Modeling Covalent-Modifier Drugs

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Abstract

In this review, we present a summary of how computer modeling has been used in the development of covalent modifier drugs. Covalent modifier drugs bind by forming a chemical bond with their target. This covalent binding can improve the selectivity of the drug for a target with complementary reactivity and result in increased binding affinities due to the strength of the covalent bond formed. In some cases, this results in irreversible inhibition of the target, but some targeted covalent inhibitor (TCI) drugs bind covalently but reversibly. Computer modeling is widely used in drug discovery, but different computational methods must be used to model covalent modifiers because of the chemical bonds formed. Structural and bioinformatic analysis has identified sites of modification that could yield selectivity for a chosen target. Docking methods, which are used to rank binding poses of large sets of inhibitors, have been augmented to support the formation of protein–ligand bonds and are now capable of predicting the binding pose of covalent modifiers accurately. The pK_a's of amino acids can be calculated in order to assess their reactivity towards electrophiles. QM/MM methods have been used to model the reaction mechanisms of covalent modification. The continued development of these tools will allow computation to aid in the development of new covalent modifier drugs.

Keywords: review, covalent modifiers, irreversible inhibition, computer modeling, docking, QM/MM, bioinformatics, pK_a, cysteine, Michael addition, kinase

1. Introduction

The general mechanism for the inhibition of an enzyme or receptor by a small molecule drug is for the drug to bind to the protein, attenuating

its activity. The canonical mode by which a drug will bind to its target is through non-covalent interactions, such as hydrogen bonding, dipole–dipole interactions, and London dispersion interactions. Kollman and coworkers estimated that small molecules that bind to proteins through non-covalent interactions have a maximum binding affinity of 6.3 kJ/mol per non-hydrogen atom [1], so these binding energies are generally sufficiently weak for the binding to be reversible. This establishes a measurable equilibrium between the bound and unbound states.

A sizable class of drugs bind to their targets by an additional mode; a covalent bond is formed between the ligand and its target. These drugs contain a moiety that can undergo a chemical reaction with an amino acid side chain of the target protein, covalently modifying the protein. Dissociating this covalent modifier from the target requires these protein–ligand bonds to be broken. In the cases where the dissociation is strongly exergonic, the equilibrium will lie so far towards the bound state that the inhibition is effectively irreversible. Some covalent protein–ligand reactions are only weakly exergonic, so covalent modification can be reversible in some instances [2]. These ligands also interact with the protein through conventional non-covalent intermolecular forces, so the total binding energy in the covalently-bound state results from both covalent and non-covalent interactions. Refs. [3, 4, 5, 6, 7, 8] are recent reviews on covalent-modifier drugs.

The covalent modification of proteins can involve many types of chemical motifs in the inhibitor and involve a variety of amino acids. Catalytic residues in the active site often have depressed pK_a's, so they are more likely to occupy the deprotonated state that is reactive towards electrophiles. Serine residues that serve as a Brønsted acid in an enzymatic catalytic cycle have been a popular target. Two of the most famous covalent modifier drugs (Figure 1) act by acylating this type of active-site serine residue; aspirin targets Ser530 of cyclooxygenase and penicillin targets DD-transpeptidase. Catalytic serines, threonine, and cysteine residues have all been targeted by covalent modifiers [9].

In recent years, there have been extensive efforts to develop covalent modifier drugs that undergo reactions with the thiol group of non-catalytic cysteine residues. Cysteines are relatively rare amino acids, comprising only 2.3% of the residues in the human proteome [10]. This limits the number of off-target reactions that are possible. These non-catalytic residues are less likely to be conserved within a family of proteins, which creates opportunities to select for a specific target in a large family of proteins. Although this type

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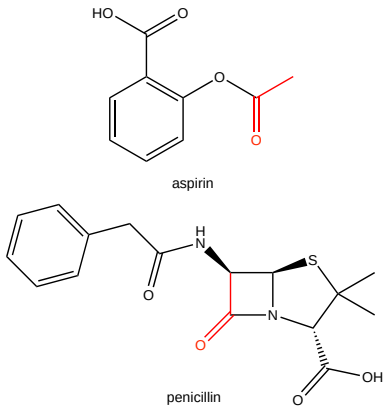

Figure 1: Aspirin and penicillin are early examples of covalent modifier drugs. These drugs bind to their target by acetylating a catalytic serine residue. The reactive moiety is drawn in red.

of covalent modification does not inactivate the catalytic residues directly, the covalent linker serves to anchor the drug binding site and achieve a stronger binding affinity. Large-scale screens have shown that reactive fragments have unexpectedly high specificity for individual proteins, suggesting that covalent modifiers have a lower risk of promiscuity than had been assumed [11, 12, 13].

These advantages must be balanced against the drawbacks associated with covalent protein–ligand binding. Covalent protein–ligand adducts are believed to trigger immune responses in some cases [14]. Further, the inhibitor must be carefully tuned so that it will only bind irreversibly to its target because irreversible inhibition of an off-target receptor could result in adverse drug reactions. The chemical reactivity of the warhead also creates the potential that the inhibitor will be chemically degraded in an inactive form through metabolism or other types of chemical reactions before it reaches the target. This constrains the reactivity of the warhead.

To develop drugs of practical use that have the advantages of covalent modification but avoid the disadvantages, researchers have developed targeted covalent inhibitors (TCIs). Typically, these compounds have a non-covalently binding framework that is highly selective for the target. For example, covalent modifier ibrutinib (Figure 2) shares the diaminopyrimidine scaffold that has been successfully employed in the development of Bruton's tyrosine kinase-selective non-covalent inhibitors. The reactive warhead is an acrylamide, which is a moderately-reactive electrophile. Thio-Michael

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additions are generally only weakly exergonic, so these additions are often reversible [15]. This allows the inhibitor to dissociate if it reacts with a thiol of a protein other than its target. High selectivity is achieved by the combination of selective non-covalent interactions and the additional strength of the covalent interaction between the warhead and a complementary reactive amino acid.

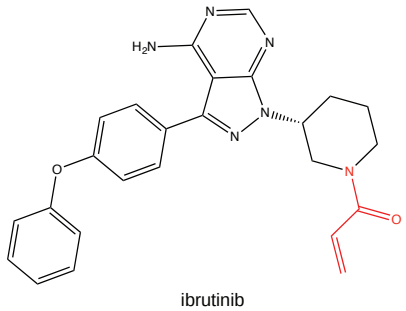

Figure 2: Ibrutinib is an example of a targeted covalent inhibitor. The reactive acrylamide warhead is indicated in red.

