period. An inhibition level greater than 50% is maintained over a period of 12 h, enabling less frequent administration. Irreversible inhibitors follow the same trend, if the rate of the permanent inactivation step (k_{inact}) is sufficiently rapid compared to the dissociation rate of the non-covalent complex and the rate of elimination of the drug (k_{out}). Under favorable conditions, a single short exposure induces permanent inhibition of the target with no need for additional doses. Of course, further parameters, such as target degradation and the protein resynthesis rate, will have to be considered as part of a complete PD study.

This simulation illustrates that even a short period of exposure can provide blockage of a given target over a long duration. This simplifies optimization of the ADME properties since long optimization for a long-lasting drug exposure can be reduced. Furthermore, it limits the time with systemic drug in the body, which, in

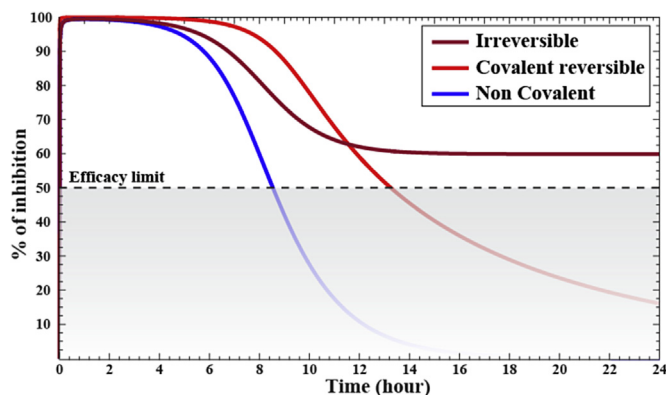


Fig. 14. Simulation (using Matlab) of the effect of pharmacokinetics (PK) on the fractional occupancy of a given target by different types of inhibitors. The parameters are such that k_{in} and k_{out} are 1 h^{-1} . Non-covalent inhibitor: $K_i = 1 \text{ nM}$, $k_1 = 1.107 \text{ s}^{-1} \text{ M}^{-1}$. Reversible covalent inhibitor: $k_1 = 1.107 \text{ s}^{-1} \text{ M}^{-1}$, $k_{-1} = 0.01 \text{ s}^{-1}$, $k_2 = 0.1 \text{ s}^{-1}$, $k_{-2} = 0.01 \text{ s}^{-1}$. Irreversible inhibitor: $k_1 = 1.107 \text{ s}^{-1} \text{ M}^{-1}$, $k_{-1} = 0.01 \text{ s}^{-1}$, $k_2 = k_{inact} = 0.1 \text{ s}^{-1}$, $k_{-2} = 0 \text{ s}^{-1}$.

turn reduces the risk of off-target liabilities, drug-drug interactions and associated toxicity. Binding kinetics data can also lead to target-tailored optimizations of the interactions in order to lead to the desired biological effect.

Thus, kinetic parameters play a major role in the effective inhibition of a target. Their characterization allows experimentalists to make more educated decisions of whether to pursue the development of a given chemical series or not. Kinetic parameters also provide a better understanding of the global inhibition process, from absorption to biological effect. In order to take advantage of these new handles for optimal drug design, one needs to understand how structural or reactivity changes affect the different parameters described above.

4. Covalent drugs and drug discovery process

4.1. Discovering a covalent drug

The drug discovery process begins with the identification and validation of one (or more) biological target(s) and continues to the final optimization of an identified lead. In this regard, covalent drugs have some unique considerations (Fig. 15), as discussed below.

4.2. Choosing a target for covalent inhibition

4.2.1. When covalent inhibition can be advantageous

The energetic aspects of covalent drug binding have been reviewed and discussed by Houk and co-workers [4]. They proposed that non-covalent affinities can be directly estimated from the surface area of the interaction, meaning that high affinities (tight binding) for small molecules/protein complexes are difficult to achieve without a covalent interaction involved in the process. Given the extra binding energy of a covalent bond, covalent inhibitors compete more efficiently with endogenous substrates (80% of the drug targets have an endogenous substrate that competes with the associated drug [46]). It should be noted that blocking a biological pathway often induces a build-up of the natural substrate that eventually leads to the displacement of the inhibitor. In the case of an irreversible covalent bond between the target and the drug, no displacement is possible, providing a more efficient biological effect.

Similarly, covalent drugs can also compete with unusually

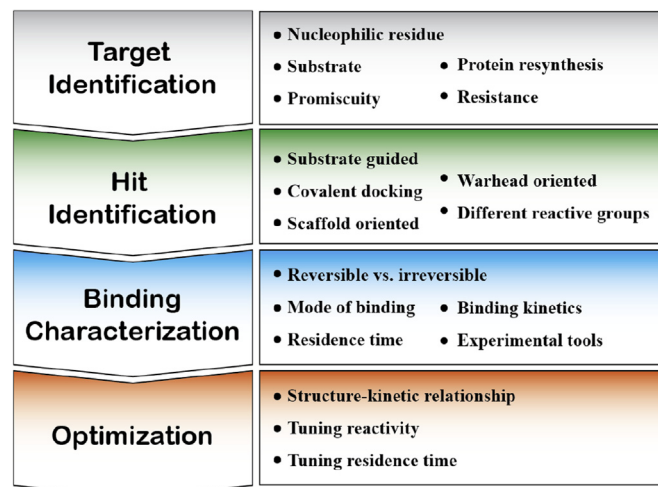


Fig. 15. Overall process of a covalent drug discovery program.

concentrated, or high affinity natural ligands, opening an entire class of new “druggable” targets such as those involved in protein-protein interactions. A recent example is the inhibition of cathepsin K (catK), a cysteine protease responsible for the remodeling of bones and an emerging target for the treatment of osteoporosis [47]. CatK had previously been labeled as an undruggable target [48] due to its very small and solvent-exposed active site. Handles available for non-covalent interactions are scarce and reaching high affinity was foreseen as challenging. Recently, a covalent inhibitor (Odanacatib) [49] was developed by Merck and has reached the final stage III clinical trials with promising clinical results (Fig. 16).

Not only can covalent inhibitors target biological entities not thought to be accessible in the past, but they also provide us with a unique tool to combat drug resistance. Resistance is often due to one or more mutations that modify the affinity of a given drug for the targeted protein. For irreversible inhibitors, complete inhibition will be achieved regardless of any differences in the rate of formation induced by a mutation, given a sufficiently long exposure. Thus, covalent drugs can be less prone to the development of resistant strains than are non-covalent inhibitors. This advantage has been briefly discussed above in the section *Marketed covalent drugs* in the context of an EGFR covalent inhibitor, Afatinib. In this example, a mutant of the receptor enhances the affinity for ATP (endogenous substrate of EGFR). It is believed that this effect alone is responsible for the resistance observed towards Gefitinib, a non-covalent inhibitor. Contrastingly, Afatinib (irreversible inhibitor) activity is not affected by the mutation since the covalent bond formation makes it less sensitive to the concentration of the natural substrate ATP [50].

