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COMPREHENSIVE REVIEW

Docking covalent targets for drug discovery: stimulating the computer-aided drug design community of possible pitfalls and erroneous practices

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Abstract

The continuous approval of covalent drugs in recent years for the treatment of diseases has led to an increased search for covalent agents by medicinal chemists and computational scientists worldwide. In the computational parlance, molecular docking which is a popular tool to investigate the interaction of a ligand and a protein target, does not account for the formation of covalent bond, and the increasing application of these conventional programs to covalent targets in early drug discovery practice is a matter of utmost concern. Thus, in this comprehensive review, we sought to educate the docking community about the realization of covalent docking and the existence of suitable programs to make their future virtual-screening events on covalent targets worthwhile and scientifically rational. More interestingly, we went beyond the classical description of the functionality of covalent-docking programs down to selecting the 'best' program to consult with during a virtual-screening campaign based on receptor class and covalent warhead chemistry. In addition, we made a highlight on how covalent docking could be achieved using random conventional docking software. And lastly, we raised an alert on the growing erroneous molecular docking practices with covalent targets. Our aim is to guide scientists in the rational docking pursuit when dealing with covalent targets, as this will reduce false-positive results and also increase the reliability of their work for translational research.

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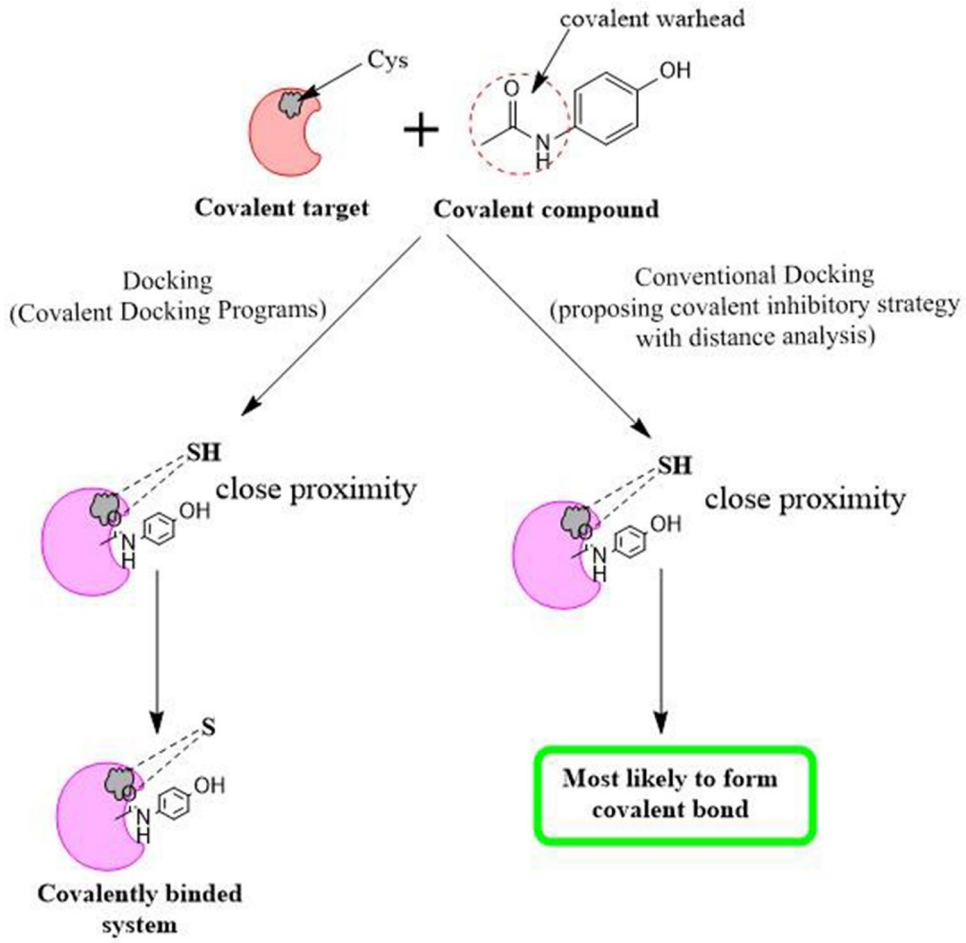
Graphical abstract



Keywords Covalent docking · Drug discovery · Medicinal chemistry · Covalent targets · Ligand warhead chemistry

Introduction

The accidental discovery of covalent agents and their unique ability to induce irreversible and full inhibition of drug targets has resulted in a paradigm shift in disease treatment away from noncovalent therapeutics [1, 2]. This has resulted in a considerable advancement in the field of covalent targeting, allowing for a better understanding of their inhibitory mechanisms and the development of new covalent binders for 'undruggable' targets [3, 4]. Docking experiment has evolved over time and has made a substantial contribution to drug development by replicating biological occurrences and so providing insights into the molecular and geometrical transitions involved with biomolecular systems [5–10]. Molecular docking is a simulation used in structure-based rational drug design to discover proper conformations of small-molecule ligands and to evaluate the strength of the interaction between binary complexes, which typically involves a receptor target and one chemical compound [6, 8, 11]. The prediction of protein–small molecule interactions, commonly using molecular docking, is a crucial step in the rational drug discovery process. There are a variety of docking tools available to assess the interaction between receptor targets and chemical entities. However, most of these conventional docking programs lack the intrinsic functionality to account for covalent inhibition mechanisms between covalently bound complexes and, hence, only focus on the docking between two molecules through noncovalent interactions. Nevertheless, recent years have witnessed the development of docking programs and protocols with enhanced functionality to delineate the biophysical interaction between covalently bound complexes [12–14]. Despite the establishment of these covalent-docking software/protocols, the computational drug discovery community has experienced limited use of these programs for covalent drug discovery applications, majorly due to a lack of technical

and methodological approaches to target covalent receptors. Hence, by discussing the concepts, technicalities, challenges, and identification of ideal covalent-docking tools for virtual-screening campaigns, we intend to aid the docking community in the design of covalent drugs. Finally, as a wake-up call to retrospective studies on erroneous covalent drug discovery practices, we aim to sensitize our audience to being cautious when dealing with covalent biomolecular systems.

Covalent inhibition in biomolecular system

Covalent inhibition is typically a mechanism in which small-molecule compounds reversibly or irreversibly inactivate their targeted protein receptors. Generally, a two-step dependent process is required for covalent inhibition (Fig. 1). To begin with, an inhibitor forms a reversible association with the target enzyme, bringing the inhibitor's chemical warhead into close proximity with a specified reactive amino acid residue of the enzyme. The second stage involves the formation of a covalent link between the inhibitor and the enzyme's reactive component [3, 15]. Reversible inhibitors are distinguished from covalent inhibitors by the absence of the second step [16]. After a length of time, a covalently conjugated inhibitor may undergo additional chemical transformations to be released from its target enzyme. Additionally, it may form an irreversible reaction with the target, thereby confining the enzyme in an inactive state. Since the late nineteenth century, when Frederick Bayer began manufacturing aspirin as a painkiller and anti-inflammatory therapeutic, the use of chemical entities as covalent inhibitors to target functionally essential enzymes in cells has been implemented [17]. Although aspirin has been on the market since the early twentieth century, its mechanism of action was not discovered until the 1970s, when Roth and colleagues demonstrated that it irreversibly blocked cyclooxygenase-1 (COX1), an enzyme critical for prostaglandin formation [18]. On binding with COX1, aspirin covalently modifies an active site serine residue through an acetylation mechanism, thereby inhibiting its activity (Fig. 2) [19–21]. Along with aspirin, acetaminophen was developed in the late nineteenth century and soon adopted as a painkiller in the clinic. Although its method of action is unknown, the electron-rich nature of acetaminophen renders it susceptible to oxidation, resulting in quinine-like compounds. These quinone-like compounds are prone to attack by nucleophilic protein/enzyme residues, resulting in protein/enzyme inhibition [22]. As a result, acetaminophen can be regarded as a covalent inhibitor as well. Penicillin is another early-identified covalent agent. Penicillin's accidental discovery as an antibiotic can be considered one of the most momentous discoveries in the history of drug development. Numerous penicillin analogs have been approved for human use. They all have a similar mechanism of action to penicillin and contain a beta-lactam as the chemical warhead. This beta-lactam combines with a serine residue in the active site of D-Ala-D-Ala transpeptidase, which is involved in bacterial cell wall production, and so inactivates it, resulting in the disruption and lysis of the bacterial cell wall structure [23].