1.1. Physical Parameters of Covalent Modification

The strength of the binding of a non-covalent inhibitor to its target can be quantified by the equilibrium constant, K_I, for the association of the ligand (inhibitor, I) and its target (enzyme, E) to form a protein–ligand complex (E·I). This association can also be defined in terms of the Gibbs energy of binding through the relation $\Delta G_{non-covalent} = -RT \ln K_I C^\circ$, where C° is the standard state concentration [16]. The binding of a covalent modifier involves additional steps. The protein–ligand complex (E·I) undergoes reaction to form the covalent adduct (I–E). The rate of this process is characterized by its rate constant, $k_{inact.}$. In some cases, this reaction is reversible and the covalent adduct can revert to the non-covalent protein–ligand complex with the rate constant $k_{-inact.}$. If the reaction is strongly exergonic, $k_{inact.}$ will be much larger than $k_{-inact.}$, so the binding will effectively be irreversible. The total binding energy of the ligand results from both the covalent and non-covalent protein–ligand interactions ($\Delta G_{non-covalent}$).

The rate at which an inhibitor reacts with the target ($k_{inact.}$) can be calculated using transition state theory. Conventional transition state theory is the simplest and most widely used theory, which relates the rate of reaction

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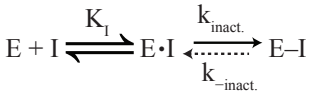

to the Gibbs energy profile along the reaction coordinate[1]. Using transition state theory, the rate of reaction can be calculated by the Gibbs energy of activation ($\Delta G^{\ddagger}$ of the rate limiting step [18]),

$$k_{TST} = \frac{k_B T}{h} \exp\left(\frac{-\Delta G^{\ddagger}}{k_B T}\right). \quad (1)$$

The mechanism of covalent modification can be complex and involve multiple reaction steps. For example, the mechanism of covalent modification of a cysteine by a Michael acceptor involves the deprotonation of the thiol to form a thiolate, formation of an enolate intermediate, and protonation of the enolate to form the thioether product (Figure 4). A comprehensive model for covalent modification would require calculation of $\Delta G_{non-covalent}$, $\Delta G_{covalent}$, as well as the rate-limiting barriers of the chemical reaction ($\Delta G^{\ddagger}$). The rates binding, unbinding, inactivation, and activation govern the drug residence time, which has been proposed to be a better determinant of in vivo pharmacological activity than the binding affinity [19, 20, 21].

1.2. Computer Modeling in Drug Discovery

Computer modeling is used extensively in the pharmaceutical industry to aid in the development of new drugs. The membrane permeability of a drug can be estimated by empirical computational methods or molecular simulation [22, 23, 24]. Docking algorithms are used to rapidly screen large databases of compounds for their ability to bind a protein or nucleic acid that is targeted for inhibition [25, 26, 27]. Other methods, such as free energy perturbation (FEP), are used to calculate the binding affinities of a drug to a protein ($\Delta G_{non-covalent}$) [28, 29, 30, 31]. These methods are generally based on molecular mechanical force fields or other simplified representations of the protein and ligand, which typically only describe the intermolecular component of protein–ligand binding. Covalent modification inherently involves the making and breaking of chemical bonds, so these methods must be

[1] A discussion of the limitations of this model in enzymatic reactions is available in Ref. 17.

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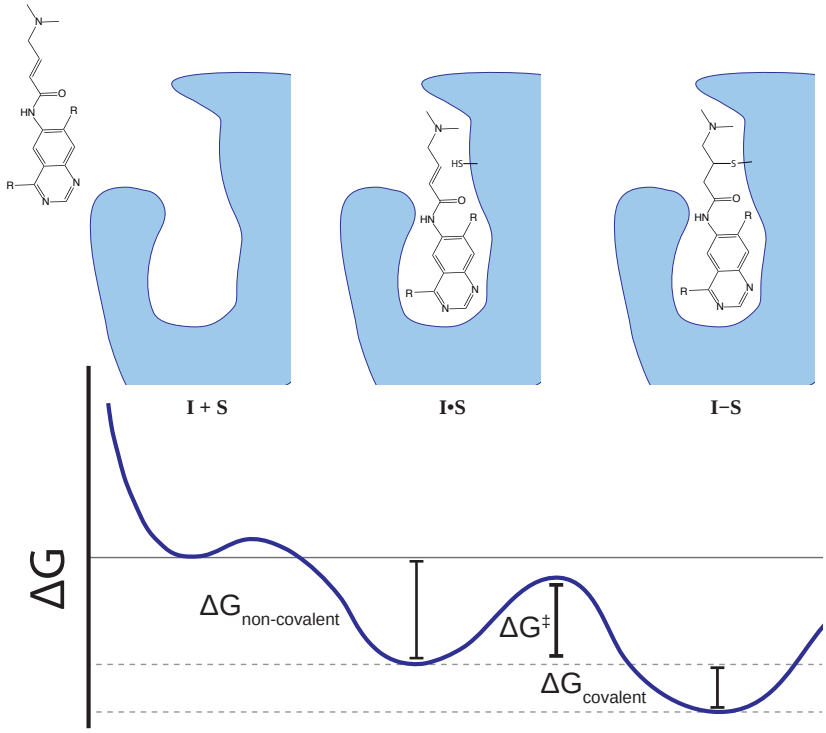

Figure 3: Schematic binding profile for the formation of a covalent protein–ligand complex. In this example, afatinib binds non-covalently to the active site of EGFR (I·S), then undergoes a chemical reaction with the thiol group of Cys-797 to form a covalent thioether adduct (I–S).

adapted to describe this mode of binding. In this review, we present methods for modeling covalent-modifier drugs. Another recent review is available in Ref. [32].

2. Structural Analysis

The Protein Data Bank (PDB) now contains over 100,000 biological macromolecular structures [33]. These data have been invaluable for the development of covalent-modifier drugs. Cocrystals showing covalent bond formation between covalent-modifier drugs have helped confirm these drugs act as covalent modifiers and unambiguously indicate the site of modification. For example, Figure 5 shows the covalent binding of ibrutinib to *Toxoplasma gondii* calcium-dependent protein kinase 1 (TgCDPK1). The availability

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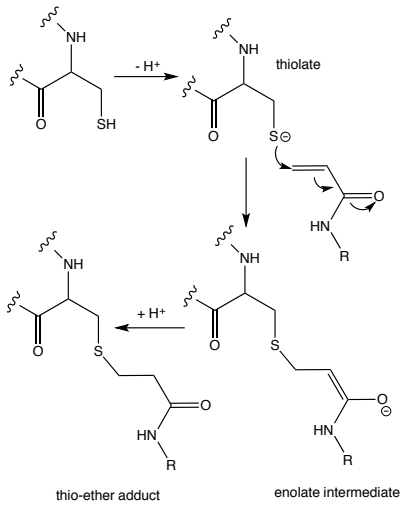

Figure 4: The mechanism of the addition of an acrylamide warhead to a cysteine thiol.

of these structures has enabled structure-based design of covalent modifiers through docking and mechanistic modeling.