4.2.2. Limitations

Despite the tremendous potential of covalent inhibition, there are several potential pitfalls and limitations of this class of drug that should be kept in mind. While sustained occupancy of the target can produce beneficial biological effects, longer residence time can also lead to toxicity. A good illustration is the irreversible inhibition of the P2Y₁₂ receptor by Clopidogrel, a drug prescribed for thrombosis (Fig. 6). While the blockade of this receptor inhibits platelet aggregation (the main factor in thrombosis), extended inhibition also leads to excessive bleeding time, which could generate adverse clinical outcomes if the drug is used on a long-term basis [51].

Conversely, when the cell rapidly resynthesizes the target, improving residence time beyond the time of resynthesis will not lead to any substantial improvement of the efficiency, and may only

increase the potential toxicity. The same conclusions apply when the pharmacological event triggered by a drug/target interaction occurs over a finite period of time. For instance, the interactions between antigens and receptors require endocytosis to produce an immune response. The half-life for endocytosis has been estimated to be 8.5 min [52], therefore any association event that would lead to a residence time over this time would not necessarily lead to an improved biological response.

Those limitations illustrate the importance of understanding the mechanism of action and the biological relevance of a given target to decide whether or not covalent inhibition is a suitable/desirable approach to pursue. We have seen that covalent drugs could be effective against previously inaccessible therapeutic targets. On the other hand, some biological targets where the biological response and toxicity derive from the same binding interaction should probably be treated with more care, residence times should be carefully monitored, and irreversible covalent inhibitors probably avoided. An example of this intertwined toxicity and activity is cisplatin which can cause toxicity issues when it binds to mitochondrial DNA of healthy cells, and not only to DNA of rapidly proliferating cancer cells [53].

4.3. Design and discovery of covalent inhibitors

4.3.1. Covalent inhibition in a drug discovery process

Designing a covalent drug can be challenging, given the paucity of knowledge existing on optimal approaches. As discussed above, the covalent nature of some of the currently available covalent drugs was discovered after their development. Nevertheless, whether the covalent nature of these drugs was obtained accidentally or by design, lessons can be learned from these examples. In this section, we will discuss briefly specific considerations in the discovery and design process for covalent inhibitors.

Strategies for discovering a covalent drug are in many regards similar to strategies employed for any given medicinal chemistry project: structural and mechanistic knowledge about the target is of paramount importance. Given this information, discovering a covalent drug may follow the same path as with any other drug discovery program: large assay screening, virtual screening, and rational design are some of the possible strategies. All those fields are now well established in the drug discovery community and discussing them in details is beyond the scope of this review. A section below will be dedicated to computational methods. Interestingly, given the extra amount of energy provided by the formation of a covalent bond, it is believed that high potency can be achieved earlier in the drug development process [4].

4.3.2. High throughput screening (HTS) for the discovery of covalent binding drugs

While high throughput screening (HTS) is very common in the early stages of drug discovery, it is expected to be a lot more complex to apply to covalent drug discovery. In general, the covalent mode of action has been discovered during the process [54,55] and there exists very few studies where libraries of potential covalent compounds were specifically screened [56]. In this context, it is critical to distinguish true covalent inhibitors from Pan Assays Interference Compounds (PAINS). Although PAINS are false positives, which must be discarded, many of such compounds are still reported as new promising hits for a variety of targets [57,58]. A strategy is to rely on PAINS alerts to remove compounds which contain common PAINS substructures from HTS screening [59]. However, a small molecule featuring any given substructure alert, may not necessarily be a PAINS and potential covalent inhibitors would also be discarded [60]. Similarly, covalent inhibitors may appear as false positives in HTS campaigns and additional

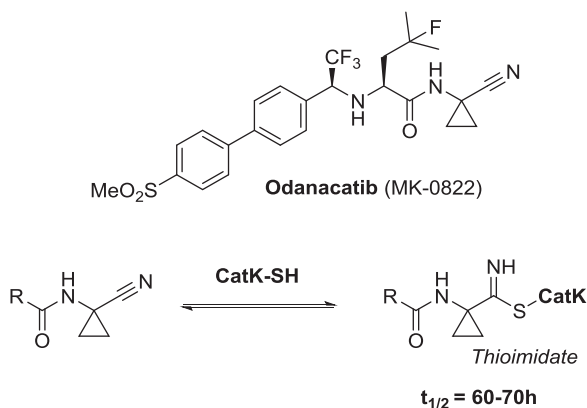


Fig. 16. Odanacatib inhibits CatK, an enzyme previously described as highly challenging.

characterization or orthogonal screens are required.

4.3.3. Activity-based protein profiling

Activity-based protein profiling (ABPP), a technique used to characterize enzyme function directly in their biological systems, relies on the covalent interaction of a probe (ligand) and enzymes. In the past years, ABPP has emerged as a major field where covalent modifiers play a prominent role [61–66]. For instance, Orlistat, a commercially available covalent drug (marketed under Xenical®, Fig. 3), is a natural product-derived irreversible inhibitor of pancreatic lipases [67]. It has also been found to inhibit fatty acid synthase (FAS), an essential enzyme in the growth of tumor cells [68–70]. Orlistat has been modified with reporter molecules such as biotin or rhodamine in order to conduct ABPP [71]. This allowed identification of eight different potential targets of Orlistat, such as HepG2 cells which are involved in tumor cell proliferation and Annexin A2 which is involved in cell proliferation and division. This work highlights the importance of ABPP in early stage identification of potential off-target effects. ABPP has been successfully used to identify active covalent inhibitors including oxime esters blocking RBBP9 enzyme, an enzyme implicated in cancer [72,73] and acrylonitriles as inhibitors of protein phosphatase methylesterase-1, a potential target for cancer and Alzheimer's disease therapeutics [74]. Different flavors of ABPP have been reported and reviewed in Ref. [75].

4.4. Optimizing covalent and non-covalent interactions

When an inhibitor has been identified, or is already available, it can interact with the target either covalently or non-covalently. It is often at this stage of a drug discovery project that most of the covalent modifiers are discarded or, in some cases, repurposed for mechanistic investigations of the target [65]. When a covalent inhibitor is identified in the screening process, it is important to ensure that the binding does not rely exclusively on a non-specific reaction of a reactive group with a nucleophilic residue in the active site. Building specific interactions around a reactive functional group can be in some regard related to *de novo* design, which comes with its own challenges and pitfalls [76]. One notable example is the development of a multitarget antipyroptotic compound that was found by screening a library of diverse α,β -unsaturated electrophilic warheads. All the compounds shared the same core warhead and the library was then built by varying many non-covalent substituents (Fig. 17) [56].

Despite the success of this study, the vast majority of designed covalent inhibitors started from a non-covalent hit or lead molecule [77]. In fact, starting from a known reversible inhibitor ensures that the derived covalent analogues will have optimal selectivity and allows for less reactive functional groups to be used further improving its safety.

4.5. Reactive functional groups

4.5.1. Typical warheads used for irreversible inhibitors

As discussed above, the distinction between reversible and

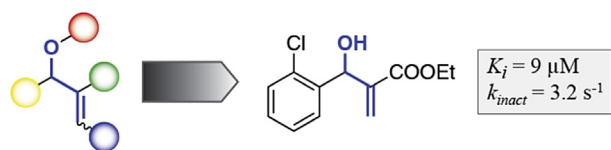


Fig. 17. Warhead-based design of irreversible inhibitor of the NLRP3 inflammasome [56].

irreversible inhibition is key. In this section, we will present some of the most widely used reactive groups in either reversible or irreversible inhibition.