Additional covalent antibiotics include beta-lactamase inhibitors such as clavulanic acid [24], sulbactam [25], and tazobactam [26]. Although covalent inhibitors have long been used to treat intervening human health conditions, the concept of covalent inhibition did not gain widespread acceptance until the 1970s, when it was discovered that many covalent drug metabolites have adverse effects on human health. For example, it was discovered that acetaminophen's cellular metabolites are hepatotoxic [27, 28]. Acetaminophen is oxidized by cytochrome P450 to highly reactive quinone intermediates (NAPQI and benzoquinone), which undergo covalent alteration when they combine with glutathione (GSH) or the sulfhydryl group of cysteine residues in proteins [16]. Unwanted immunogenic reactions in patients may result from nonspecific covalent drug-protein adducts. Despite this disadvantage, numerous factors have rekindled the pharmaceutical industry's interest in developing covalent medicines as therapeutic agents. First, there were successful covalent medications on the market, such as aspirin and penicillin. Second, not all covalent drugs become hazardous when metabolically activated. Additionally, certain natural compounds act as covalent inhibitors [29]. Furthermore, covalent inhibitors have several advantages over reversible inhibitors, including high target affinity and prolonged residence time in patients. Consequently,

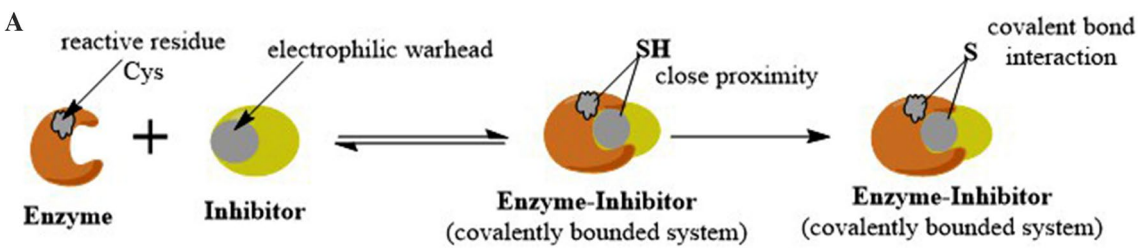


Fig. 1 Two steps required for covalent inhibition of enzyme

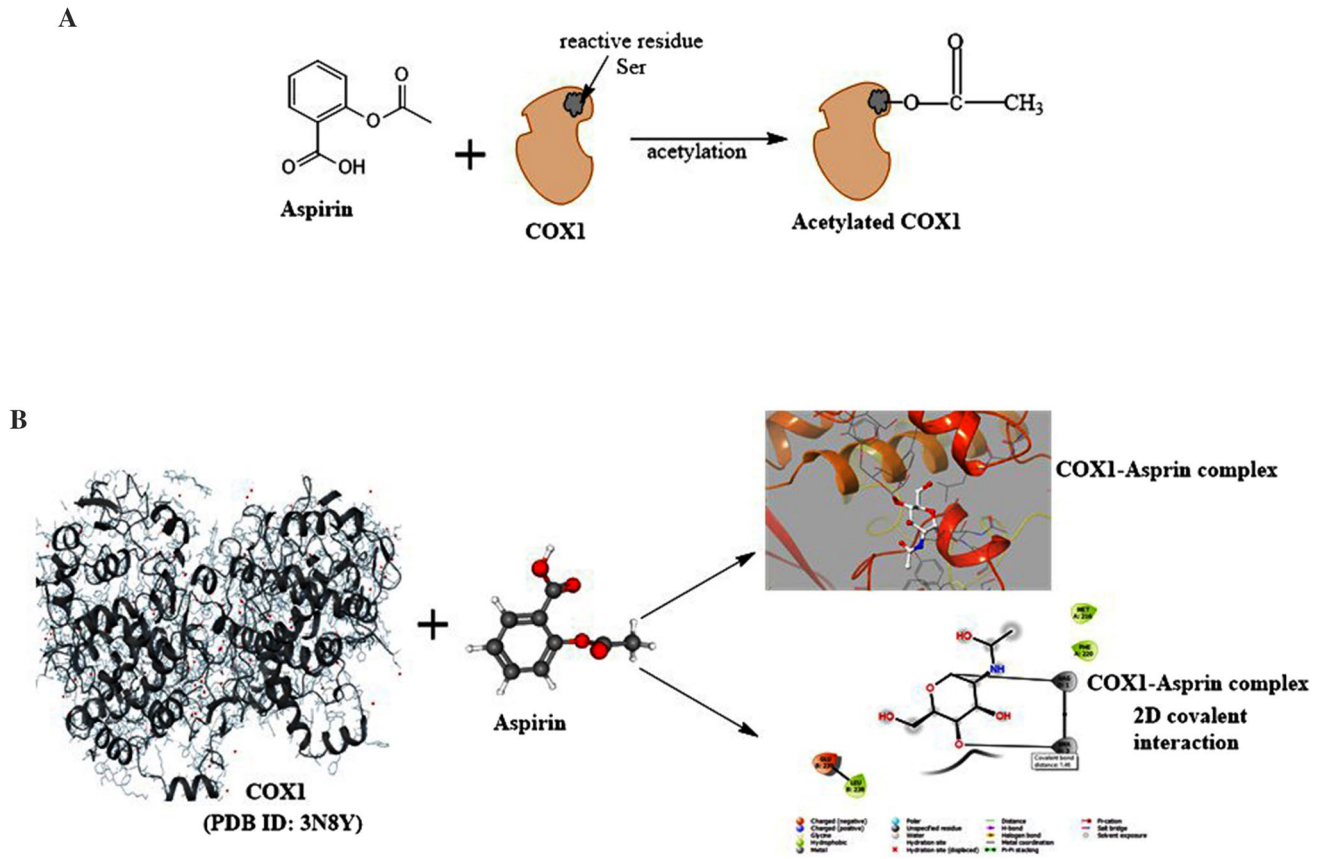


Fig. 2 **A** Acetylation of Aspirin by COX1. **B** 3D and 2D presentation of COX1-Aspirin complex after covalent reaction

unwanted pharmacokinetic features can frequently be tolerated because the inhibitors' pharmacodynamic properties outlast their detectable plasma concentration. On the basis of these data, it has been argued that if the reactivity of a covalent inhibitor's warhead can be regulated, there should be no major problems with employing it therapeutically. As a result of these factors, the development of covalent inhibitors has accelerated significantly over the last two decades (Table 1 consist of FDA-approved covalent drugs in recent years). Covalent inhibitors are generally composed of electrophiles that react with nucleophilic residues in enzymes. To date, compounds with a range of electrophilic warheads have been found as covalent inhibitors, including halomethyl carbonyl, vinyl sulfonyl, phosphonate, aldehyde, ketone, vinyl carbonyl, boronic acid, and many more (Table 2) [30]. A novel class of inhibitors dubbed "sulfur tethers" has also garnered interest due to its potential to covalently conjugate cysteines in enzymes [31]. Three steps are typically required to build a covalent inhibitor for a given enzyme target. First, structural analysis of the target reveals which nucleophile (e.g., cysteine, serine) is present in or near a potential binding pocket. The nucleophile must be unique within that protein family; otherwise, selectivity will be low. Second, a reversible inhibitor with some potency (IC50 values ranging

Table 1 Lists of FDA-approved covalent drugs in recent years

No.	Covalent drugs	Targeted disease	Warhead group	Date of approval
1	Acalabrutinib	Cancer	α,β-Unsaturated proparglycamide	10/31/2017
2	Neratinib	Cancer	α,β-Unsaturated carbonyl	7/17/2017
3	Dacomitinib	Cancer	α,β-Unsaturated carbonyl	9/27/2018
4	Selinexor	Cancer	α,β-Unsaturated carbonyl	7/3/2019
5	Zanubrutinib	Cancer	α,β-Unsaturated carbonyl	11/14/2019
6	Remdesivir	COVID-19	Aldehyde	10/22/2020
7	Sotorasib	Cancer	α,β-Unsaturated carbonyl	5/28/2021

Table 2 Common electrophilic warhead groups found with covalent ligands and their possible mechanism of action

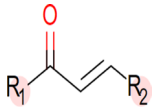
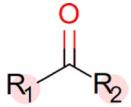
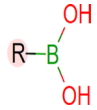
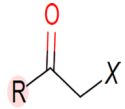
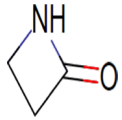
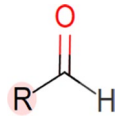
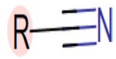
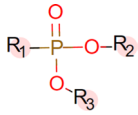
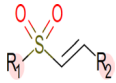
No.	Warhead class	Warhead SMARTS	Warhead structure	Example of ligand with warhead group	Type of pre-reactive mechanism
1	Vinyl Carbonyl	[C]=[C]-[CX3](=[O])[*]		Sotorasib	Michael Addition, Composite reaction, Imine Condensation, and Cyclohemiaminoacetalization
2	Ketone	[#6][CX3](=[O])[#6]		Boceprevir	Composite Reaction, Hemiaminalization, Hemi(thio)acetalization, and Imine Condensation
3	Boronic acid	[#6]-[B]([O])[O]		Delanzomib	Borylation and Composite Reaction
4	Halomethyl Carbonyl	[*][CX3](=[O])[CX4]-[F,Cl,Br,I]		ML175	Borylation, Composite Reaction, Imine Condensation, Nucleophilic Acyl Substitution and Nucleophilic Aliphatic Subsititution
5	Beta-Lactam	[O-0X1]=[C]1[C][C][N]1		Amoxicillin	Beta-Lactam Addition, Composite Reaction, Michael Addition and Nucleophilic Aliphatic Subsititution
6	Aldehyde	[#6][CX3H1](=[O])		16-epi-Vellosimine	Composite Reaction, Cyclohemiaminoacetalization, Hemiaminalization, Hemi(thio) acetalization, and Imine Condensation
7	Nitrile	[C,c]-[C,c]#[N,n]		Saxagliptin	Nucleophilic Addition to a Triple Bond, Nucleophilic Aliphatic Subsititution, and Nucleophilic Aromatic Substitution
8	Epoxide	[C;r3][O;r3][C;r3]		Carfilzomib	Composite Reaction, Cyclohemiaminoacetalization and Epoxide Opening
9	Phosphonate	[#6][P]([O])([O])=O		BCL-1-(R)	Phosphorylation
10	Vinyl Sulfonyl	[C]=[C]-[S](=[O])(=[O])[*]		FT827	Michael Addition

Table 2 (continued)