This structural data is particularly valuable for designing drugs that are selective for one member of a family of structurally similar targets. The protein kinase family is a prototypical example of this. This family contains hundreds of members with a large degree of structural similarity in their kinase domains. Due to their roles in cell signaling and division, individual members of this family are drug targets, but there is a significant risk of adverse drug reactions due to inhibition of other kinase proteins with a structurally-similar active-site [34]. Several kinase-targeting covalent modifiers have been developed, which have the potential for higher affinity and selectivity [35, 36]. Structural analysis of the available kinase structures has been essential for identifying poorly-conserved druggable residues within the active site of the target, which allows a covalent-modifier to be designed with a warhead in a complementary position.

Cohen et al. reported an early structural study where a covalent-modifier drug was designed to selectively inhibit the RSK1 and RSK2 protein kinases [37]. Proteins in the kinase family have been selectively targeted by non-covalent inhibitors because a residue, known as the gatekeeper, can block binding of the ligand to a hydrophobic pocket in the active site. Inhibitors with a hydrophobic group that binds to this pocket will be selective for

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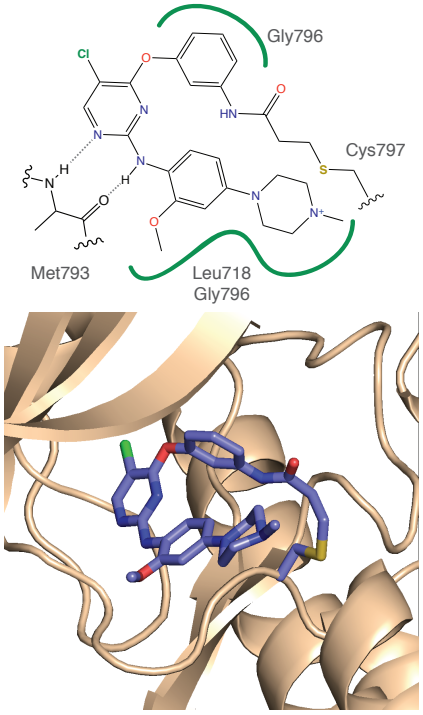

Figure 5: Ibrutinib bound to TgCDPK1 (PDB ID: 4IFG). The acrylamide warhead has undergone a Michael addition with the thiol group of Cys-797, forming a covalent thioether adduct between the protein and the ligand. Non-covalent interactions also stabilize the protein–ligand complex.

these kinases. The RSK1, RSK2, and Src kinase all have a small threonine gatekeeper, so they all bind inhibitors of this class with similar affinity. A non-conserved cysteine is present in the glycine-rich loop on the N-terminal lobe adjacent to the binding site in RSK1 and RSK2, which is absent in Src. A pyrrolopyrimidine ligand with a fluoromethylketone warhead was synthesized, where the non-covalent interactions are optimal for a threonine-gatekeeper binding site and the warhead is situated such that it will react with the loop cysteine residue of RSK1 and RSK2. This molecule was found to selectively inhibit RSK1 and RSK2 by covalent modification, but had a lower affinity for other Src kinase proteins that lacked a cysteine residue at the targeted position. This is illustrated by docked structures in Figure 6.

Zhang et al. performed a more extensive bioinformatic analysis of the human kinase family and found that approximately 200 members of the family

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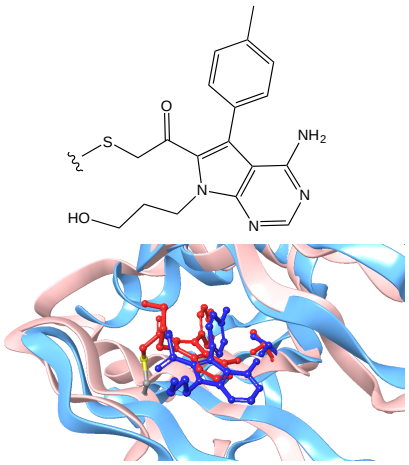

Figure 6: Docked structures of the pyrrolopyrimidine fluoromethylketone ligand (fmk) of Cohen et al. [37] bound to RSK2 (red, PDB ID: 2QR8) and c-Src kinase (blue, PDB ID: 3F6X). Both proteins have a threonine in the gatekeeper position, limiting the selectivity that can be achieved by existing non-covalent inhibitors. Fmk binds selectively to RSK2 by forming a covalent bond to with a non-conserved cysteine residue (Cys-436). A valine residue (Val-281) holds this position in c-Src.

had a cysteine residue in the vicinity of the binding site [38]. These proteins were divided into 4 groups based on the general areas of the binding site where the cysteine is located. These residues are not highly conserved across the family, so covalent modification that targets one of these residues will be selective for a small subset of the kinase family.

A structural analysis by Leproult et al. of the active sites of kinase proteins also showed that there was significant variation in the location of active site cysteine residues [39]. The available crystal structures of human kinases were grouped into three conformational classifications: active, inactive C-helix-out, and inactive DFG-out. This analysis showed that there is a large number of targetable cysteines in the kinase family, so the strategy of designing a targeted covalent inhibitor could have wide application for these drug targets. Non-covalent inhibitor imatinib binds to the DFG-out conformation of ABL1, KIT, and PDGFRα kinases, but has the highest affinity for KIT kinase. The authors synthesized variants of imatinib with different electrophilic warheads. These inhibitors had a lower affinity for ABL1, but had a higher affinity for KIT and PDGFRα. The new inhibitors were shown to bind non-covalently to these proteins.

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These structural studies of the kinase family highlight the potential of TCIs. The catalytic-domain binding sites of the human protein kinase family has limited structural variation, so it is difficult to design a selective inhibitor. Targeted covalent modifiers can be designed by adding an electrophilic warhead to existing non-covalent drug scaffolds. This warhead can only form a covalent linkage with a cysteine residue at a complementary position. Only a small number of proteins in the kinase family have a cysteine residue at this position, so binding to this site by a covalent addition is selective for a small number of kinases. This design principle may also be effective to achieve selectivity for a target in a family of similar proteins.

Wu et al. generalized this type of analysis by constructing a web-based database of known covalently-bound structures collected from structural and literature databases[40]. At the time of its public release, the database contained 462 protein structures and 1217 inhibitors. This searchable database hyperlinks to entries in the PDB for the protein–ligand complex and to chemical database entries (e.g., UniPROT) of the ligands. For each druggable cysteine, the solvent-accessible surface area (SASA) and conservation within its family are also reported. The calculated pK_a of the residue is also reported, although these calculations were performed using the PROPKA3.1 package, which has poor accuracy for cysteine residues [41]. The database is accessible through the URL: `http://www.cysteinome.org/`.