A variety of reactive groups that form irreversible links with a target are available for use in covalent inhibitors (Fig. 18). Some functional groups such as epoxides, aziridines, halomethyl- and acyloxymethyl-ketones are often regarded as too reactive for potential drug applications. Nonetheless, examples of selective and relatively safe inhibitors bearing those functionalities exist. Fosfomycin (Fig. 3), a highly reactive epoxide, demonstrates that even highly reactive groups can be used for pharmaceutical applications. Extensive study has revealed that it forms a covalent bond with the target protein, UDP-N-acetylglucosamine-3-enolpyruvyltransferase (MurA), an essential enzyme for the bacterial cell wall biosynthesis [78,79]. Similarly, other scaffolds take advantage of ring strain to achieve irreversible inhibition. This includes scaffolds such as aziridines (azinomycin, Fig. 3), cyclopropanes, β -lactones (Marizomib in Fig. 18) or β -lactams (Marizomib in Fig. 18 and penicillin in Fig. 3). For β -lactone such as Marizomib, the covalent adduct may be hydrolyzed, although this process is slow and binding can be considered irreversible. Similarly, the Michael addition to some covalent inhibitors may be reversible as discussed below.

Although cyclopropanes are poor electrophiles, they may react

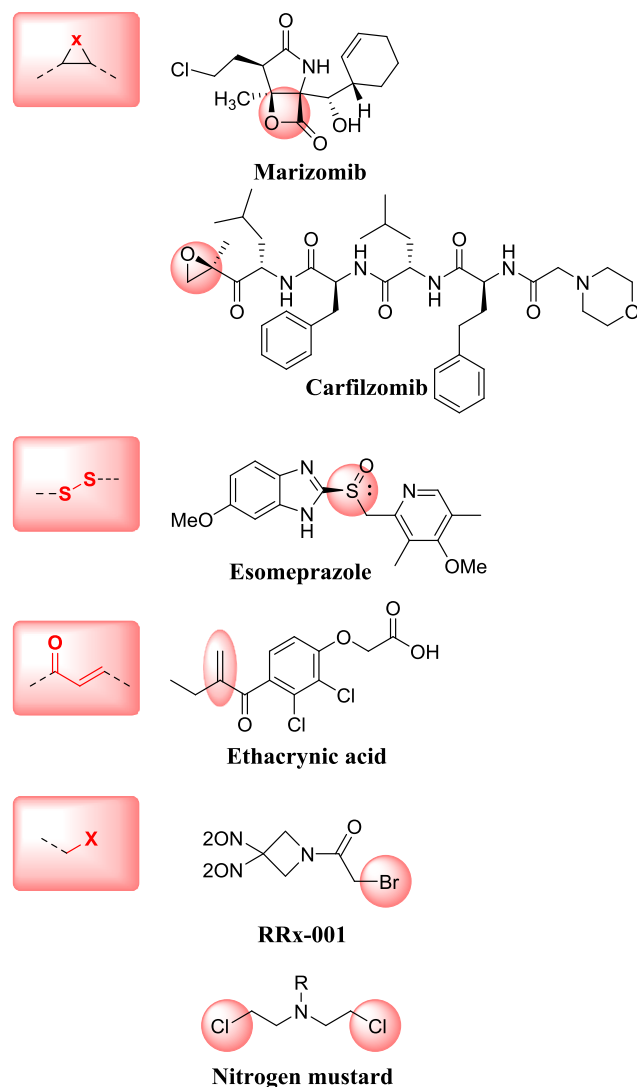


Fig. 18. Examples of reactive groups forming an irreversible bond with their target.

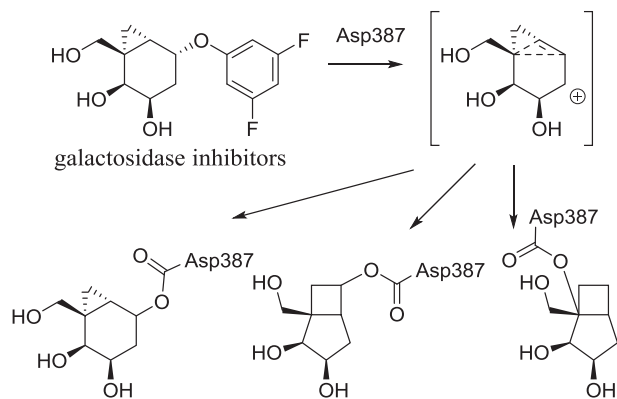


Fig. 19. Proposed mechanism of action for α -galactosidase inhibitors.

under specific conditions. For instance, Bennett and co-workers have discovered galactosidase inhibitors which are converted into carbenium ions which are trapped by an aspartic acid of the catalytic site (Fig. 19) [80].

Alkylating agents are another well-established class of irreversible covalent drugs, although they are generally associated with significant toxicities. Alkylating agents generally bind non-selectively to DNA or RNA which can lead to off-target effects. Some very famous historical examples are nitrogen mustard and its various analogues which were used in treatment of tumors, lymphomas and leukemia following their use as a chemical warfare in World War I [81]. Some recent efforts have been made as to improve the selectivity of alkylating compounds, such as the compound RRx-001 (EpicientRx) a prooxidative vascular anticancer agent, which is currently in phase II clinical trials [82].

Another functional group that involves permanent linkage is the formation of a disulfide bridges. This is well exemplified by the blockbuster drugs Omeprazole (Losec[®]) and Clopidogrel (Plavix[®]) which were discussed in a section above. However, this may be facing other potential issues such as cell reducing environment and the presence of glutathione, a cysteine-containing detoxifying peptide.

Finally, one functional class attracting considerable interest in recent drug discovery programs comprises the α,β -unsaturated carbonyls that are often referred to as Michael acceptors. The target of choice for these electrophiles has been thiols from cysteine residues which are the most nucleophilic among non-activated residues. Targeting catalytic or non-catalytic cysteines in an active site is becoming a popular strategy in covalent inhibition. This approach is more commonly referred as targeted covalent inhibition (TCI) [3,83]. Notable examples of the use of α,β -unsaturated carbonyls is in the field of kinase inhibition with inhibitors such as Afatinib (Fig. 4 and Marketed covalent drugs) [84].

4.5.2. Typical warheads used for reversible inhibitors

Reversible covalent inhibitors possess reactive moieties that react to produce labile cross-linked products. For example, aldehydes may react with catalytic serine residues forming hemiacetals (Fig. 20 and Fig. 21). As this transformation is reversible, the free aldehyde and cross-linked hemiacetals are in dynamic equilibrium. Similarly, esters and carbamates can form reversible linkages due to the possible hydrolysis of the newly formed bond, although in this case, the inhibitors have been hydrolyzed and do not re-react. Other possible reversibly-reacting groups include ketones, nitriles and boronic acids. Those groups usually react with a serine or cysteine of the biological target (Fig. 21).