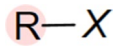
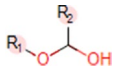
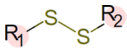
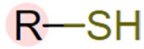
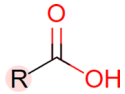
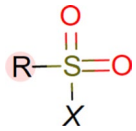
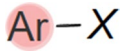
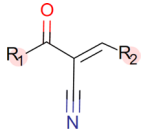
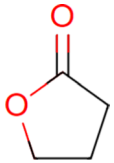
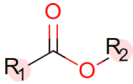
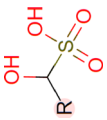
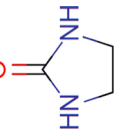
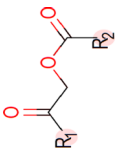
No.	Warhead class	Warhead SMARTS	Warhead structure	Example of ligand with warhead group	Type of pre-reactive mechanism
11	Alkyl Halide	[CX4]-[F,Br,Cl,I]		(2S)-2-amino-N-(5-bromopentyl)propanamide	Composite Reaction, Nucleophilic Acyl Substitution and Nucleophilic Aliphatic Subsititution
12	Hemiacetal	[OX2H][CX4][OX2][#6]		2-deoxy-2-fluoro-D-glucuronic acid	Composite Reaction, Nucleophilic Acyl Substitution, and Nucleophilic Aliphatic Subsititution
13	Disulfide	[#6][S][S][#6]		Ajoene	Disulfide Formation
14	Thiol	[CX4,c]-[SX2H1,SX1-]		ergothioneine	Disulfide Formation
15	Carboxylic Acid	[#6]-[CX3](=O)-[OX2H1,OX1-]		Caffeic acid	Composite Reaction, Nucleophilic Acyl Substitution, and Nucleophilic Addition to a Double Bond
16	Sulfonyl Halide	[#6][S](=O)(=O)[F,Cl,Br,I]		AEBSF	Sulfonylation
17	Aryl Halide	[c][F,Br,Cl,I]		1,5-Difluoro-2,4-dinitrobenzene	Nucleophilic Aromatic substitution
18	Aziridine	[C;r3][N;r3][C;r3]		*N*-acetyl cyclophellitol aziridine	Aziridine Opening
19	Alpha-Cyanovinyl Carbonyl	[C]=[C]([C]#[N])-[C](=[O])[*]		Rao-IV-151	Micheal Addition
20	Gamma-lactone	O=[#6]-1-[#6]-[#6]-[#6]-[#8]-1		GR157368	Lactone Addition
21	Ester	[#6][CX3](=[O])-[O]-[#6]		Gabexate	Nucleophilic Acyl Substitution and Nucleophilic Addition to a Double Bond

Table 2 (continued)

No.	Warhead class	Warhead SMARTS	Warhead structure	Example of ligand with warhead group	Type of pre-reactive mechanism
22	Alpha-hydroxy sulfonic Acid	[#6]([OH1])[S](=[O])(=[O])[O]		(R,S)-GC376	Hemi(thio)acetalization
23	Imidazolidinone	[O]=[C]1[N][C][C][N]1		Avibactam	Imidazolidinone Opening
24	Acycloxymethyl Carbonyl	[*][CX3](=[O])[C][O][CX3](=[O])[*]		(3R)-3-[2-(acetylsulfanyl)acetamido]-5-(2,6-dichlorobenzoyloxy)-4-oxopentanoic acid	Nucleophilic Aliphatic Subsitution

from M to mM) is identified and its binding manner and interactions are elucidated. This inhibitor may be derived from previously developed inhibitors for related enzymes. Lastly, an electrophilic 'warhead' is placed in a reversible inhibitor of interest to react precisely with the enzyme target's chosen nucleophile. Typically, isosteric substitution and analog synthesis are used to generate candidates of active covalent inhibitors [32].

Targeted covalent inhibitors and "warhead" reactivity

Despite the widespread use of covalent inhibitors in therapeutics, medicinal chemists have typically avoided developing compounds that permanently alter their target receptors [2, 33]. The introduction of a reactive electrophilic species necessitates the possibility of indiscriminate alteration and idiosyncratic toxicity, as has been established with some routinely used covalent medicines such as acetaminophen [27, 28]. However, these effects are frequently caused by highly reactive metabolites, and with a better knowledge of the reactivity requirements for covalent inhibitors, interest in the topic has resurged [1]. Indeed, the recent establishment of the covalent database (COVPDB) from the protein data bank (PDB) has been hailed as a paradigm shift in the covalent drug development paradigm [30]. Unlike prior covalent databases, which lacked covalent complexes, the CovPDB has a large number of high-resolution co-crystal structures of biologically relevant covalent inhibitors attached to their protein targets. For the curated protein–ligand complexes, Gao and his colleagues manually annotated the covalent inhibitors' chemical structures, defined their pre-reactive electrophilic warhead groups, and covalent-binding mechanisms with their targets [30].

When compared to typical reversible ligands, covalent inhibitors offer various advantages, including extended residence duration, greater potency, and, when the target residue is poorly conserved across the proteome, enhanced selectivity. Notably, these improvements have been proved to be clinically meaningful, and highly specific-targeted covalent inhibitors (TCIs) are now being introduced into the market [34, 35]. These molecules are invariably dependent on the accessibility of oxygen or sulfur atom in residues such as serine, threonine, or cysteine found within a small-molecule-binding site. It should be highlighted, however that the mere presence of a reactive residue near a ligand-binding site does not necessarily justify a covalent method; the target residue's local chemical environment must be carefully assessed to establish its suitability [36, 37]. While cysteine's strong nucleophilicity makes it an attractive candidate for the development of TCIs, its rarity in proteins limits its potential applications; this is exacerbated further by the fact that many

cysteines are located behind disulfide bridges and, therefore, inaccessible for covalent modification [38]. As a result, various research groups have lately investigated the design of novel warheads capable of interacting with additional nucleophilic amino acid side chains in order to generate irreversible inhibitors. While this concept is not novel [39, 40], it is now possible to evaluate these approaches in the context of commercially available covalent medicines. When evaluating a typical two-stage covalent-binding event, acrylamides (the common warhead used for cysteine modification) typically benefit from relatively quick rates of cysteine modification (k_{inact}) and very low (or non-existent) rates of warhead hydrolysis/decomposition (k_{hyd}). As a result, including an appropriately placed acrylamide into ligands with poor k_{on} or k_{off} rates may nevertheless result in functional target inhibition. In comparison, non-cysteine residues generally exhibit lower k_{inact} rates, necessitating the use of more reactive warheads. However, increasing reactivity often results in increased k_{hyd} rates, necessitating careful tuning of both reactivity and noncovalent-binding interactions (thereby optimizing k_{on} and/or k_{off}) in order to achieve the optimal target inhibitory profile [36, 41].

The intrinsic reactivity of warheads can be determined experimentally or computationally by employing a nonspecific covalent modifier (glutathione) and a specific covalent modifier (cysteine) [37, 42–44]. Additionally, there was a report devoted to deciphering the reactivity of a single covalent warhead [45]. However, by examining a variety of covalent warheads (Table 2 represents 24 common warhead groups found with covalent inhibitors) and aminoacids, one can have a greater understanding of their relative reactivity. To this end, Martin and his colleagues developed an NMR-based assay for determining the rate of reactivity of amino acids with possible covalent warhead formation property [37]. The researchers determined the reactivity of a variety of commonly known covalent warheads against cysteine and serine, the most commonly targeted amino acids. Additionally, a number of other possibly reactive amino acids were explored. Interestingly, it was discovered that a covalent modifier's reactivity was largely dependent on the amino acid residue. When the researchers compared the reactivity of several covalent compounds (including electrophilic warhead benzoxaborole, sulfonyl halide, and acrylamide/vinyl carbonyl) with cysteine and serine, they discovered that the reactivity of the various warheads with both amino acid residues followed a very distinct pattern. This is largely explained by the fact that serine is a considerably "harder" nucleophile than cysteine (as the oxygen nucleophile in Ser has a smaller atomic radius than sulfur in Cys and therefore its electron cloud is less susceptible to distortion) and hence reacts more quickly with "harder" electrophiles (benzoxaborole and sulfonyl fluoride). In comparison, it was discovered that the acrylamides (12, 15, and 16) are softer electrophiles and react more swiftly with the "softer" cysteine. Intriguingly, cysteine reacts with these Michael acrylamides at a rate that is at least two orders of magnitude faster than serine. In the majority of situations, the "hard" or "soft" character of the electrophiles was sufficient to determine the relative reactivity of cysteine and serine. Additionally, it demonstrates how a drug having a covalent warhead can be tailored to a specific project. A less reactive chemical entity can be used if a covalent chemical entity is found to be excessively reactive and exhibits adverse side effects. On the other hand, if the covalent warhead is not sufficiently reactive to create a covalent bond with the target, a more reactive warhead can be chosen [37].

Given the stringent nature of targeted covalent inhibitors, identifying drug leads or candidates is contingent on the library being screened. On one side, the choice of the appropriate warheads determines the selectivity and gives the molecule's fundamental inherent reactivity. On the other hand, ultimate reactivity is also influenced by the chemical characteristics of nonreactive components, which can be distinguished prior to the reaction conditions being satisfied [46].

Irreversible warheads can serve as a starting point for developing temporary binders; by providing appropriate chemical modifications, irreversible warheads' reactivity can be altered to make them reversible [47]. Bonatto et al. predicted the binding-free energies of reversible covalent inhibitors using free energy perturbation procedures and evaluated their binding affinity [48]. Fanfrlk and colleagues demonstrated the principle of rational design of reversible covalent vinylsulfone inhibitors using an enhanced quantum mechanics-based scoring function (QM) [49], whereas Schirmeister and co-workers used a hybrid approach combining quantum mechanics and molecular mechanics (QM/MM) for the design of covalent vinyl sulfone-based inhibitors [50].