Most recently, Bourne and coworkers applied the functional-site interaction fingerprint (Fs-IFP) method to classify the binding modes of known kinase inhibitors. The Fs-IFP method analyzes the crystallographic structure of a protein–ligand complex into a bit-string, allowing diverse protein–ligand complexes to be classified systematically [42]. The researchers analyzed 2774 structures of protein–ligand complexes of kinases [43]. 1599 structures were found to have one or more cysteine residues in the binding site, which corresponded to 169 different kinases that could, in principle, be targeted by a covalent modifier. 17 cysteine residues were found to be in close contact with an inhibitor in the published structures. Based on this analysis, cysteine residues on the P-loop, catalytic group, DFG loop, roof, and front of the kinase binding sites were identified as being accessible for covalent modification. Conversely, cysteine residues on the hinge region were concluded to have a poor orientation or to be too inaccessible to be suitable targets.

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3. Docking Algorithms

There are many established codes that can screen databases of small molecules for the ability to bind to a protein. For large numbers of compounds to be screened in a tractable period of time, these methods use highly efficient algorithms to estimate if a molecule has the appropriate geometry to bind to the target site. These docking algorithms also assign "scores" to the various binding poses based on estimates of the intermolecular interactions in the bound state. To allow high throughput screens of a large databases of compounds, these models are generally highly simplified, so the calculated interaction scores serve to rank the poses approximately and are not rigorous Gibbs energies of binding.

Conventional docking methods were developed to describe non-covalent protein–ligand interactions, so the stabilization that occurs through the covalent bond formation is not included by these algorithms. Moreover, covalent linkage places the ligand at a short distance from the covalently-modified residue, which is strongly disfavored by the standard steric repulsion terms in non-covalent docking algorithms. The covalent linkage also imposes additional constraints on the pose due to the geometry of warhead-residue bond. To address these issues, new methods have been developed to model the covalent docking of a ligand with the protein (Table 1).

Table 1: Algorithms for predicting covalent-binding poses and the programs they are implemented in.

Algorithm	**Program**	**Reference**
MacDOCK	DOCK/MIMIC	44
FITTED	FORECASTER	45
DOCKovalent	DOCK Blaster (web)	46
CovDock	Glide/Prime	47
CovDock-VS	Glide	48
Two-point attractor / flexible side chain	AutoDock	49
DOCKTITE	MOE	50

Support for covalent docking has been implemented into the FITTED modeling suite [51, 52, 45]. Compounds containing appropriate reactive warheads are identified from the ligand database. Poses where the warhead is in close proximity to a reactive amino acid are automatically identified and used to construct a covalently-bound adduct. This approach uniquely allows

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both covalent and non-covalent binding models to be identified simultaneously and automatically. The binding poses are ranked by the RankScore and MatchScore algorithms. This methodology was used to identify novel covalent inhibitors of prolyl oligopeptidase that exhibited high selectivity and affinity to Ser554 [53, 54, 55].

Del Rio et al. developed a computational workflow for evaluating the binding of a covalent modifier to a protein [56]. A database of kinase proteins with non-covalently-bound structures was constructed from the Protein Data Bank. From these structures, inhibitors bound near cysteine residues that had strong non-covalent binding energies were selected. These structures were used as scaffolds for the design of new inhibitors with electrophilic warheads that bind near cysteine residues. The covalently-bound protein–ligand complexes for these compounds were constructed and simulated using molecular dynamics. Inhibitors which required a large distortion in the protein structure in order to form the covalent ligand were rejected.

Ouyang et al. developed CovalentDock by modifying AutoDock5.2 [57]. This method is distinct from some other codes because ligand–protein interaction potential explicitly includes the energy of the covalent linkage. This linkage is described using a Morse potential, where the potential energy minimum forms a covalent bond but the bond can form or dissociate dynamically. This program is also available through a web-based interface, CovalentDock Cloud [58].

London et al. developed the DOCKovalent docking algorithm for covalent docking using the web-based docking server DOCK Blaster [46]. This server uses a library of electrophile-containing ligands, including α, β-unsaturated carbonyls, aldehydes, boronic acids, cyanoacrylamides, alkyl halides, carbamates, α-ketoamides, and epoxides. These ligands were modified to assume the geometry they will hold when covalently bound to the target. A set of likely rotamers is generated for each compound. To search for stable binding poses, protein–ligand complexes are generated where the protein–ligand linkage is constrained to its ideal geometry. The DOCK3.6 scoring algorithm is used to rank the ligands. This method was successfully applied to a novel inhibitor of AmpC β-lactamase, where a covalent bond is formed between a boronic acid warhead and the catalytic serine residue. It was also effective at finding drugs capable of binding to kinase proteins RSK2, MSK1, and JAK3 through reaction of ligands containing a cyanoacrylamide warhead with non-catalytic cysteine residues in or near the active site (Cys436 in RSK2, Cys440 in MSK1, and Cys909 in JAK3).

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Bianoco et al. recently implemented two covalent docking algorithms into AutoDock4, a widely-used docking program [59]. The first algorithm is the two-point attractor method, where an artificial potential is defined that favors overlap between two artificial sites attached to the warhead of covalent modifiers to the C_β–O sites of a target serine or the C_β–S sites of a target cysteine. This allows the drug to move freely in the active site, while favoring conformations where the ligand is in a covalently-bound pose. The second algorithm treats the ligand as a flexible side chain of the protein. The flexible side chain method predicted the RMSD of bound poses in better agreement with the experimental structures than the two site model.

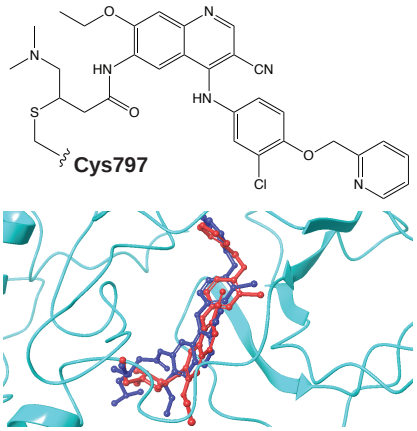

Figure 7: Binding of neratinib (HKI-272) to EGFR modeled using CovalentDock. The RMSD of the non-hydrogen atoms of the ligand are 2.14 Å with respect to the experimental crystallographic structure (PDB ID: 2JIV). The experimental and predicted ligand poses are colored in red and blue, respectively.

The Glide docking program developed by Schrödinger Inc. has also been modified to support modeling covalent binding ligands [47]. This algorithm mutates the target residue into an alanine residue and performs a conventional docking simulation to generate a library of hundreds of poses. These poses are filtered to select those where the warhead is within 5 Å of the target residue based on a rotamer library. Covalently-bound structures are generated from these bound poses. Further optimization and clustering is performed on these poses using the Prime refinement program [60, 61], which are ranked to yield a final optimal binding pose. Ligands can be screened by calculating an apparent affinity, which is estimated from the average of the scores for the covalently-bound and non-covalently-bound poses. On a

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test set of 38 covalently-bound protein–ligand complexes, this method was able to predict the binding pose with an RMSD of 1.52 Å from the experimental crystallographic structure. An example of the predicted pose of a covalently-bound inhibitor using this method is presented in Figure 7.