Aldehydes have been a valuable tool for discovering potential

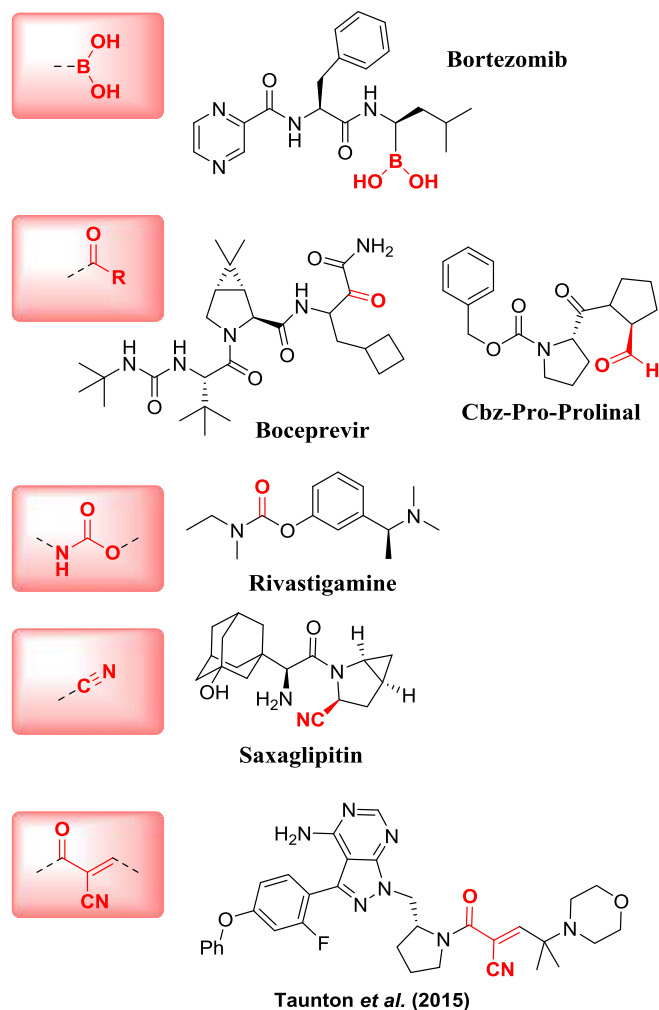


Fig. 20. Examples of reactive groups used in reversible covalent inhibition.

covalent inhibitors. However, although aldehydes are common in natural products (cinnamaldehyde is found in cinnamon while vanillin is extracted from vanilla beans), aldehydes are believed to be too reactive for safe drug development. However, there are several examples in which, inhibitory activity was discovered with an aldehyde reactive group which was subsequently substituted with a milder electrophile. The development of Bortezomib (Fig. 20), an FDA-approved drug that covalently inhibits the 20S Proteasome, reflects this concept. While the first inhibitors of the proteasome were aldehyde-containing peptides, they were also poorly selective over other proteases. Substitution of the aldehyde by a boronic acid moiety led to an improved selectivity profile [85]. Boronic acids are in fact widely used as warheads, especially in protease inhibitors, and have also been used as anticancer, antibacterial and antiviral agents [86,87].

Nitriles are also receiving much attention in covalent drug design [88]. For example, Saxagliptin is a nitrile-containing covalent inhibitor of dipeptidyl peptidase IV (DPP IV) (Fig. 20) [89]. Their popularity is mainly due to their milder reactivity that requires proper positioning in the active site in order to trigger covalent bond formation via a (reversible) Pinner reaction. As a result, the chemical nature of the binding site controls the equilibrium shown in Fig. 21c by stabilization of the iminoether. Fine-tuning the reactivity of the nitrile and/or stability of the iminoether (i.e., modulating the equilibrium shown in Fig. 21c) provides some control of the residence time of the designed inhibitors.

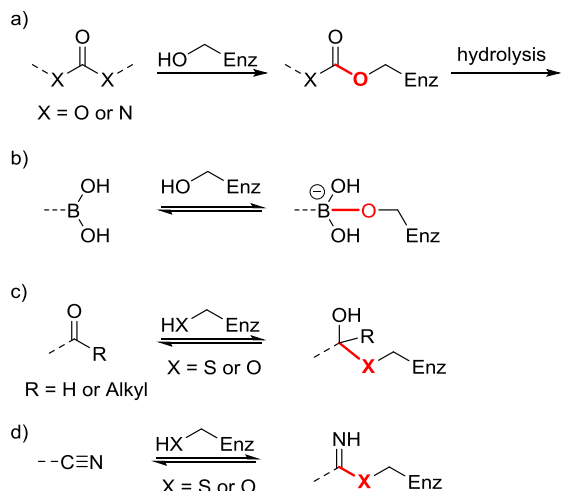


Fig. 21. Mechanisms of reversible inhibition. a) carbamates and carbonates; b) boronic acids; c) ketones and aldehydes; d) nitriles.

Another possible class of reversible covalent warhead is the α,β -unsaturated carbonyls mentioned above as a group for irreversible inhibition. Indeed, if the electronic environment is tuned properly, the Michael addition can be reversible. For example, introduction of a nitrile at the α position converts an irreversible into a reversible inhibitor (Fig. 20) [52]. This property is of particular importance for the tuning of the reactivity of warheads as discussed below.

4.6. Modulation of reactivity and residence time

Structure-based design of inhibitors has become increasingly popular over the past decades, with the growing advances in structural biology. This approach enables medicinal chemists to visualize directly the possible structural modifications that can be made to a ligand to optimize its interactions with the targeted biomolecule.

Similarly, experimental tools to study the kinetics of binding are gaining popularity and ease of use. Those techniques along with the increasing interest in covalent inhibition should induce large-scale kinetic and structural assessment of potential bioactive compounds. These Structure Kinetic Relationships (SKR) studies will, similarly to more traditional Structure Activity Relationship (SAR) investigations, increase our knowledge of the impact of structural modifications on kinetic parameters.

In fact, examples of such endeavors have already been reported. Covalent inhibition of kinases has flourished in recent years [52,84,90–97]. Most of these covalent inhibitors feature an irreversible acrylamide Michael acceptor warhead (Fig. 22). Although proven to be suitable and fairly selective, irreversible inhibition always raises safety concerns, especially in such a large family of similar proteins (over 500 different kinases in the human kinome).

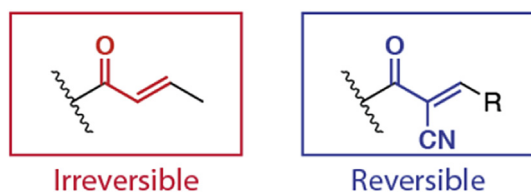


Fig. 22. Irreversible and reversible warheads used in kinase inhibition.

It has been shown that many of the kinases feature a non-catalytic cysteine in close proximity to the active site [98], making them good candidates for targeted covalent inhibition.

Potential liability could arise from irreversible binding to an off-target kinase and/or other proteins containing reactive cysteines [99]. Examples exist where acrylamide has been replaced with cyanoacrylate in order to confer reversibility to the addition of thiols (Fig. 22). Activation of this Michael acceptor by both a nitrile and a methyl ester, two electron-withdrawing groups, increase the acidity of the α proton, increasing both the rate of thiol addition and the rate of thiol adduct elimination. In a first report of this new warhead class [52], Taunton and co-workers developed reversible RSK2 kinase inhibitors. They discovered that cyanoacrylate forms a tight, yet reversible, covalent bond with the targeted kinase. They also demonstrated that although the ligand reacts rapidly with exposed cysteines, such as the one found in glutathione, isolation of the resulting adducts was not possible due to the rapidly reversible nature of this association. The stability of the covalent complex (hence the residence time) is governed by the stabilization of the non-covalent interaction and the lower accessibility of the acidic hydrogen for water (Fig. 23). The acidity of the H_α has also been linked to the stability of the complex. Calculated DFT proton affinities correlated well with the observed rate of dissociation [96].