Covalent docking with covalent-docking programs: methods and drug discovery applications

CovDock

A commercially available tool based on the Schrodinger programs Glide [51] and Prime was introduced by Zhu et al. and is referred to as CovDock [52]. The CovDock-VS virtual-screening mode, which was developed specifically for this application, was also implemented [13]. Pre-reactive species are docked to a mutant receptor in which the reactive residue has been changed to an alanine, and the covalent attachment site is formed, followed by structural refinement of the resultant complex in accordance with typical noncovalent-docking techniques. It does not necessitate any parameter fitting for the investigation of new covalent reaction types but

rather depends on a force field that is appropriate for dealing with the covalent bond. In contrast, the bond formation is heuristic, which means that the reacting atoms are predefined and simply joined after traditional noncovalent docking of the pre-reactive species, and selection of relevant poses is performed. The resulting complexes are structurally refined, and the poses are grouped and scored. Different compounds are ranked based on their apparent binding affinity score, which is defined as the average Glide score of the binding mode of the pre-reactive ligand species and the approximate Glide score obtained in the covalent complex. According to the logical assumption, an optimum covalent inhibitor should have favorable noncovalent interactions with the protein both before and after the process. This conclusion can be reached. Because the reactivity of the warhead is not taken into consideration in this strategy, it works best when a specific warhead is provided and the emphasis is placed on optimizing the remaining portion of the covalent inhibitor in order to increase the apparent binding affinity.

The research of Alamri and colleagues was focused on developing a pharmacophore model based on the X-ray crystallographic structures of MERS-CoV 3CLpro in a complex with two covalently bound antagonists, which was then tested in mice [53]. Following an in silico screening of a covalent chemical library including 31,642 compounds, 378 compounds were identified, which satisfied the pharmacophore searches. After that, the Lipinski rule of five was employed in order to select only small-molecule compounds with the best physiochemical properties for use as orally bioavailable therapeutics. In order to assess their binding energy scores, 260 compounds were generated and subjected to covalent-based virtual screening using the COVDOCK-VS software. The top three candidate compounds, which were demonstrated to adopt binding modes that were similar to those of the previously described covalent ligands, were chosen. The binding mechanism and stability of these compounds were validated using a 100 ns molecular dynamic simulation followed by a computation of the binding-free energy using the MM/PBSA. It was concluded that the hits discovered could aid in the rational design of novel covalent inhibitors of the MERS-CoV 3CLpro enzyme.

The CovDock-VS was used in a virtual-screening protocol to find covalent inhibitors of the immunoproteasome's 5i subunit [54]. A commercial library of 100,000 boronic acids with desirable characteristics was screened in a multistep procedure. First, Glide noncovalent docking was used to constrain the boronic acid group at the reactive site. Similarly to CovDock, the targeted catalytic Thr1 was altered to alanine to provide room for the ligand warhead model. The top 10% of compounds were then covalent docked by CovDock-VS while reverting the mutation of the nucleophilic residue. The final phase of the procedure uses Cov-DockLO to covalently dock the top 10% scoring compounds. A total of 32 commercially accessible hits were chosen for experimental testing after visual assessment, clustering, and the implementation of diversity filters. Five substances with IC50s in the micromolar range (best IC50 = 34 mM) block the immunoproteasome's functional activity. The time-dependent inhibition of two hits validated their covalent binding.

Paul et al. used Autodock Vina to virtually screen around 1400 cysteine-focused ligands in order to identify the top candidates that can act as effective inhibitors of Mpro [55]. According to the authors' findings, the selected ligands exhibit strong interactions with the critical Cys145 and His41 residues. Covalent docking with the Schrodinger software suite (COVDOCK) was used to explore the mode and mechanism of inhibition of the selected candidates having the acrylonitrile group, which can establish a covalent connection with Cys145. To validate the docking contacts, an all-atom molecular dynamics (MD) simulation was performed on the four inhibitors L1, L2, L3, and L4. Additionally, the authors' results were compared to those obtained using a control ligand, a-ketoamide (11r). Principal component analysis of the structure and energy data from the MD trajectories reveals that L1, L3, L4, and a-ketoamide (11r) are structurally comparable to the Mpro apo-form. For pattern recognition of the best ligands, a quantitative structure–activity relationship method was used, which revealed that ligands comprising acrylonitrile and amide warheads can exhibit superior performance. According to the ADMET analysis, the identified drug candidates appeared to be safer compounds.

In another example, Chowdhury and colleagues employed COVDOCK to identify new covalent inhibitors of cathepsin L, identifying its catalytic Cys25 [56]. A library of 1648 chemical fragments was constructed by filtering the ZINC-purchasable database for carboxylic acids with desirable physicochemical characteristics. A vinylsulfone warhead was then attached to the acids and the library was tested against the target by COVDOCK, but the selected module was not stated explicitly in this case. Among the 33 compounds having good scores and docking positions, five compounds were identified and purchased for experimental investigation. Finally, one vinylsulfone fragment displayed time and dose-dependent inhibition ($K_i = 146$ μM), whereas others could not be evaluated due to solubility challenges. Further exploration of various analogs resulted in specific and effective covalent cathepsin L inhibitors.

In another research, Al-Khafaji and colleagues used COVDOCK (MMGBSA module in Schrodinger suite 2020-1) to evaluate the efficacy of FDA-approved medications that can form a covalent bond with Cys145 inside the SARS-CoV-2 main protease (Mpro) binding site utilizing a covalent-docking-based screening technique [57]. Saquinavir, ritonavir, remdesivir, delavirdine, cefuroxime axetil, oseltamivir, and

prevacid all had the highest MMGBSA binding energies of − 72.17, − 72.02, − 65.19, − 57.65, − 54.25, − 51.8, and − 51.14 kcal/mol, respectively. For saquinavir, ritonavir, and remdesivir, a 50 ns molecular dynamics simulation was performed to determine their stability inside the binding pocket of the SARS-CoV-2 Mpro. The work delivers compelling in silico results that can be utilized in conjunction with or in place of conventional therapy to treat SARS-CoV-2.

FLEXX

FLEXX [58] is a commercial software that can perform covalent docking only if the covalent link between the ligand and the receptor is defined manually. On the receptor side, the ligand structure input file is extended to include the two atoms nearest to the covalent linkage. The first placement is accomplished by superimposing these atoms on their receptor structure sites. According to a stepwise evaluation by the software's empirical scoring function, the software's standard incremental construction method results in the ultimate placement in the most suitable parts of the pocket. To our knowledge, we have found no report of the usage of this software for covalent virtual-screening studies.

GOLD

GOLD is an automated commercial docking tool based on genetic algorithms developed at the Cambridge Crystallographic Data Centre (CCDC) [59–61]. Its covalent implementation relies on the superposition of a "link-atom" in both the ligand and protein structures to utilize post-docking ligand conformations. Manual modification of the input files is required to bind the reactive sulfur or oxygen atom type of the targeted amino acid residue (Cys, Ser, or Thr) to the ligand's electrophilic atom. The ligand's link-atom is forced to fit onto the protein's link-atom, and a bending energy term is generated from the associated parameter file [62]. Finally, this energy term is added to the fitness docking score to verify that the covalent linkage geometry is appropriate. Other factors that contribute to the final score are the typical noncovalent interactions created by sampling the ligand's torsional degrees of freedom within the binding pocket. The GOLD Fitness Score is used to rank ligand docking conformations. The simulation's best docking result is given the highest score.

The GOLD software has been used to conduct various covalent-docking research in the past. For example, Schröder and his colleagues embarked on a virtual-screening campaign by employing the use of the GOLD program to identify novel inhibitors of cathepsin K. To accomplish this, the authors applied an automated substructure searching and warhead preparation method for compounds containing electrophilic chemical groups of interest found in a pool of commercial databases in order to construct a targeted library. Following the covalent docking of the targeted library into the crystal structure of human cathepsin K, a group of 44 compounds was identified as potential inhibitors of cathepsin K. After being subjected to experimental investigation, 21 chemical entities were shown to be covalent reversible binding inhibitors. Three of these inhibitors were found to have nanomolar efficacy against cathepsin K and were therefore certified as lead structures. This study's findings support the successful deployment of a high-throughput covalent-docking method in lead discovery, according to the researchers [63].

In another work, Li et al. conducted a study to identify novel covalent inhibitors of the 20S proteasome. The structures of proteasome inhibitors were manually split between a noncovalent-binding portion coming from virtual screening and a covalent-binding portion consisting of an epoxyketone group that was pre-selected. Noncovalent docking and a pharmacophore model based on the 20S proteasome were used to screen the SPECS database. After confirming the covalent conjugation, 88 epoxyketone hits were covalently docked (with GOLD) into the 20S proteasome to study the intermolecular interactions. Four compounds were chosen following extensive filtration and validation. Molecular dynamics simulations were used to determine the stability of noncovalently and covalently docked ligand-enzyme complexes and to characterize the interaction patterns of the tested inhibitors. Finally, two compounds with unique aromatic backbones were kept due to their appropriate interactions and stable covalent-binding mechanisms. These compounds could be used as lead compounds for additional biological evaluation [64].

Using GOLD [65], Zhang et al. identified covalent inhibitors of the NEDD8-activating enzyme (NAE), a target implicated in the degradation of cancer-related proteins. Covalent docking was used as a component of a hierarchical pharmacophore-based virtual-screening protocol. To begin, two pharmacophore (ph4) models were constructed: a ligand-based ph4 (LBP) and a structure-based ph4 (SBP) (SBP). GOLD covalently docked a training set of seven known NAE inhibitors with sulfamoyl groups as warheads to build an LBP model, which was enriched further by integrating a shape constraint from a crystalline covalent inhibitor (MLN4924, PDB: 3GZN). By mapping the interactions that occurred during the MD simulation of the target in complex with the unbound form of MLN4924, a dynamic SBP model was generated. Following validation, the two ph4 models were used to screen a focused library of 28,000 sulfamoyl chemicals obtained from the ZINC database. Then, a total of 256 distinct hits were submitted to covalent docking, resulting in the selection of eight compounds based on the scaffold's score and uniqueness relative to the warhead. Experiments verified the existence of three novel NAE covalent inhibitors, the most effective of which had an IC50 of 1 M.