Warshaviak modified the CovDock workflow to enable fast structure-based virtual screening of covalent modifiers [48]. In the CovDock-VS workflow, the target residue is also mutated into an alanine, but a restraint is imposed so that the warhead remains within 5 Å of the target residue during the conformational search. This restricts the initial search so that only poses where covalent bond formation is possible are identified. Covalently-bound structures are generated from these poses, energy-minimized, then clustered to identify unique poses. These poses are directly scored using the GlideScore algorithm, omitting the additional structural refinement stage in CovDock. This simplified workflow had a throughput that was 10–40 times faster than CovDock but the mean RMSD of predicted binding poses for the test set of 21 structures only increased to 1.87 Å from the 1.52 Å RMSD of the original CovDock workflow.

Ai et al. presented a technique steric-clashes alleviating receptor (SCAR) [62], where the covalently-binding residue is mutated into a sterically smaller residue, such as serine or glycine. This allows the ligand to dock in poses similar to the covalently-modified mode without experiencing a steric clash. These poses are ranked according to their non-covalent binding score. This procedure was evaluated by comparing the predicted pose to the experimental crystallographic structures of covalently-inhibited AdoMetDC. This technique predicted the binding pose with an RMSD of 3.0 Å, which is comparable to other covalent docking methods. In their complete workflow, the ensemble of docked poses was filtered to select those where the warhead was within 1 Å of the targeted residue. The mean RMSD of the top-ranked structures after imposing this constraint was reduced to 1.9 Å. This procedure was successfully applied to discover novel covalent inhibitors of S-adenosylmethionine decarboxylase.

Scholz et al. presented the implementation of DOCKTITE [50], a covalent docking method into the Molecular Operating Environment (MOE [63]) modeling suite. This method searches a database of potential ligands for molecules possessing one of 21 electrophilic warhead motifs. The structure of the ligand is adjusted to reflect its structure when covalently-bound. Constraints are imposed to force the ligand to occupy a geometry consistent with a covalent linkage and a conformational search is performed to identify the

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low-energy poses of the ligand in the receptor. Of the 76 covalent-modifier test set developed by Oyang et al., the top-ranked pose predicted by DOCKTITE was 2.4 Å, comparable to other covalent-docking methods. A recent application of DOCKTITE was reported by Schirmeister et al., who found that the relative affinities of covalently-binding dipeptide nitriles inhibitors of rhodesain were correctly predicted by the calculated affinity scores [64].

These implementations have made the docking of covalent-modifiers drugs practical and accessible, although further development of these methods is still needed. Databases containing drugs with warheads must be developed and the codes must be able to identify the potential modes of modification. In some of these codes, the user is required to provide the site of covalent modification, so the screening performed by this code is not yet fully automatic. The energy associated with covalent bond formation (i.e., $\Delta G_{covalent}$) is not immediately available in conventional scoring algorithms, so the absolute strength of binding cannot be realistically estimated by these algorithms either.

4. Calculation of the pKa's of Targeted Residues

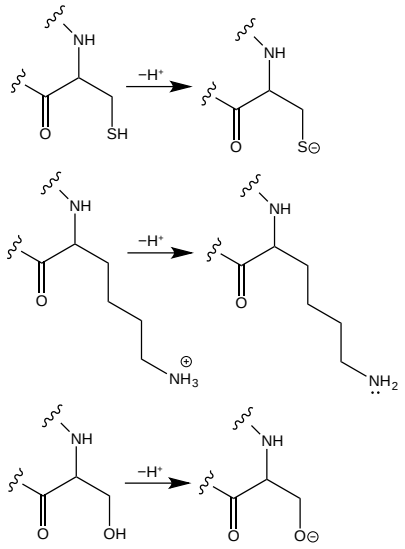

Figure 8: Deprotonation reactions involving cysteine, lysine, and serine. Covalent modification of these residues typically involves reaction in their deprotonated, nucleophilic states.

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Generally, the first step in the mechanism for covalent modification of cysteines, lysines, and serines is their deprotonation to yield their more reactive form (Figure 8). In the case of the modification of a cysteine residue by an electrophile, the thiol group of the amino acid side-chain must be deprotonated to form the reactive thiolate nucleophile. The stability of the thiolate is thus a significant parameter for the inhibition of a target site by a drug molecule.

The equilibrium between the thiol and thiolate states of a cysteine residue in a protein is defined by its pK_a. Cysteines with low pK_a's are more likely to exist in their reactive thiolate state, so they will be more susceptible to covalent modification by electrophilic inhibitors. The standard pK_a of a cysteine residue is 8.6 [65], but pK_a's of cysteines have been reported to range from 2.9 to 9.8. This broad range results from the intermolecular interactions that the thiol and thiolate states of the cysteine experience inside the protein. Catalytic cysteines in enzymes like cysteine proteases tend to have nearby cationic residues, like histidine or asparagine, which lowers their pK_a's by stabilizing the thiolate state of the cysteine [66]. Conversely, the thiolate state of the cysteine residue will experience repulsive interactions with nearby anionic residues, raising the pK_a. Amino acids buried in hydrophobic pockets of the protein can also have elevated pK_a's because they do not experience stabilizing interactions with water molecules.

Calculating the pK_a of an amino acid side chain in a protein is a long-standing challenge in computational biophysics. Traditionally, the pK_a of an amino acid side chain is estimated based on the relative stability of the charged and neutral states. Continuum electrostatic models were among the earliest methods used [67], although the approximations incorporated in their methodology limit their accuracy. Since this time, these models have been continually improved, and some methods that make use of an explicit solvent representation perform well for predicting the pK_a's of aspartic and glutamic acid residues [68, 69].

The methods for the prediction of the pK_a of cysteine residues are less established. In a recent paper, methods for calculating the pK_a's of cysteine residues in proteins were evaluated for a test set of 18 cysteine pK_a's in 12 proteins [41]. Three methods that use an implicit solvent representation were tested, namely: PROPKA, H++, and MCCE. The root-mean-square deviation (RMSD) of the calculated pK_a's with respect to experimental values were large, with some methods having essentially no predictive power. H++ was the most accurate of the three implicit methods, although the RMSD

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was still 3.4. A method using an all-atom explicit-solvent model with replica exchange molecular dynamics thermodynamic integration (REMD-TI) was more accurate. When used with the CHARMM36 force field, this method was able to predict the pK_a's of cysteine residues in the test set with an RMSD of 2.4. Plots illustrating the correlation between predicted and experimental values for these two methods are presented in Figure 9.