Similarly, Taunton et al. tackled inhibition of the Burton's tyrosine kinase (BTK) with a similar strategy [36]. After proper positioning of the reactive warhead, they looked at the influence of various “caps” on the cyanoacrylate moiety. They observed that an increase in steric bulk around the H_α is linked to an increase in residence time (Fig. 23). Those two examples are a good illustration of the knowledge that one can gain from SKR investigations.

The correlation of ligand geometry and warhead intrinsic reactivity with protein-ligand complex stability and residence time highlights the new possibilities offered in fine-tuning covalent inhibition in an educated manner. In addition, this approach is not limited to just cyanoacrylates, and can be applied to every known reactive functional group, both experimentally and computationally. The intrinsic reactivity of electrophiles has been extensively studied in the context of organic synthesis. Computational predictions of reactivity can also contribute to this effort. Indeed, despite the lack of general methods for reactivity assessment of different amino acid nucleophiles (serine, threonine, histidine, ...), methods exist for predicting reactivity of functional groups towards cysteines [100–102].

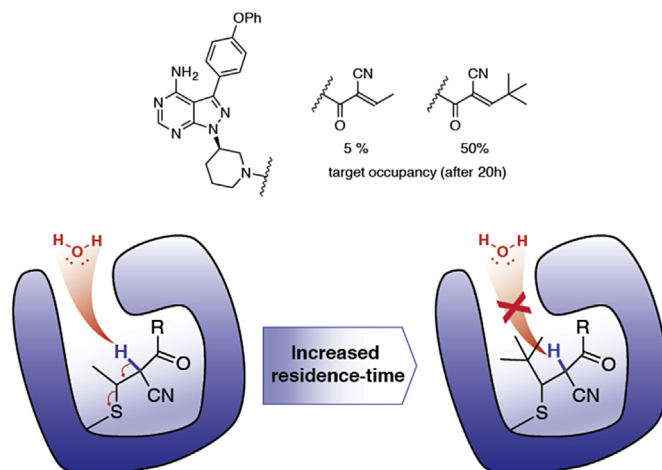


Fig. 23. Tuning of residence time through steric control in kinase inhibition [36].

5. Computer-aided covalent inhibitor design

5.1. Computational drug design

While computational tools are widely used for non-covalent drug design, computational methods for covalent drugs are scarce. Thousands of quantitative structure-activity relationship (QSAR) models and docking studies have been derived for non-covalent drugs whereas little has been reported for covalent drugs and few programs/methods are available to the medicinal chemistry community. It should be clarified that computational methods are complementary to and benefit from other structural techniques such as crystallography [103].

5.2. Quantum mechanics methods

As discussed above (Fig. 22), the reactivity of a warhead can be significantly affected by neighboring groups. Computing the reactivity of the drug and the stability of the enzyme-drug adduct would enable medicinal chemist to design and fine-tune drugs with appropriate residence time. In 2007, Oballa and co-workers used density functional theory (DFT) to assess the reactivity of nitrile-containing fragments for the design of CatK inhibitors (Fig. 24) [101]. They concluded that DFT-based approaches can be used as a tool to predict nitrile reactivity towards cysteines. Approaches like these show the potential of computational methods to predict covalent reactivity and have begun to be exploited.

More recently, Flanagan et al. and Smith et al. investigated the reactivity of various groups with a strong focus on Michael acceptors towards thiols (models of cysteine residues as found in glutathione-GluH and some kinases) [100,102]. Flanagan et al. found a strong correlation between the calculated energies of activation ($\Delta G^\ddagger$) and the measured half-lives of the substrates (Fig. 25) [100]. Similarly, Smith et al. showed that there is no correlation between the Michael adduct (Fig. 26) stability and the presence of a given group in a drug [102]. In contrast, they found that anion stability (intermediate in Fig. 26) is a good descriptor of the viability of a Michael acceptor group in a drug. Too stable an intermediate often refers to compounds that reacts readily while too unstable often refers to compounds that hardly react.

These studies illustrated the application of DFT towards predicting reactivity of compounds, a property that has not been studied or applied to enzymatic reactions extensively. This integral property of electrophilic reactivity and protein residue nucleophilicity is essential in predicting reactions which are covalent in nature (ie, covalent ligands). This method could easily be extended to the prediction of the reaction between a kinase cysteine and a covalent inhibitor.

Quantum mechanics (QM) methods have shown to be useful in

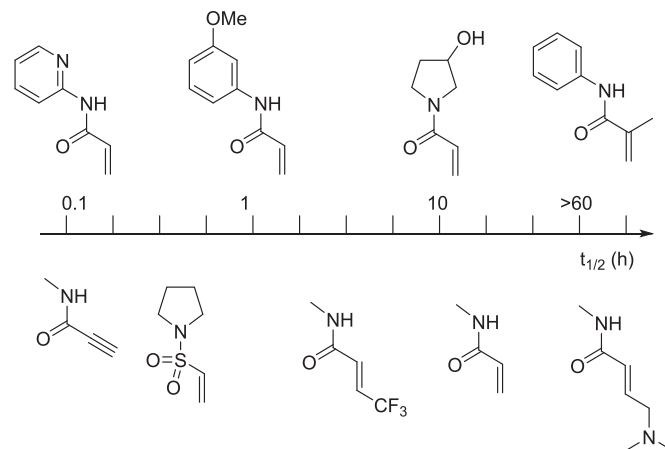


Fig. 25. selected compounds with their corresponding half-lives as ranked by Flanagan et al.

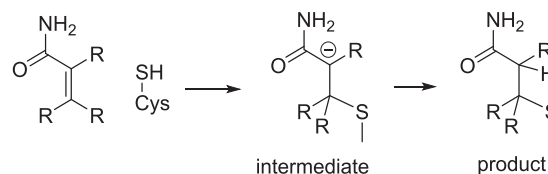


Fig. 26. Correlations of drug-like covalent fragment and stability of intermediates and products.

predicting the reactivity of covalent ligands. Although QM calculations scale poorly as the number of atoms in a system increase, extracting parameters or generating predictive models provides useful information in library screening for covalent compounds. These methods provide a basis from which other methods, such as covalent docking or virtual screening can be built upon.

5.3. Ligand-based methods

A common computational approach in drug discovery is QSAR. However, the traditional QSAR models are developed for non-covalent inhibitors and thus do not consider the contribution of the covalent bond to the binding energy. For example, models for covalent binding-related toxicity were developed without specific descriptors for reactivity [104].

In order to address this issue, Shokhen and co-workers have developed two descriptors (W1 and W2) which measure the energetics of the bond formation between a nucleophilic residue and a carbonyl [105]. While W1 measures the binding energy when the intermediate remains anionic, W2 measures the energetics of the binding with protonation (Fig. 27). These descriptors were derived from semi-empirical calculations (PM6) on model systems. As a validation, they carried out retrospective studies on thrombin and cathepsin D and found that W1 and/or W2 are critical for optimal predictions. Although this was a first step towards the use of QSAR with covalent drugs, safe covalent drugs are not only built around a carbonyl (Figs. 18 and 20) and models for other warheads are to be developed.