Sgrignani et al. used GOLD to screen a virtual library of 1385 boronic acids against the AmpC-lactamase-binding site in *Escherichia coli* [66]. The authors used a previously validated and optimized covalent-docking protocol against the same target, which included three conserved water molecules, to select six compounds that were experimentally found to inhibit AmpC β-lactamase of various species at low micromolar concentrations (best $K_i = 0.11$ M against *E. coli* AmpC β-lactamase). Additionally, by evaluating these compounds against a panel of eight lactamases, a nanomolar inhibitor of KPC-2 was identified ($K_i = 25$ nM), along with another that exhibited broad-spectrum activity by inhibiting multiple β-lactamase classes.

DOCKTITE

DOCKTITE entails the deployment of a highly adaptable covalent-docking procedure within the Molecular Operating Environment (MOE) via SVL-scripts [67], which is capable of visually screening enormous databases of possible lead structures. The innovative step-by-step procedure enables the user to maintain control over and customize each phase of a docking project [68]. Because the procedure is guided by an intuitive graphical user interface, DOCKTITE is equally suited to beginners and advanced users. The screening stage for warheads is searching a ligand database for user-defined covalent warheads that are then tagged and transformed to the bound state. A pharmacophore-based docking with optional force field refinement is performed after connecting the possible ligands to the covalently bonded residue of the receptor. Following that, the chimeric poses are cleaved and rescored using a new consensus-scoring approach that employs MOE-internal empirical scoring functions in conjunction with the external knowledge-based scoring function DSX [69].

DOCKTITE was used in a virtual-screening process to identify permanent inhibitors of the FMS-like tyrosine kinase 3 (FLT3), a protein known to possess drug-resistant mutations associated with acute myeloid leukemia (AML) [70]. By coupling four distinct warhead classes to known reversible scaffolds at favorable sites, a targeted library of 128 compounds was developed. Although the promising hits were not validated experimentally, the authors selected two scaffolds for further development, which resulted in the generation of many covalent inhibitors with enhanced cytotoxicity in FLT3-driven cell lines.

Omar et al. reported in silico screening for possible activators of G245S-mp53. The ZINC15 (13 million chemical entities) database was filtered to include only drug-like molecules with moderate to high reactivity [71]. The DOCKTITE protocol in the MOE program was used to screen the filtered database of 130,000 chemicals. Covalent docking of G245S-mp53 at Cys124 was used to identify putative activators of the mutant protein. A consensus-scoring approach was used to rank the docked compounds. The ADMET Predictor™ was utilized to evaluate the compounds' pharmacokinetics and potential toxicity. The virtual-screening approach highlighted compounds predominantly belonging to the warhead class of thiosemicarbazones and halo-carbonyls as showing the highest potential as G245S-mp53 activators. Compound 2 was determined to have the greatest potential as a G245S-mp53 activator based on its binding affinity and ADMET risk score when compared to the other top hits.

ICM-Pro

Molsoft LLC developed the commercially available docking code ICM-Pro [72], which incorporates a novel covalent-docking protocol [12]. ICM-Pro automatically turns the prereaction ligand into a "pseudo-ligand" based on the reaction type by covalently connecting the cysteine side chain to the electrophilic warhead in all stereoisomers formed upon addition. The protein's sidechain atom coordinates are used to constrain the placement of the pseudo-ligand and then deleted to avoid unfavorable collisions during docking. Monte Carlo simulations are used to sample ligand conformations in a set of pocket-specific grid maps [73]. Finally, a modified version of the usual ICM-Pro scoring function is utilized to evaluate docking conformations by disregarding pairwise interactions between atoms immediately connected to the new covalent bond.

Katritch and colleagues used iCM-pro to conduct a virtual-screening experiment using covalent docking on a comprehensive library of around 230,000 accessible ketone and aldehyde compounds [12]. After converting the warheads to sp3-hybridized post-reaction states, the database was docked against an I7L homology model. Out of 456 determined hits, 97 inhibitors of I7L proteinase activity were confirmed in biochemical experiments (approximately 20% overall hit rate). These experimental results both verify the I7L ligand-binding model and suggest a strategy for rationally optimizing poxvirus I7L proteinase inhibitors.

Molecular operating environment (MOE)

The MOE [67] suite has a built-in covalent-docking module which is only available commercially. Docking postures are created using a technique known as reaction transformation placement. At this point, the covalent link between the protein and ligand atoms is formed, and the produced poses are scored. It should be stressed that the user may import bespoke reaction schemes as RDF files if they are not currently included in the predefined list. The default covalent-docking technique refines the docking solutions obtained during the placement step using MOE's rigid receptor scheme and then rescored using the force field-based GBVI/WSA

scoring function to determine the free binding energy associated with the given pose [74]. Despite the incorporation of covalent-docking functionality in this software, till date we have found no virtual-screening experiment that directly employs MOE for covalent docking of receptors. However, it should be noted that DOCKTITE, which has been used for a couple of covalent-docking-based virtual screening [70, 71], involves the deployment of a highly adaptable covalent-docking procedure within the MOE program via SVL-scripts.

MacDOCK

MacDOCK [75, 76] combines DOCK 4.0 [77] with MIMIC [78, 79]. An incremental building approach is guided toward favorable binding modes by MacDOCK. MacDOCK compares the similarity between anchoring locations on the ligand and protein to favor those orientations that generate a good geometry for the covalent binding. The attached structure requires the initial alteration of the ligand warhead into its post-reaction form. Once the reactive functionality of the ligand is identified, the approach allows for automated procedures. MacDOCK scores ligand conformations using a modified version of its usual scoring method that excludes intermolecular interactions involving the covalent bonding atoms and those related to them. Finally, the final score incorporates a similarity weight that optimizes the initial anchor placement.

CovalentDock

In 2013, Ouyang et al. devised a model to account for the energy contribution of covalent binding as a term compatible with the noncovalent-docking scoring function. This approach was implemented as CovalentDock (available as a free webserver) using the Autodock source code. Inspired by both Autodock and GOLD, a method was developed that employs a dummy atom to perform the same function as the "link atom" in GOLD, by incorporating the proposed energy term into the Autodock scoring function and searching for docking results using the built-in grid map calculation and Genetic Algorithm. Meanwhile, a process for automatically recognizing and preparing covalently bondable chemical groups (limited to ligands reacting via Beta-lactam ring-opening and Michael addition) has been devised, enabling automated covalent docking and covalent virtual screening on a large scale [80].

In 2014, Blake and Soliman aimed to uncover and characterize novel potential inhibitors capable of irreversibly inhibiting the RecA intein splicing domain of Mycobacterium tuberculosis via the establishment of a covalent linkage with its active site cys1 [81]. Their hunt for novel leads as potential protein splicing inhibitors is centered on Michael acceptor-like compounds, which are powerful electrophiles that react covalently with the enzyme's active site's nucleophilic cysteine SH group. Autodock Vina and CovalentDock were used to accomplish structure-based virtual screening using a hybrid of noncovalent and covalent docking. Ten interesting covalent inhibitors were identified following the covalent-docking process using the CovalentDock software. Additionally, molecular dynamic simulations (MD) and thorough post-dynamic analysis were used to assure the stability of docked ligand-enzyme complexes and to provide insight into the inhibitors' binding affinities and interaction patterns. Interestingly, three unique hits demonstrated increased binding affinity when compared to experimentally determined drugs known to hinder protein splicing. Additionally, MD simulations demonstrated that the docked compounds are fairly stable in the active region of the protein.

DOCKovalent

DOCKovalent developed by London and colleagues in 2014, is a website that facilitates covalent docking [82]. A modified version of the noncovalent-docking method DOCK 3.6 [83] was created in order to enable large-scale virtual screens of electrophilic chemical libraries. DOCKovalent allows users to choose the preferred warhead-specific screening set from a list of options available on the web platform. These sets were compiled from commercially accessible compounds in the ZINC database at the time of the database's initial release. DOCKovalent makes use of a number of different programs to process input ligand structures prior to running docking simulations. Initially, compounds are converted in the post-reaction form according to the warhead chemistry utilizing OpenEye tools, which are initially converted in the SMILES form. AMSOL calculates partial charges and solvation energies [84], Epik evaluates protonation and tautomeric states [85, 86], and OMEGA generates low-energy conformations [87]. Corina is used to generate 3D conformations and stereoisomers [87, 88]. The calculations for ligand preparation are fully automated in web server applications, but conducting the ligand preparation workflow on a local system necessitates manual intervention in addition to license tokens for proprietary methods. As part of the docking procedure, several ligand postures are routinely sampled around the freshly formed covalent bond, with limitations applied to guarantee that the bond length and angles are optimal. The DOCK scoring function is then used to score and rank docking solutions after the energy contributions involving the electrophilic atom have been ignored. However, it should be noted that DOCKovalent docks the compounds in either their entire post-reaction form or the high-energy intermediate formed after covalent bond formation, depending on the warhead used to dock the compounds (e.g., aldehydes or carbamates).

Shraga et al. used Dockovalent to screen a virtual library of 117,667 acrylamides against Cys218 in the protein's

active region in order to identify selective covalent inhibitors of MKK7 [89]. Ten compounds were chosen for synthesis and testing after manually inspecting the top 500 predictions from each sub-library. Three compounds inhibited MKK7 (one of two direct MAP2Ks that activate JNK) in an in vitro kinase activity assay, with IC50 values of 11, 502, and 873 nM. These chemicals were designed to be effective inhibitors of JNK phosphorylation in cells at low micromolar concentrations. The crystal structure of a lead compound bound to MKK7 established that docking correctly recapitulated the binding mode. The authors established the inhibitors' selectivity at the proteome level and against a panel of 76 kinases, as well as their on-target activity using knockout cell lines. Finally, they demonstrated that the inhibitors suppress lipopolysaccharide-induced activation of primary mouse B cells. It was then proposed that MKK7 tool compounds would facilitate JNK signaling research and could serve as a starting point for therapeutics.