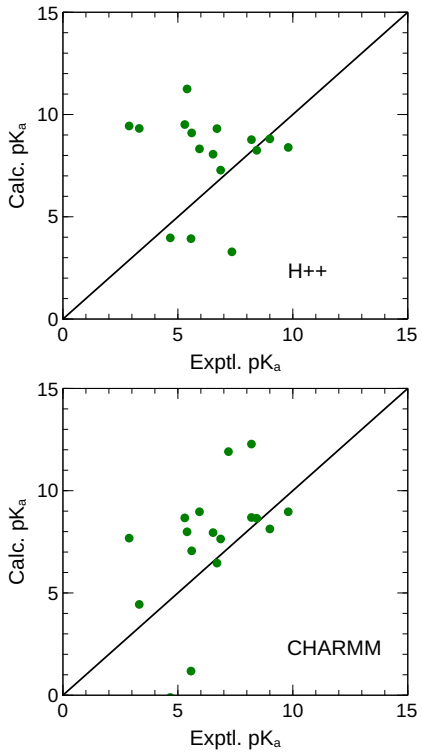

Figure 9: Predictions of cysteine pK_a's using implicit-solvent H++ method and an explicit-solvent replica-exchange molecular dynamics free energy calculation method using the CHARMM force field. Adapted from Ref. [41].

Although REMD-TI gives reasonably accurate cysteine pK_a's, new methods for calculating the pK_a's of cysteines will be needed to allow the reactivity of a cysteine residue to be predicted quantitatively. Improved force fields and the use of algorithms capable of describing variable protonation states of other residues within the protein may improve the accuracy of these methods. Additionally, experimental measurement of more cysteine pK_a's in

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proteins will allow for a more thorough and comprehensive evaluation of existing pK_a methods. Of particular importance are the pK_a's of noncatalytic residues in protein active sites, which are typically the target of TCIs.

5. Quantum Chemical Methodology

The docking and free energy calculation methods described thus far rely on molecular mechanical methods to describe the protein and inhibitor. Typically, these methods do not describe the interactions associated with chemical bonding, so terms like $\Delta G_{covalent}$ and $\Delta G^{\ddagger}$ cannot be calculated by these methods. This has led researchers to employ quantum chemistry to model the mechanisms, kinetics, and structures involved in covalent modification. Density functional theory (DFT) is widely used for modeling biological systems because of its ability to describe large chemical systems with quantitatively accurate energies and structures.

Early models of electrophilic thiol additions were unable to identify the enolate/carbanion intermediates that occur in the canonical mechanism for a thiol-Michael addition. The failure of conventional DFT methods to describe these reactions stems from an issue in contemporary DFT known as delocalization error [70, 71, 72, 73]. DFT calculates inter-electron repulsion in a way that erroneously includes repulsion between an electron and itself, which must be corrected for in an approximate way through the exchange-correlation functional. The result of this effect is a spurious delocalization of electrons to reduce their self-interaction.

Delocalization error is an issue when DFT is used to model thiol additions. The thiolate intermediate features a diffuse, sulfur-centered anion. When some popular DFT functionals are used (e.g., B3LYP or PBE), self-interaction error causes the energy level of the highest occupied molecular orbital (HOMO) to be positive, making the anionic electron formally unbound. When the thiolate is complexed with a Michael acceptor, delocalization error spuriously stabilizes a non-bonded state where electron density is transferred from the HOMO of the thiolate to orbitals of the Michael acceptor. For some electrophiles, this complex is the most stable form and these methods predict that there is no enolate/carbanion intermediate.

One popular method to define the exchange functional in density functional theory calculates the Hartree–Fock exchange energy using the DFT Kohn–Sham orbitals, a technique known as exact exchange. Hybrid DFT

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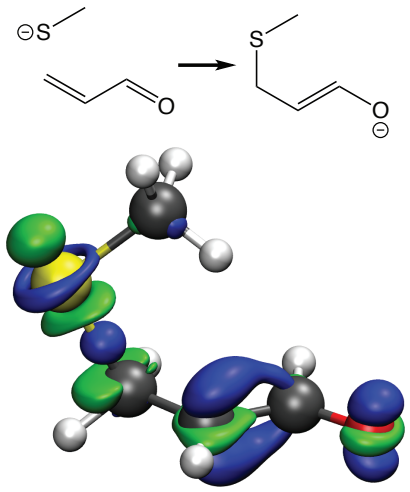

Figure 10: The charge transfer between a thiolate and Michael acceptor calculated using ωB97X-D/aug-cc-pVTZ. Charge is transferred from methylthiolate (top) to the acrolein Michael acceptor (bottom). Areas in blue indicate an increase in charge density while areas in green correspond to a decrease in charge density when the two fragments interact. Charge is lost from the thiolate anion and gained in the space between the S–C_α sigma bond, the π molecular orbital of the C_α–C bond, and the p_z orbital of the O atom, corresponding to an oxygen-centered anion.

functionals have been developed where part of the exchange energy is calculated by exact exchange. These functionals generally outperform "pure" functionals that do include an exact exchange component.

Issues with delocalization error have led to the development of range-separated DFT functionals, where the exchange-correlation functional uses a large component of exact exchange for long-range inter-electron exchange-correlation. Smith et al. showed that range separated DFT functionals such as ωB97X-D predicted a stable thiocarboanion intermediate, while popular methods like B3LYP predicted that this intermediate could not exist as a distinct species (Figure 11). This result was corroborated by highly accurate CCSD(T) calculations [74]. Some hybrid functionals that have a high component of exact exchange globally, such as PBE0 or M06-2X, also predicted a stable carbanion intermediate.

The reaction energies of thiol additions are also sensitive to the DFT functional used. Krenske et al. studied the addition of methyl thiol to α,β-unsaturated ketones [75]. The calculated reaction energies were sensitive to the quantum chemical method used, but the M06-2X and B2PLYP func-

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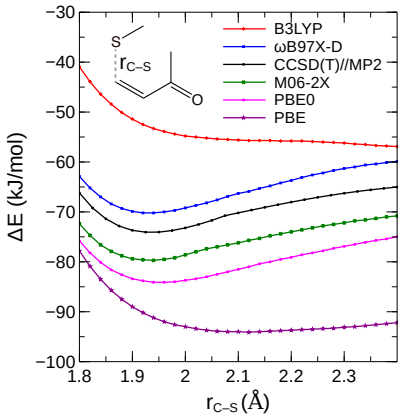

Figure 11: The potential energy surfaces for the addition of methylthiolate to methyl vinyl ketone, calculated using DFT and ab initio methods. The PES for the B3LYP and PBE functionals fail to predict a stable enolate intermediate. High-level ab initio (CCSD(T)), range-separated functions (e.g., ωB97X-D) and hybrid functionals (e.g., PBE0) predict a moderately-stable enolate intermediate with a minimum near C_β–S = 1.9 Å.

tionals provided results in close agreement with high-level ab initio results (CBS-QB3). Smith et al. showed that the ωB97X-D and PBE0 functionals also provide accurate thiol-addition reaction energies [74].