Others have used QM calculations to derive QSAR models for reactivity. For example, following up on their QM investigations of the addition of thiols to double bonds described above [102], Smith and Rowley developed a QSAR model which can predict the reactivity from easy-to-compute descriptors such as the orbital energies of the double bond. Thus, an electrophilicity index, a

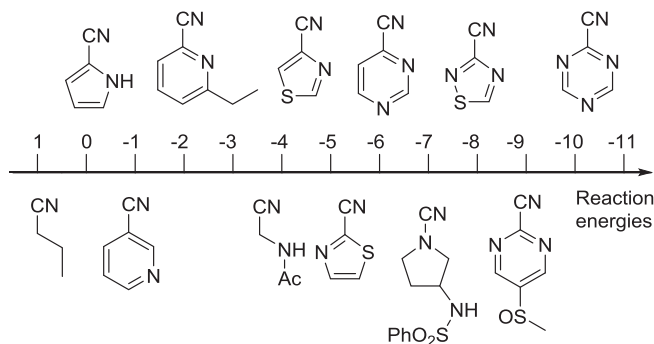


Fig. 24. CatK inhibitor fragments.

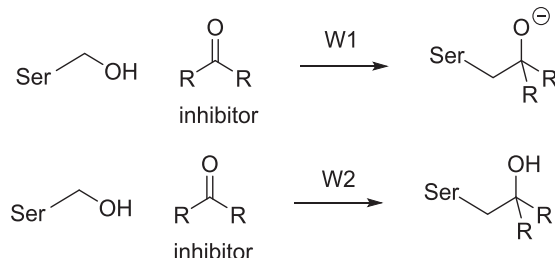


Fig. 27. Descriptors for covalent inhibition in QSAR models.

resonance stabilization index, and a steric hindrance descriptor were combined into a model predicting Michael acceptor reactions with glutathione [106]. This approach was found to be very predictive with a high correlation between the predicted and experimental rate constants. Once more, this reactivity model can certainly be extended to covalent inhibition.

5.4. Docking: modeling covalent bonding

5.4.1. Docking covalent inhibitors-status

Docking-based drug design has become a common practice in numerous academic and industrial medicinal chemistry groups. Docking programs predict binding modes and relative affinities of ligands and rely on two fundamental steps: 1) sampling the conformational space of the ligand-protein complex (flexible ligand and sometimes flexible protein); 2) ranking the sampled conformations with a scoring function to predict the likely binding mode or, in the case of virtual screening, distinguish active from inactive compounds. Each docking program utilizes its own methodology in order to determine the most promising drug candidates. These algorithms are beyond the scope of this review and have been exhaustively reviewed in Ref. [107]. This review will focus on the methods employed to dock and score covalent ligands and on the limitations of current covalent docking methodologies.

5.4.2. Bond formation

In the past, covalent drug development was under-looked and most drug discovery programs focused on the development of non-covalent ligands. This has resulted in virtual high throughput screening (vHTS) and docking methods tailored towards non-covalent compounds. In contrast, methods to efficiently identify covalent actives were underdeveloped and not as accurate as those that can identify non-covalent ligands. The rising interest in covalent drugs has resulted in a surge in the development of docking methods to bolster their discovery and design [108]. As a result, several of the most popular docking programs have been modified to accommodate covalent docking and several new docking programs that focus on covalent docking have also emerged. Examples of these include DOCKoalent (from DOCK) [109], DOCKTITE [110], CovDock [111], AutoDock [112], FlexX [113], FITTED [114,115], GOLD [116], and CovalentDock (in AutoDock) [117].

One of the fastest routes to include covalent compounds in previously developed programs was to modify methodologies to accommodate bond formation between the target and ligand, allowing for identification of covalent poses (predicted binding modes) that can then be scored and ranked. Several covalent bond forming procedures have been developed. One of the simplest is the link-atom approach implemented in GOLD [116]. This method essentially forces the superposition of the manually defined “link-atom” on both the nucleophilic residue in the protein and the electrophilic moiety of the ligand. If the serine oxygen in the active site of a protease is chosen as the link atom, this oxygen atom is also

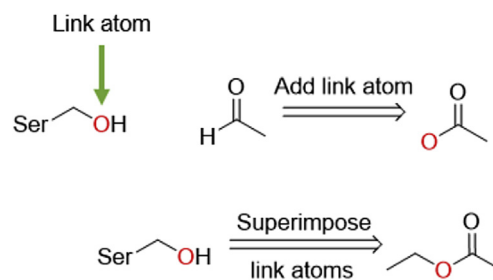


Fig. 28. Link atom approach.

added to the electrophile on the ligand in the desired position (Fig. 28). Superposition of these two atoms now simulates a covalent bond. This procedure in GOLD has been validated on a set of 76 covalent complexes, however the average RMSD was found to be quite poor at 3.69 Å while RMSD below 2 Å is usually considered as a criterion for successful docking [97].

A disadvantage of the link-atom approach is the requirement for manual definition of link-atoms, an approach hardly applicable to large libraries of small molecules. A second generation of programs appeared that addressed this issue. For example, this has been overcome in DOCKTITE through the use of database searching for specific electrophilic warhead containing compounds. The electrophilic atom is tagged which allows automatic recognition of reactive groups (Fig. 29). These reactive groups are linked to various reaction types that are used to modify the ligand to its bound conformation, followed by application of a tag to the electrophilic atom. By fully automating this process, DOCKTITE has the ability to be applied to screening of large libraries. DOCKTITE has been validated on 35 protein-ligand complexes with an average RMSD of 1.7 Å [91].

DOCKoalent [109], integrates a method to automate bond formation through the use of SMARTS patterns (Fig. 30). These patterns are used to match reactive groups to reactions in order to generate bound structures. This methodology has been used to discover reversible covalent ligands which bind to several nucleophilic proteins including AmpC β -lactamase, and RSK2, MSK1 and JACK3 kinases [109].

Another common disadvantage of docking programs is the inability to screen a library consisting of both covalent and non-covalent binders. This has been addressed in the docking software FITTED [95,114,115,118]. A diverse range of functional groups can be identified in an automated fashion by FITTED such as Michael acceptors, aldehydes, nitriles, and boronic acids. A more recent implementation (unpublished) considers the reactivity of these groups, although further training is required to encompass a large

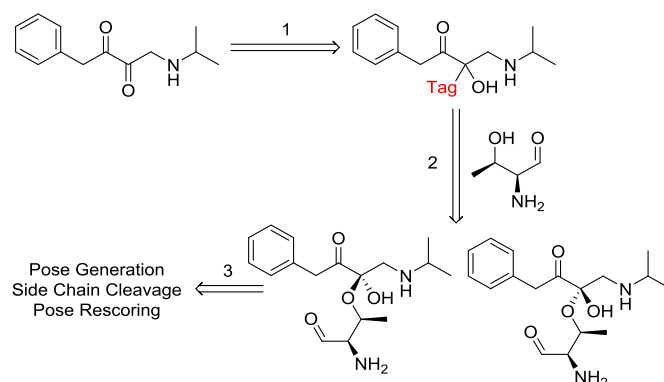


Fig. 29. DOCKTITE procedure for covalent bond formation.