DOCKovalent was used by London et al. to screen huge virtual libraries of electrophilic small compounds [82]. They used the software prospectively to identify reversible covalent fragments directed against a variety of different protein nucleophiles, including the catalytic serine of AmpC-lactamase and noncatalytic cysteines in RSK2, MSK1, and JAK3 kinases. The authors identified submicromolar to low-nanomolar hits with excellent ligand efficiency, cellular activity, and selectivity, including the first covalent inhibitors of JAK3 described to date. The crystal structures of inhibitor complexes with AmpC and RSK2 validate docking predictions and provide guidance for further development.

DOCKovalent was also used to screen a library of 60,300 acrylamide-based compounds for irreversible KRAS G12C covalent antagonists. The researchers tested covalent docking and empirical electrophile screening against the extremely dynamic KRAS G12C target. While both approaches achieved a similar overall hit rate, they were able to rapidly advance a docking hit to a powerful irreversible covalent binder capable of modifiying the inactive, GDP-bound state of KRAS G12C. The protein kinetics of chemical binding to the switch-II pocket and subsequent destabilization of the nucleotide-binding region were investigated using hydrogen–deuterium exchange mass spectrometry. Contrary to previous switch-II pocket inhibitors, these novel chemicals seemed to accelerate nucleotide exchange via SOS. This work demonstrates the efficacy of covalent docking as a technique for identifying chemically unique hits against difficult therapeutic targets [90].

AutoDock 4

AutoDock4 [91] is a docking tool developed at the Scripps Research Institute that is available as an open-source project. The two kinds of covalent-docking methods supported in AutoDock4 are the two-point attractor and the flexible side chain approaches [14]. Both utilize grid maps that have been precalculated with atom probes in order to speed up the scoring operation, but they differ in how they mimic ligand conformations during the scoring method. The two-point attractor approach converts ligands in the post-reaction state by attaching two dummy atoms to the two terminal atoms of the nucleophilic side chain, which correspond to the two terminal atoms of the nucleophilic side chain. In this step, side chain atoms are removed from the protein, and an attractive Gaussian potential is used to drive the insertion of the dummy atoms into their original positions in the protein structure during the untethered docking step, which is performed in the absence of the protein. The flexible side chain technique, on the other hand, makes advantage of tethered docking to mimic the bound form of covalent ligands within the pocket. The electrophilic core of the ligand must be linked to the two terminal nucleophilic protein atoms in order for this approach to work. By defining the relevant SMARTS pattern, these two atoms are subsequently superposed to the respective residue atoms in the protein, resulting in the desired structure. The bound ligand is then considered as a completely flexible residue, and its conformations in the pocket are sampled using the conventional AutoDock4 technique for flexible residues.

Amendola and colleagues recently launched a virtual-screening campaign against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) Mpro chymotrypsin-like cysteine protease using an in-house database of peptide and non-peptide ligands characterized by various types of Michael acceptors [92]. The scientists predicted the creation of a covalent compound with the target protein using the AutoDock4 docking software. In vitro validation of the most promising candidates' inhibitory capabilities resulted in the identification of two novel lead inhibitors that merit further development. From a computational standpoint, this work illustrates AutoDock4's predictive capability and suggests its use for in silico screening of huge chemical libraries of putative covalent binders against the SARS-CoV-2 Mpro enzyme (Fig. 3).

Selecting an ideal covalent-docking program for virtual-screening campaign of covalent targets

It has been suggested that for an ideal covalent-docking tool, the success rate should not be dependent on protein classes, ligand warhead classes, residue types, and reaction mechanisms [93]. However, the performance of the present covalent-docking tools is largely dependent on these aforementioned criteria. Nonetheless, in a covalent virtual-screening

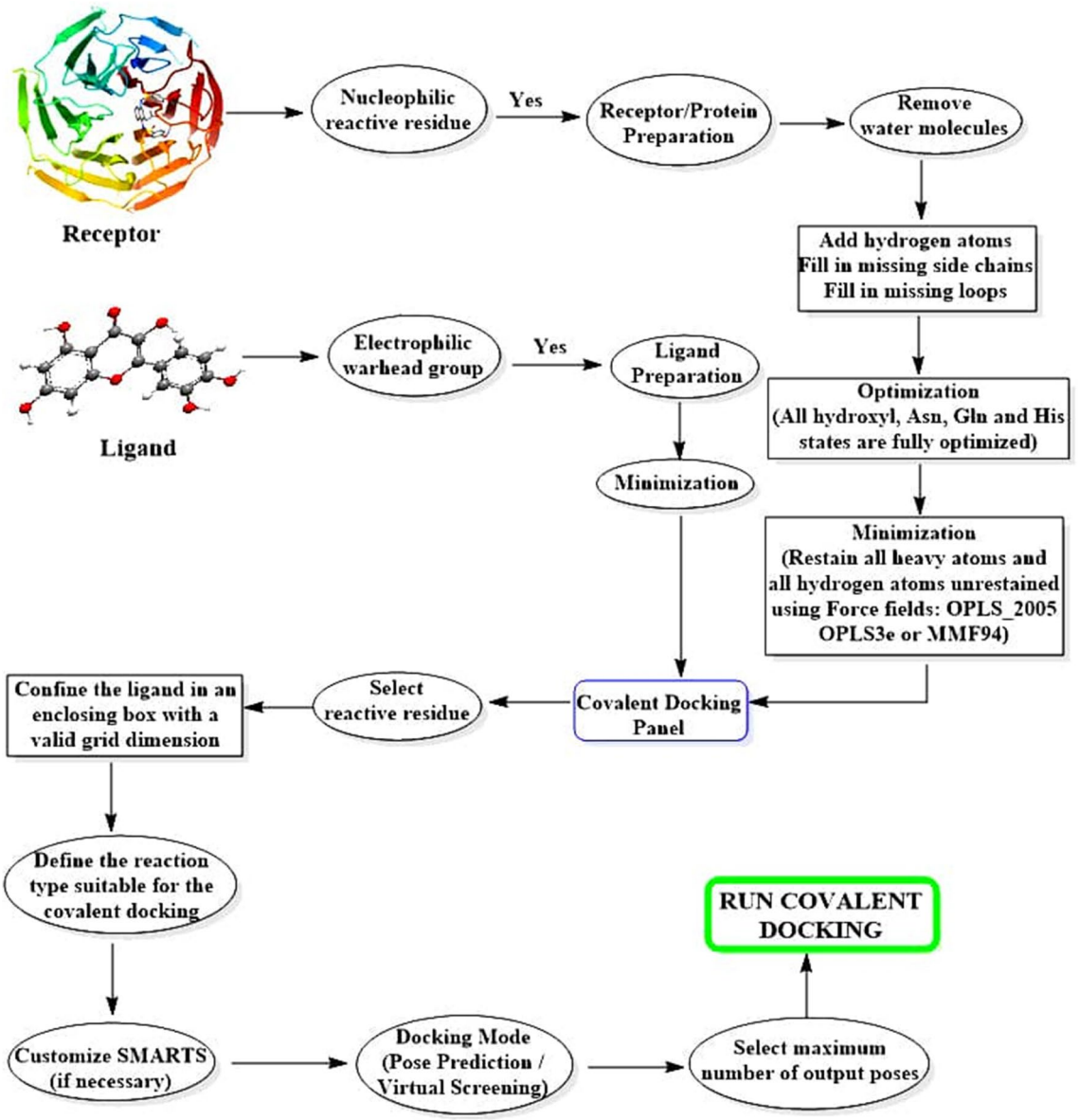


Fig. 3 Typical workflow for conducting covalent-docking simulation

study, a researcher may select a covalent-docking program that has been reported to achieve a high success rate for his specific receptor/residue target, ligand warhead or reaction types. Hence, as a guideline to aid the docking community in choosing an ideal covalent-docking program for their virtual-screening experiments, we survey studies that are focused on comparative evaluation of the "front runner" covalent-docking tools [94].

Currently, there are three research works that have benchmarked the performance of the available covalent-docking tools. First was the study of Keserű and colleagues, which evaluated six covalent-docking tools (FITTED, Autodock4, MOE, GOLD, COVDOCK, and ICM-Pro) against a dataset consisting of 207 protein–ligand complexes with diverse electrophilic warhead groups and receptor types [95]. Secondly, Wen et al. assessed the performance of four covalent-docking programs (COVDOCK, MOE, ICM-Pro, and GOLD) by employing a dataset from the BCDE set, which consists of 330 diverse ligand scaffolds and 104 receptor targets [93]. Lastly, Wei and co-workers developed a hybrid covalent-docking method known as COV_DOX and compared its performance to the previously identified covalent-docking tools (MOE, ICM-Pro, COVDOCK, and GOLD) [94]. Although, COV_DOX was validated to have a higher performance rate than all other covalent-docking tools (MOE, GOLD, ICM-Pro, and COVDOCK), the hybrid

method is considered "not feasible" for a virtual-screening experiment because of its very high computing time, which is as a result of its GSA (Generalized simulated annealing) quantum mechanical calculation method [94]. Therefore, the comparative studies by Keserű et al. [95] and Wen et al. [93] on covalent-docking tools are the only focus of this review section.