6. Warhead Design

An additional factor in the design of covalent modifiers is the selection of an appropriate functional group for reaction with the target residue of the protein. This warhead is typically an electrophilic group. The type of amino acid undergoing modification is the first design criteria. Covalent modifiers of cysteine residues often feature acrylamides or other electron-deficient alkenes, which can undergo Michael additions to the cysteine residues to form thioether adducts.

Quantum chemistry has been used to model the reaction mechanisms of covalent modification, providing information about the reaction kinetics and thermodynamics for various warheads. For Michael additions to cysteines, a model thiol (e.g., methylthiol) has commonly been used to represent the cysteine residue. The transition state and carbanion intermediate stability can theoretically be used to estimate the rates of reaction ($k_{inact.}$). The Gibbs energy for the net reaction determines whether the addition is spontaneous or non-spontaneous and the degree to which it is reversible.

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Taunton and coworkers have pursued a line of development of cysteine-targeting covalent inhibitors with acrylonitrile warheads with electron donating aryl or heteroaryl groups at both the α and β positions [76]. The most effective electrophiles formed a carbanion intermediate with a high proton affinity. This warhead was successfully applied to develop a high-affinity 1,2,4-triazole-activated acrylonitrile covalent modifier that was selective for RSK2 kinase.

Smith and Rowley implemented an automated workflow to assess the stability of the carbanion intermediate and thioether product for the addition of a model thiol (methylthiol) to a large set of substituted olefins [77]. All combinations of $-$H, $-CH_3$, $-C(=O)NH(CH_3)$, $-$CN, and $-C(=O)OCH_3$ substituents on an ethene scaffold were evaluated. The lowest energy conformations of each species were identified by a replica exchange molecular dynamics method [78]. Generally, it was found that substitution of the alkene core has a large effect both on the stability of the intermediate and products, but conventional warheads fell into a narrow range where the addition was weakly spontaneous and went through a moderately stable intermediate (e.g., 120–160 kJ/mol). This is illustrated in Figure 12, with the approximate range of appropriate warheads highlighted in red. Dimethyl fumarate and N-methylacrylamide, which are established covalent warheads, are indicated. The complete set of calculated reaction energies and intermediate stabilities for the full set of warheads are available in the supporting information of Ref. 77.

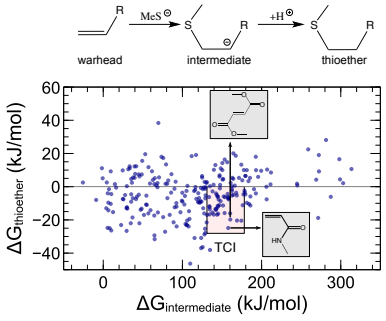

Figure 12: The calculated stability of the carbanion intermediate vs. the stability of the thioether product for the full data set of model thiol additions from Smith and Rowley [77]. The region of potential TCI warheads is highlighted, where the thiol undergoes a weakly exergonic addition ($\Delta G_{thioether} < 0$) through a moderately-stable carbanion intermediate (130 kJ/mol $< \Delta G_{intermediate} <$ 180 kJ/mol).

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The calculated reaction energies of warheads used in successful TCIs are weakly exergonic, which makes these reactions reversible. This allows them to dissociate from non-targeted thiols should they react with an off-target thiol in the cell prior to reaching their target. This is consistent with an emerging principle of TCI design, which is that the warhead should only be moderately reactive in order to avoid promiscuous modification [79, 80, 20]. Because thiol additions to warheads like acrylamides are reversible, modification of an off-target protein can be reversed. The moderate rate of reaction due to a moderately stable enolate intermediate favors the formation of the covalent bond only after the inhibitor has complexed to its target through selective non-covalent interactions. Of the hundreds of putative warheads evaluated in this study, only a small number have the appropriate thiol-addition kinetics and thermochemistry to serve as a therapeutic TCI.

Krenske et al. studied the addition of methyl thiol to α, β- unsaturated ketones [75]. α-methyl, β-methyl, and α-phenyl ketones were found be more readily reversible than the unsubstituted vinyl ketone. A subsequent study by Krenske et al. used DFT calculations to study the Michael acceptor warheads with aryl groups at the α-position and electron withdrawing groups at the β-position [81]. These calculations were consistent with the experimental observation by Taunton et al., who observed that electrophiles with two electron withdrawing groups at the α-position (e.g., amide and cyano) and an aryl at the β-position yielded a warhead that reacted covalently but reversibly with thiols [20].

Zhao and coworkers calculated the potential energy surfaces for the addition of a model thiol to an α, β-unsaturated aldehyde in a range of dielectric environments [43]. The barrier to a direct 1,2 addition and ammonia-assisted addition was higher in low-dielectric environments, suggesting that the rates of covalent modification through these mechanisms will be lower in cysteine residues buried in hydrophobic binding pockets. It should be noted these mechanisms are distinct from the canonical Michael addition mechanism, where the reaction proceeds through a thiolate and carbanion intermediate. These calculations used the B3LYP exchange-correlation functional, which tends to underestimate the stability of carbanion intermediates and thioether products [74].

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7. QM/MM Models of Covalent Modification

Studies of covalent modification using model reactants in the gas phase or using a continuum solvent model do not provide a rigorous description of how the protein environment affects the reaction between the protein and the inhibitor. Paasche et al. found that continuum solvent models provided limited success in describing the cysteine–histidine proton transfer reactions associated with cysteine protease functoin [82]. Describing the full enzyme, inhibitor, and solvent using a quantum mechanical model would be prohibitively computationally demanding, so it is not practical to apply these methods naively to model the covalent modification of a protein.

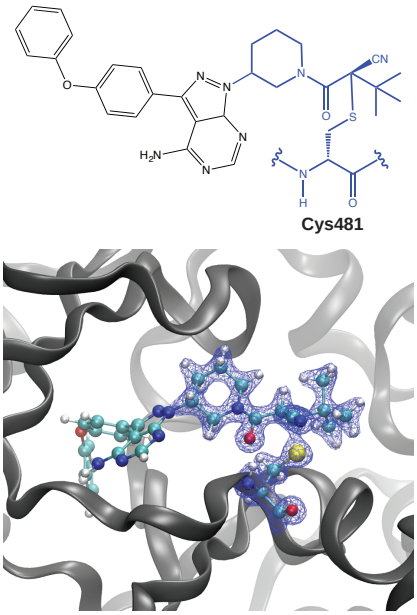

Figure 13: An example QM/MM model of Bruton's tyrosine kinase in complex with a covalent modifier (Ref. 20, PDB ID: 4YHF). The covalently-modified Cys481 residue and the cyanoacrylamide warhead define the QM region. The calculated electron density of the QM region is represented by the blue mesh. The remainder of the protein (gray) and inhibitor comprise the MM region.