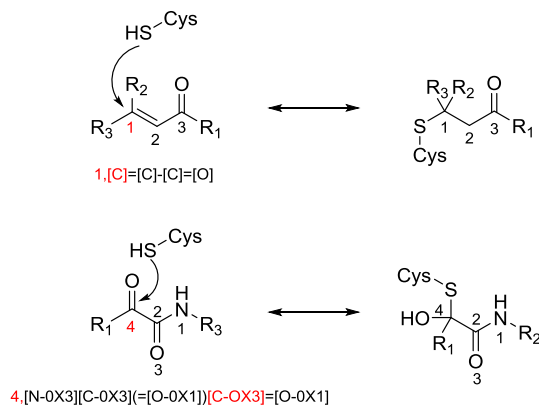


Fig. 30. SMARTS pattern under the electrophilic ligand identifies the reactive atoms. A) Michael addition reaction where the index number (1) specifies the block in the SMARTS pattern with the reactive carbon. B) Nucleophilic addition to an α-ketoamide, the index number (4) identifies the fourth block in the SMARTS pattern corresponding to the reactive ketone.

number of effects. For example, electron-withdrawing groups in the proximity to the warhead affect its reactivity. When the ligand is docked in close proximity to the reactive residue, FITTED switch to the covalent mode and the bond is formed. As a validation of the currently available version, covalent prolyl oligopeptidase inhibitors were designed. The most promising compounds were then synthesized and demonstrated nanomolar activities [114,118,119].

CovalentDock, also handles libraries of both covalent and non-covalent ligands but only supports two covalent reactions, Michael additions and ring opening of β-lactams. During pose generation, the covalent bond is not forced to occur, but is instead accounted for in poses which have formed the bond and are ranked alongside the non-covalent poses. Molecular mechanics geometrical terms consider the level of formation of this bond (i.e., if a Michael adduct is formed, the terminal carbon becomes sp³). For β-lactams, CovalentDock takes the opened structures as input with a dummy oxygen (i.e., the one from the catalytic serine) completing the valence of the carbonyl and enabling optimization of the

geometry around the new bond (Fig. 31). CovalentDock has been utilized to discover novel covalently binding Michael acceptor-like structures which bind to proteins, such as RecA, involved in growth and survival of *Mycobacterium tuberculosis* [120]. This program has also been validated on a set of 76 covalent complexes, yielding an average RMSD of 1.68 Å.

5.4.3. Scoring

The largest challenge in current docking methodologies is the accurate scoring and ranking of the docked compounds. Many factors are entirely ignored in the scoring process most notably those involved in binding kinetics. Some docking programs contain functions that take into account thermodynamic parameters such as the binding energy. Accurately parameterizing binding thermodynamics and kinetics for the massive chemical space covered by drug-like species is virtually impossible due to the sheer diversity caused by reacting groups, mechanisms of binding, interacting residues, water molecules and other factors. Desired ligand properties also vary drastically depending on the target. For instance, in some cases long residence times are desired whereas in other cases short residence times are desired [121]. These factors must all be taken into account when screening covalent ligands. In order to improve docking, methods that can generally rank the relative electrophile reactivities, residence times, and covalent bonding energy must be developed. The effect of peripheral groups on the inhibitor – such as electron withdrawing or electron donating groups – can then be included to further improve scoring once more general trends have been identified. The next evolution of scoring functions will be a result of accurate parameterization of thermodynamic and kinetic parameters for covalent ligands.

One approach to include some of the previously mentioned parameters has been developed and implemented in CovalentDock. This method attempts to parameterize a covalent bonding energy term through the use of a modified scoring function from AutoDock; this function uses a Morse potential to describe the enthalpy change associated with bond formation and also estimates entropy change with simulated QM results (from the Gaussian electronic structure package) [117].

CovDock (Fig. 32) has tried to incorporate the entire binding process of covalent ligands in order to improve covalent docking without the need for direct parameterization. This process includes both (1) non-covalent binding followed by (2) covalent bond formation.

The rationale behind this approach is that prior to bond formation, the ligand first orients itself non-covalently within the active site. By finding this pose, the geometry of the bound complex can be found more accurately. This is done by first docking the ligand non-covalently to the protein and then removing poses that do not have the electrophilic region of the ligand aligned with the nucleophilic region of the reactive residue. After these poses have been identified, the covalent bond is formed and the complex is rescored. In order to allow for VS, a modified version of CovDock, CovDock-VS, has been developed which circumvents some of the most time-consuming steps. The program has been validated with 76 covalent complexes, showing an average RMSD for the best in the top ten poses of 1.4 Å [122]. Poses of known actives to 4 targets, Cathepsin K, HCV NS3 Protease, EGFR and XPO1, were tested against CovDock and CovDock-VS with only a negligible loss in accuracy [122].

As mentioned above, kinetics of ligand binding is largely ignored in current *in silico* screening methods for covalent ligands. One kinetic factor that has been revealed as a crucial factor in characterization of the bioactivity covalent ligands is residence time. Identifying trends in the residence time of various electrophilic warhead – nucleophilic residue pairs, would allow for encoding

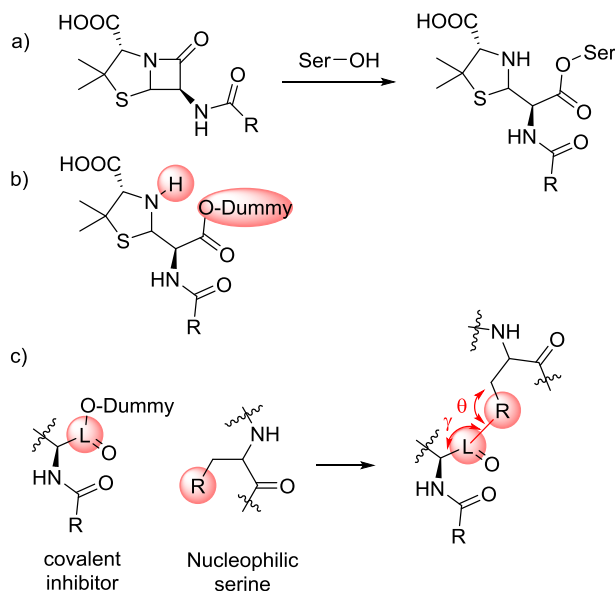


Fig. 31. a) β-lactamase inhibition by penicillin. b) pre-processed ligand used by CovalentDock. c) covalent bond geometry consideration by CovalentDock.