Based on the original RMSD data reported by Keserű et al. and Wen et al., we formulated some results (as shown in Tables 3 and 4) for the identification of ideal covalent-docking tools for protein types and ligand warhead chemistry that may be useful in a virtual-screening experiment. While Keserű and co-workers used Top1 (best scoring pose) and Top10 (best RMSD among top10 scoring poses) as a metric for determining the best covalent-docking program, Wen et al. utilized best scored pose (top-ranked binding mode) and best sampled pose (the conformation with the minimum root-mean-square deviation values with the crystal pose among all docked ligand poses) as a determinant for ranking the best performing docking software. According to our own statistics of the Table S1 RMSD data of Keserű et al. (https://pubs.acs.org/doi/suppl/10.1021/acs.jcim.8b00228/suppl_file/ci8b00228_si_001.pdf), ICM-Pro was ranked as the best performing covalent-docking program (both TOP1 and TOP10) for receptor types that belong to the hydrolase and transferase families (Table 3). In contrast, the study of Wen et al. revealed that CovDock is the docking program with the best scored pose and best sampled pose for both receptor types. Overall, the comparative analysis of the data of both groups of scientists for hydrolase, transferase, and other protein types (Table 3) showed inconsistency and a high discrepancy of results, which may be due to the preference of test sets considered while conducting their benchmarking studies [94]. Similarly, this is also the case for the common

Table 3 Identifying ideal covalent-docking program for various receptor types through comparative studies from benchmark studies

S/N	Receptor types (Wen et al.)	Number of test sets	Software with best scored pose (Average RMSD)	Software with best sampled pose (Average RMSD)	Reference
1	Hydrolase	204	COVDOCK (1.71 Å)	COVDOCK (1.39 Å)	[93]
2	Transferase	83	COVDOCK (1.3 Å)	COVDOCK (1.06 Å)	[93]
3	Ligase	3	COVDOCK (1.08 Å)	COVDOCK (0.84 Å)	[93]
4	Metal binding protein	5	MOE (1.72 Å)	MOE (1.04 Å)	[93]
5	Oxidoreductase	6	ICM-Pro (1.13 Å)	GOLD (1.06 Å)	[93]
6	Transcription	18	COVDOCK (2.15 Å)	MOE (1.6 Å)	[93]
S/N	Receptor types (Keserű et al.)	Number of test sets	Top 1 pose (average RMSD)	Top 10 pose (average RMSD)	Reference
1	Hydrolase	126	ICM-Pro (2.65 Å)	ICM-Pro (1.32 Å)	[95]
2	Transferase	53	ICM-Pro (1.28 Å)	ICM-Pro (1.03 Å)	[95]
3	Ligase	4	FITTED (1.46 Å)	FITTED (0.96 Å)	[95]
4	Metal binding protein	3	Autodock 4 (1.91 Å)	COVDOCK (1.15 Å)	[95]
5	Oxidoreductase	3	Autodock 4 (0.87 Å)	Autodock 4 (0.73 Å)	[95]
6	Transcription	4	MOE (1.39 Å)	FITTED (0.58 Å)	[95]

Table 4 Rationalizing the selection of an ideal covalent-docking program for ligand warhead chemistry through comparative studies of Keserű and Wen et al.

S/N	Common reaction types	Keserű et al.'s result [95]	Wen et al.'s result [93]	Rational opinion
1	Nucleophilic substitution	ICM-Pro	ICM-Pro	The agreement of both group's results makes ICM-Pro suitable
2	Addition to nitrite	Autodock 4	GOLD (Cys) and COVDOCK (Ser)	Autodock4 (because of larger dataset tested). However GOLD and COVDOCK can still be used for Cys and Ser targets respectively
3	Ring opening	GOLD	MOE	MOE should be considered because of larger data set tested. However authors may also choose GOLD
4	Disulfide formation	GOLD	MOE	With larger test set, MOE may be more suitable than GOLD
5	Michael addition	COVDOCK	ICM-Pro	COVDOCK may be considered because of larger dataset but ICM-Pro can also be used (also tested with large dataset)

reaction types found with ligand warhead chemistry groups, with the exception of nucleophilic substitution (Table 4). For instance, in the study of Keserű et al., GOLD was ranked as the best performing software for disulfide formation and ring-opening reaction types, while MOE emerged as the best performing tool considering the same reaction types in the work of Wen et al.

However, considering the larger and more diverse BCDE test set of Wen et al. (covering both Cys and Ser targeting warheads) when compared to Keserű's test set (covering only Cys targeting warheads), it may be rational to consider the results of Wen and colleagues for the selection of covalent-docking program in a virtual-screening study involving both receptor types and ligand warhead chemistry (Tables 3 and 4). For example, the front runner in Wen et al.'s work (COVDOCK) for hydrolase should be prioritized than Keserű's front runner tool (ICM-Pro) in covalent-docking research due to the larger and more diverse test set (as shown in Table 3). On the other hand, since the vast majority of Keserű's ligand datasets were made in favor of addition reaction mechanisms, their result of the comparative assessment of the existing covalent-docking tool should be considered more during a covalent-docking-based virtual-screening experiment involving ligand warhead groups with only addition reaction (Table 4). In spite of the discrepancy, both research investigations found that docking programs had varied accuracy when dealing with diverse receptors and ligand warhead chemistries, indicating that this is a prevalent flaw in empirical models.

Challenges of covalent docking

Modeling covalent bond

One of the major problems encountered for covalent-docking simulations is how to deal with the creation of covalent bonds between the protein nucleophile and the ligand electrophile. This covalent reaction cannot be explained using the traditional intermolecular potentials commonly utilized in docking but rather through physics-based quantum mechanics. Quantum mechanics (QM) enables the modeling of the electronic rearrangements that occur during a covalent chemical reaction between an electrophilic ligand warhead and a nucleophilic protein residue [96]. Bond formation, breakage, and rearrangement all require an explicit treatment of electronic degrees of freedom and, hence, the need for a QM calculation. While energetic effects from QM methods are increasingly being incorporated into docking programs, such as in QM/MM on-the-fly docking [97], a full QM treatment is still impractical in routine applications due to the size of the molecular systems and the large number of configurations and ligands to be taken into consideration [98].

Deficient scoring function

A robust covalent-docking method should be capable of weighing all important contributions to binding interactions, including those resulting from covalent bond formation. Unfortunately, a significant disadvantage is the inability to efficiently score the energy involved in bond formation. As described by Scarpino et al., modeling the chemical reaction needs time-consuming quantum mechanical (QM) calculations that are not yet suited for fast docking techniques [99]. The scoring function of covalent-docking systems is deficient in terms of bond formation, preventing the evaluation of ligands with varying degrees of reactivity and hence could have a negative impact on the success rate of virtual-screening campaigns in the identification of covalent inhibitors [92, 99]. Till date, the only covalent-docking program with more accurate scoring function with very high quantum mechanical calculation is COV_DOX (81% success rate) and it is not surprising that the hybrid method performed better than all other covalent-docking front runners 40–60% in performance) [94].

Higher computing time

Unlike conventional-docking programs that typically require a lower time scale, covalent-docking software/webservers generally require higher computing time due to the quantum mechanical and free energy calculations required to evaluate a covalent bond formation between the electrophilic ligand warhead and the nucleophilic center of the residue. Examples of this could be found in the Schrödinger COVDOCK program (COVDOCK-LO) and COV_DOX, which are considered not fast and unfeasible for structure-based virtual-screening studies, respectively [13, 94]. However, Toledo and colleagues have successfully addressed the longer time scale associated with the latter program by modifying the default Covdock workflow to suit the requirements of conducting covalent-docking-based virtual-screening campaigns in a shorter time period (COVDOCK-VS) [13].

Can covalent docking be achieved using conventional-docking programs?

Theoretically and experimentally, it has been proposed that covalent systems with no direct covalent bonds could have the potential to induce covalent linkage if the electrophilic warhead group and the side chain of the nucleophilic receptor target are found in close proximity (Fig. 4) [32, 100–102]. While there have been developments of covalent-docking protocols to simulate a covalent biomolecular system, some conventional noncovalent-docking programs/protocols have also been employed in the modeling of

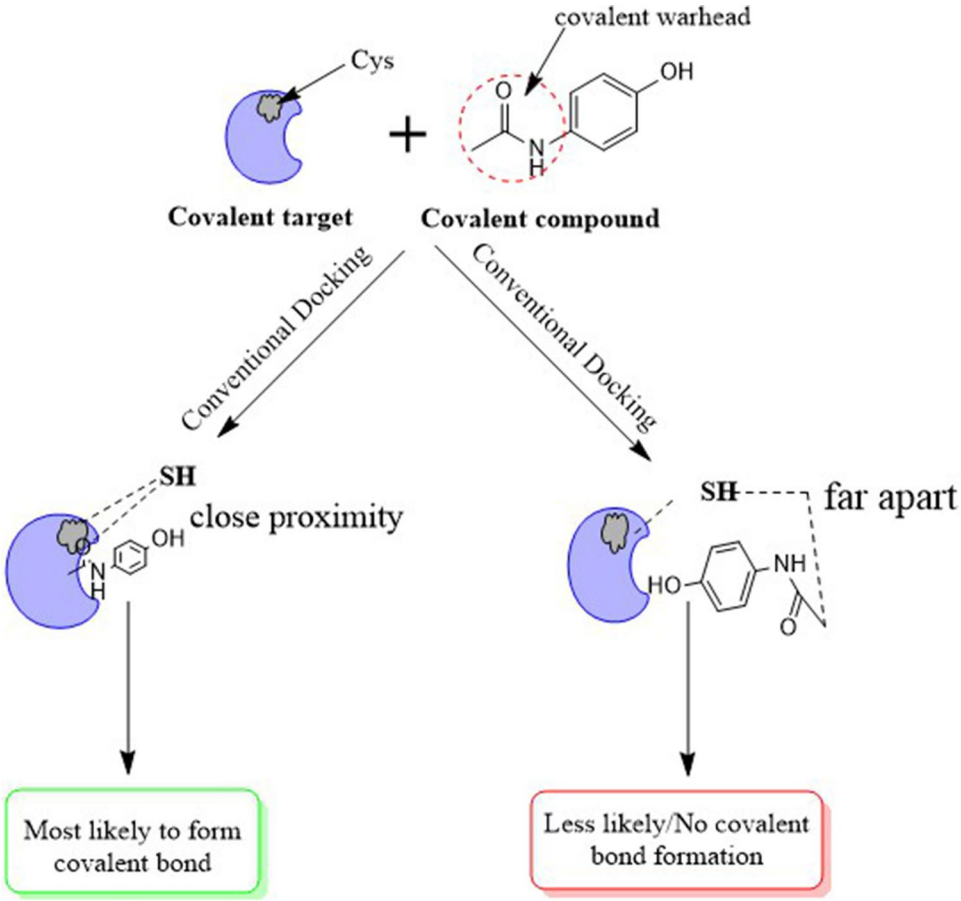


Fig. 4 Proposing covalent inhibition with distance analysis between warhead group and nucleophilic reactive protein residue