Quantum mechanics/molecular mechanics (QM/MM) methods allow for a critical component of a chemical system to be described using a quantum mechanical model, while the rest of the system is represented using a molecular mechanical model (Figure 13). As the size of the QM region is reduced

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to a relatively small size, the computational expense of these QM/MM calculations is tractable. This is well-suited for modeling chemical reactions involving proteins, like enzymatic reaction mechanisms, where the chemical reaction only directly involves a small number of atoms, but the rest of system provides an essential environment. Analogously, the covalent modification of proteins can also be described using a QM/MM model, where the reactive warhead of the inhibitor and the residue being modified are described using QM, while the balance of the system, such as the solvent and the rest of the protein are described using an MM model. If needed, additional sections of the inhibitor and protein can be included in the QM region. QM/MM methods are now available in codes such as Gaussian [83], CHARMM [84], Amber [85], NAMD [86], and ChemShell [87, 88].

One example of QM/MM modeling of covalent modification probed the mechanisms involved in the covalent modification of Ser530 of cyclooxygenase by aspirin. Tosco and Lazzarato [89] constructed a QM/MM model where an aspirin molecule and the nearby amino acid residues in the enzyme active site (e.g., Ser530, Tyr348, and Tyr385) were modeled using semiempirical SCC-DFTB QM method and the balance of the system was modeled using the CHARMM force field. The aim was to propose a putative reaction mechanism for the irreversible inactivation of cyclooxygenase by aspirin. The results obtained suggests that acetylation of Ser530 in cyclooxygenase by aspirin occurs under intramolecular general base catalysis conditions, where the vicinal carboxylate group of aspirin abstracts a proton from the hydroxy group of Ser530 in cyclooxygenase, followed by a nucleophilic attack by the O_γ of Ser530 on the acetyl carbonyl carbon of aspirin. This forms an anionic tetrahedral intermediate, which is stabilized by Tyr385.

Tóth et al. [90] also reported a QM/MM study of the reaction mechanism, transition state, and potential energy surface of the inhibition reaction of cyclooxygenase by aspirin. In their approach, static ONIOM-type QM/MM methods were used, where the HF, B3LYP, MP2, and B97-D methods were used to describe the QM region, which was comprised of the aspirin molecule and surrounding amino acids. The authors concluded that the transesterification reaction of aspirin and cyclooxygenase occurs through a concerted mechanism.

QM/MM methods have also been employed in studying the mechanism of enzyme inhibition in peptide cleaving enzymes, such as cysteine proteases [91, 92]. A wide range of warheads will react readily with the acidic catalytic cysteine, so covalent-modifiers have frequently been used to inhibit these

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targets. QM/MM studies have examined the inhibition of cysteine proteases by epoxides [93], aziridines [94], peptidyl aldehydes [95], vinyl sulfones [96], and nitroalkene-based inhibitors [97] of cysteine proteases, among others. It should be noted that some of these studies used DFT methods (e.g., B3LYP) that underestimate the stability of the enolate intermediate [77].

Engels and coworkers have published a series of computational studies [92, 94, 98, 99, 100] that explore the factors governing the kinetics, regiospecificity and stereoselectivity of epoxide- and aziridine-based inhibitors of cysteine proteases. QM/MM and molecular dynamics (MD) methods were used to investigate basic mechanistic principles and inhibition processes of these enzymatic reactions. Hydrogen bonds between the inhibitor and other residues in the active site were found to affect the reaction mechanism significantly, demonstrating that the explicit representation of the active site was necessary to describe the reaction mechanism. These studies aided the design and synthesis of new, potent inhibitors of this family of enzymes [94, 101, 102].

Schirmeister et al. [103] developed QM/MM-based workflow for the development of covalent inhibitors. Using an existing protein-inhibitor structure, the substituents on the warhead are systematically varied. A QM/MM model of the protein-inhibitor complex is used to calculate the potential energy surface of the covalent modification, which provided the activation energy and reaction energy. The docking programs FlexX and DOCKTITE were used to identify variants on the drug scaffold that would improve the non-covalent component of the binding energy. The covalent competence of this new warhead were tested by a second round of QM/MM calculations. This workflow was used to develop novel covalent vinyl sulfone-based inhibitors of rhodesian, a parasite protease belonging to the papain family of cysteine proteases. The halogenated warhead designed by this procedure was predicted to be a reversible covalent modifier, which was confirmed experimentally.

Another QM/MM workflow for the design of covalent inhibitors was reported by Fanfrlik et al. [96], which uses a QM-based scoring function based on a hybrid QM/semi-empirical QM model to describe the process of covalent binding in protein–ligand complexes. The performance of the algorithm was evaluated on a series of vinyl sulfone-based inhibitors of *S. mansoni* cathepsin B1. The calculated Gibbs energy difference between the non-covalently-bound state and the covalently-bound state was found to correlate to the log of the experimental IC_{50} with a coefficient of determination of 0.69.

QM/MM modeling has the potential to play a significant role in under-

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standing and predicting the mechanisms, kinetics, and thermodynamics of covalent modification. More accurate QM methods, improved algorithms to interface the QM and MM regions, and more extensive configurational sampling will be needed to make these methods quantitatively accurate. In combination with other simulation methods to calculate the non-covalent binding and deprotonation steps, QM/MM methods can be used to calculate the kinetic and thermodynamics terms of covalent modification rigorously. Ultimately, the integration of these methods will make it possible to model the action of covalent modifier drugs in a comprehensive way through the calculation of $\Delta G_{non-covalent}$ and $\Delta G^{\ddagger}$.

8. Conclusions

Computational methods for modeling the covalent modification of proteins have developed rapidly over the last 10 years. Docking programs such as AutoDock, Glide, and MOE now include functionality to find poses of docked covalent modifiers. The design of reactive warheads has been aided by quantum chemical modeling of model reactions. One of the most promising areas of this field is the use of computer modeling to examine the reaction mechanisms of covalent modification; pK_a calculations can be used to determine the reactivity of targeted residues and QM/MM models can be used to elucidate the reaction mechanism, activation energy, and covalent binding energy. With further developments in computing power and more accurate computational methods, these methods may eventually allow the estimation of rates of inactivation and the covalent component of the binding energy. The maturation of these methods will allow computer modeling to contribute to the development of covalent-modifier drugs to the same degree that they have contributed to the development of non-covalent drugs.

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