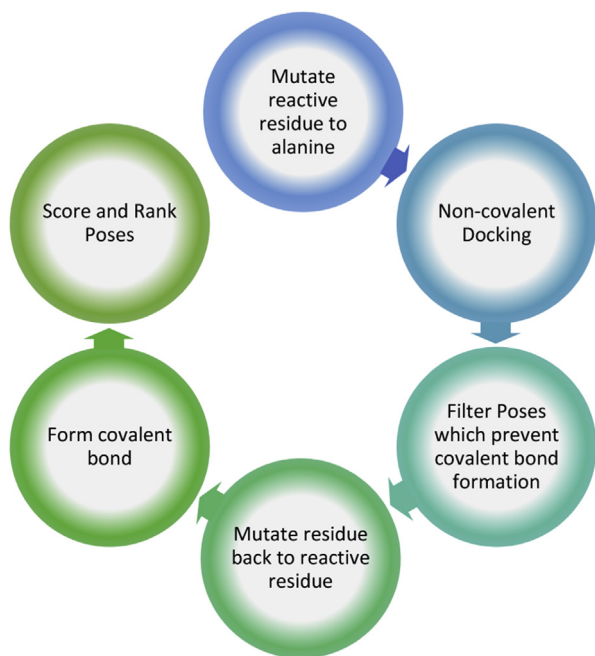


Fig. 32. Overall CovDock workflow.

and application of this parameter to increase accuracy in prediction of bioactivity of covalent ligands. Intrinsic reactivity of electrophilic and nucleophilic pairs is another factor that has only begun to be studied [100]. The study of the intrinsic reactivity of electrophiles towards glutathione described above correlates with *in silico* prediction of the reaction barrier for various electrophilic groups to the intrinsic reactivity of the group. To extend this method to protein – ligand binding, it would be necessary to examine the activation energy for bond formation. A lower activation energy would correspond to a more reactive electrophile-nucleophile pair. This type of study may be useful in parameterizing electrophilic reactivity of chemically reactive groups towards specific targets. The development of such a scoring function will likely result in a vast increase in the predictive ability of covalent docking programs.

5.4.4. Pros and cons

These docking methods have been modified or developed to support covalent docking, and their application to the discovery of novel bioactive inhibitors (e.g., DOCKoalent-guided design of JAK3 reversible inhibitors and FITTED-guided design of prolyl oligopeptidase inhibitors) demonstrates the potential of these methods. However, further improvement is necessary. For example, only FITTED and CovalentDock can screen both covalent and non-covalent ligands in a single run, CovalentDock only supports two reaction types.

Additionally, some of these methods suffer from important limitations, both in terms of usability and versatility. CovDock/CovDock-VS require that the reactive residue is first mutated to alanine, then subsequently mutated back to wild-type, the docking being done on the alanine mutant, an approximation that may be detrimental to the docking accuracy. CovalentDock, DOCKoalent and GOLD require the complex modification of scoring functions to include covalent bonding energetics or to remove repulsive terms between the ligand electrophilic atoms and the protein nucleophilic atom. [109,112], DOCKTITE also forces a complete side chain disconnection with the addition of multiple constraints to model bond formation. These modifications are all highly case-dependent and quite complicated and may discourage application to targets

which have not been specifically trained by the software. Due to the vast number of potential targets, covalent docking protocols which can provide accurate and reliable predictions to a wider range of targets is desirable. In addition, the complexity of these methods deters non-experts from using them.

5.5. Quantum mechanics/Molecular mechanics investigations

Quantum Mechanics/Molecular Mechanics (QM/MM) methods take advantage of the high accuracy of QM and the scalability of MM. QM/MM methods utilize QM for areas of the protein and ligand where high level of detail is required and MM for the rest. Consequently, QM/MM methods enable to study detailed binding mechanisms, energy barriers and interactions in enzymes with covalent ligands with high accuracy and moderate computational costs. This provides a route to address limitations of docking methodologies that fail to include some complex factors involved in binding kinetics and thermodynamics with the use of QM. QM/MM docking methods have also been shown to provide significantly improved results over classical docking protocols on systems where strong polarization or formation of covalent bonds is present [123].

To address challenges of treating polarization and covalent interactions in docking, Rohrig et al. developed a QM/MM on-the-fly docking algorithm. This methodology uses pose generation through classical docking procedures, but then takes these poses and re-optimizes and re-scores them with QM/MM. Their algorithm was validated on three data sets, Astex Diverse, a zinc data set and a hemeprotein data set. Their algorithm performed significantly better on the zinc and hemeprotein data sets than classical docking procedures (AutoDock vina and GOLD) when starting with randomized ligand conformations at a moderate computational cost [123].

Another example of QM/MM used synergistically with docking procedures to improve the drug development process involves the irreversible vinyl-sulfone based inhibitor, K11777, for rhodasin [124] (Fig. 33). Calculations showed that reversible and shorter residence time derivatives of K11777 will be more biologically active [124]. In order to develop potential drug candidates, the authors screened various derivatives of K11777 with QM/MM protocols and identified those which are only slightly exothermic (those which will be reversible) after binding to rhodasin. The molecules predicted to be slightly exothermic/reversible through QM/MM and QM modeling are then taken and docked non-covalently. This non-covalent complex is then further optimized to improve the inhibitor affinity. After this step, QM/MM or molecular dynamic simulations are complete again to further support the modifications made. After the design stage, experimental verification is carried out. This protocol was successfully employed to identify nanomolar affinity inhibitors for rhodasin. The authors suggest that their QM/MM protocol could be very informative in

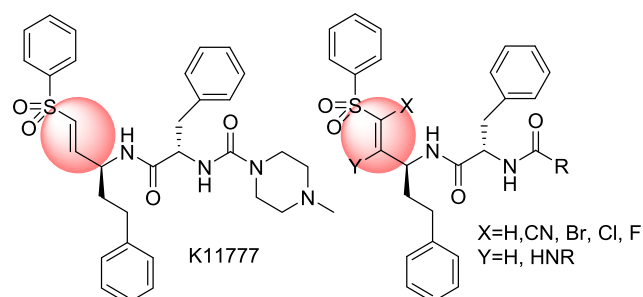


Fig. 33. Irreversible rhodasin inhibitor and its derivatives modeled/tested.

investigating and optimizing the most promising candidates from a covalent docking screen. A major limitation of this protocol is the requirement for a crystal structure with the covalent inhibitor bound.

QM/MM methods are only beginning to be utilized to their full potential. They show significant promise in application to covalent inhibitor design. Protocols are currently being developed and are being used to overcome limitations of classical docking protocols. Obviously, their time requirement make these approaches unusable for screening more than a few dozens of compounds.

6. Conclusion and perspectives

Whether discovered serendipitously or by design, many successful examples of drugs acting through a covalent mode of action are currently on the market. Although many aspects of the discovery process are shared by non-covalent and covalent drugs, there are several important additional factors, including residence time, nucleophilic reactivity, and covalent bond formation energy. The choice of target and reactive group, along with the proper characterization of those inhibitors with the appropriate techniques, are important for making informed decisions on whether to pursue or end the exploration of a given chemical series. Binding kinetics, in particular, are of utmost importance in this matter. Furthermore, the kinetics of covalent inhibition need to be considered in the bigger picture of pharmacokinetics and pharmacodynamics (PK/PD) evaluation. Indeed, while covalent inhibition can greatly reduce the time spent on improving pharmacokinetic parameters of drugs, its importance, along with pharmacodynamics, is not diminished. The knowledge gained in PK, binding kinetics and PD should be combined in order to improve the translation of *in vitro* efficacy into *in vivo* efficacy and ultimately clinical success [125].

It is therefore essential to perform structure kinetics relationship analysis in order to improve our understanding of how structural changes affect the binding kinetics of covalent inhibitors. As described above, this knowledge can lead to improved covalent inhibitors, for instance against various kinase targets. We believe that this knowledge can be used for further development of computational tools in order to accelerate the discovery of safe and effective covalent inhibitors. As of today, available computational methods have not yet reached the accuracy and usability required for medicinal chemists to use them on a daily basis. However, with minimal training and understanding of the limitations of the methods, computational methods have been successfully used to design novel enzyme inhibitors.

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