covalent binders that involves assessing the bond distance between the pre-reactive covalent warhead of the compound and the nucleophilic protein group. This approach involves examining the distance between the electrophilic moiety of the ligand and the nucleophilic center of the amino acid side chain to determine whether they are in close proximity to potentially form a covalent linkage. Undoubtedly, this is a rational strategy to predict the chances of the ligand warhead group forming a covalent adduct with its targeted nucleophilic reactive residue. A typical example of this could be found in the work of Soulère and co-workers [103]. The researchers conducted a docking-based virtual screening of covalent inhibitors with the use of the Arguslab program. To investigate the possibility of covalently inhibiting SARS-CoV-2 MPro, docking of the covalently co-crystallized inhibitors within the protease was examined in order to establish the structural basis for covalent inhibition. The distance between the electrophilic moiety and the thiol of Cys145 was examined in particular. Thus, docking tests on these three molecules were performed. This investigation corroborated the crystallographic results since the electrophilic core of the compounds, namely the methylene group of the chloro-acetamide functional group, is positioned near Cys145 with distances between the carbon and thiol atoms varying from 2.83 to 3.19 Å. Based on docking investigations, the structural foundation for covalent inhibition of Mpro was characterized as the ligands binding the protein with a high affinity and having an electrophilic moiety oriented appropriately toward Cys145 at a distance less than 4 Å from the sulfur. Following that, flexible docking investigations were conducted on a targeted approved covalent drug library consisting of 32 compounds with a variety of electrophilic functional groups. Among them, the calculations identified four compounds capable of interacting with the protein's binding site and, secondly, their potential to act as covalent inhibitors, as the distance between the sulfur and the electrophilic center varied between 2.98 and 3.78 Å (values less than 4 Å indicating a complex capable of forming a covalent bond) [101].

In another study, Ai and colleagues described a method called steric-clashes alleviating receptor (SCAR), in which the covalently bound residue is altered to a sterically smaller residue, such as serine or glycine [104]. This enables the ligand to dock in a conformation comparable to that observed in the covalently modified form without encountering steric clashes. These poses are classified according to the strength of their noncovalent bonds. The generated pose was compared to the experimental crystallographic structures of covalently

inhibited "AdoMetDC" to assess this approach. This technique predicted the binding pose with an RMSD of 3 Å, which is similar to that of other covalent-docking techniques. The ensemble of docked complexes was screened throughout their workflow to identify those in which the warhead was within 1 Å of the targeted residue. After imposing this limitation, the mean RMSD of the top-ranked structures was lowered to 1.9. This strategy was then effectively used to identify new covalent inhibitors of *S*-adenosylmethionine decarboxylase.

Finally, a virtual-screening strategy to detect covalent binders using cysteine reactivity data was introduced as WIDOCK [99]. The approach was inspired by Backus et al.'s reactive docking approach for identifying ligandable cysteines in the proteome [105]. In order to overcome one of the primary intrinsic constraints of covalent-docking technologies, WIDOCK was developed. WIDOCK focuses on the noncovalent interactions that occur in the binding site and includes a reactivity-scaled reward for compounds that may position the warhead near to the targeted cysteine. The reward is a pseudo-Lennard–Jones potential added to the noncovalent AutoDock4 scoring system. WIDOCK allows screening of chemical sets with a variety of warhead types and reactivities, prioritizing the most promising discoveries for experimental validation. WIDOCK was first tested against three targets, each representing a different warhead class and reaction pattern. Oncogenic mutant KRASG12C was screened against a collection of 20 chemicals equipped with multiple warheads and a shared scaffold. WIDOCK correctly predicted ten of the twelve known actives, outperforming AutoDock4's flexible side chain method (TPR = 75%).

Erroneous drug discovery docking pursuits involving covalent targets in the last few years

As discussed earlier in this review, there are two rational approaches to docking covalent proteins. First is the application of covalent docking programs/protocols for predicting the ability of a ligand warhead to form a covalent

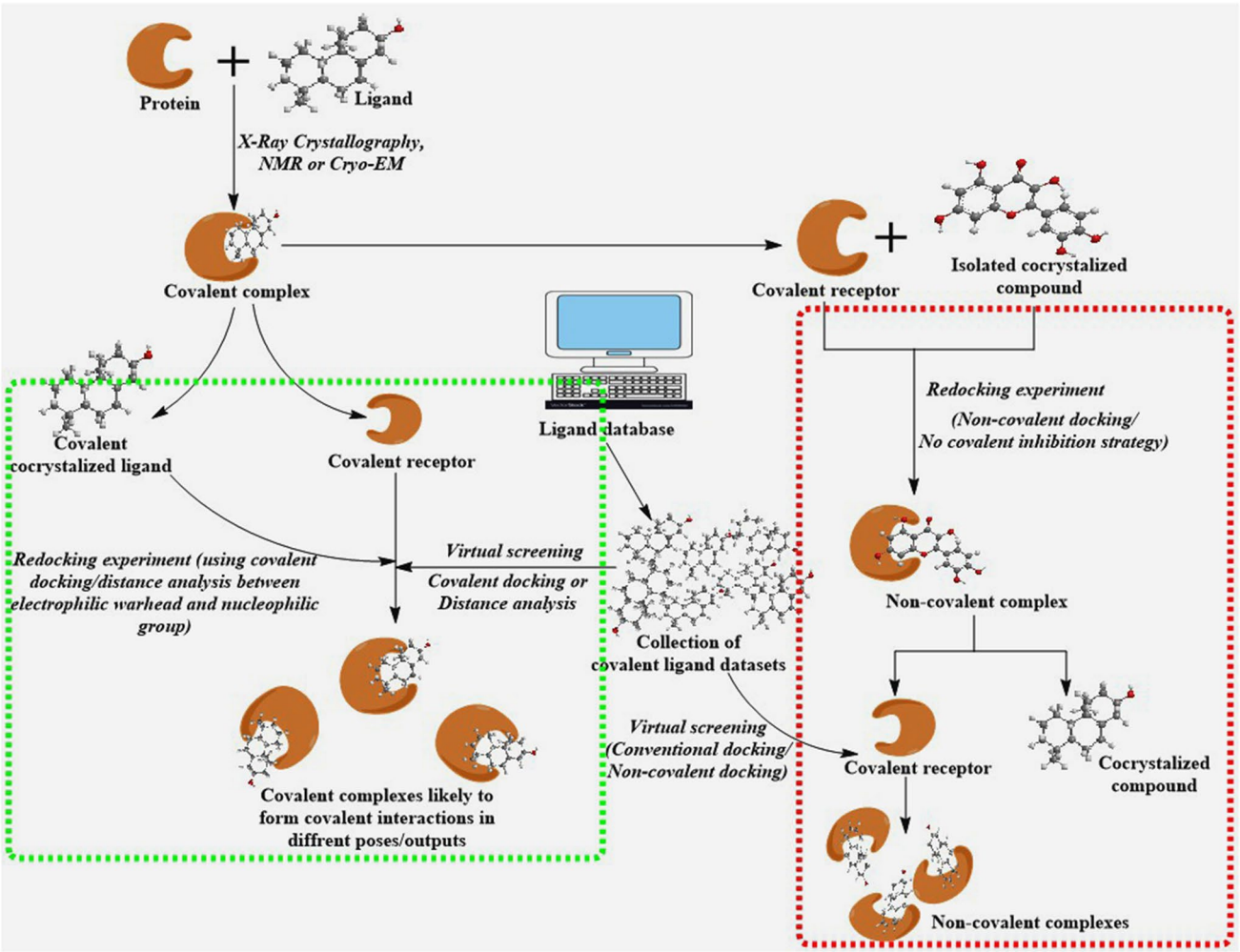


Fig. 5 A workflow depicting the rational (green box) and irrational (red box) drug discovery stages

linkage with the nucleophilic side chain of the receptor target. Secondly, the evaluation of the distance analysis between the electrophilic warhead group and the nucleophilic moiety of the protein also represents a rational strategy for modeling covalent systems when a conventional-docking program is employed. Hence, studies that involve docking covalent compounds with covalent targets without consideration of these approaches could be considered irrational and, therefore, may be an erroneous drug discovery practice resulting in false-positive results (Fig. 5 depicts the rational and irrational drug discovery steps). For example, the x-ray crystallographic experiment revealed by Jin et al. showed that the SARS-CoV2 Mpro (PDB ID: 6LU7) is a covalent target of the peptidomimetic inhibitor N3 [106]. It was reported that the co-crystal compound binds covalently with the thiol group of the protein's Cys145 sidechain, representing a crucial mechanism of inhibition of the peptidomimetic compound (Fig. 6). Therefore,

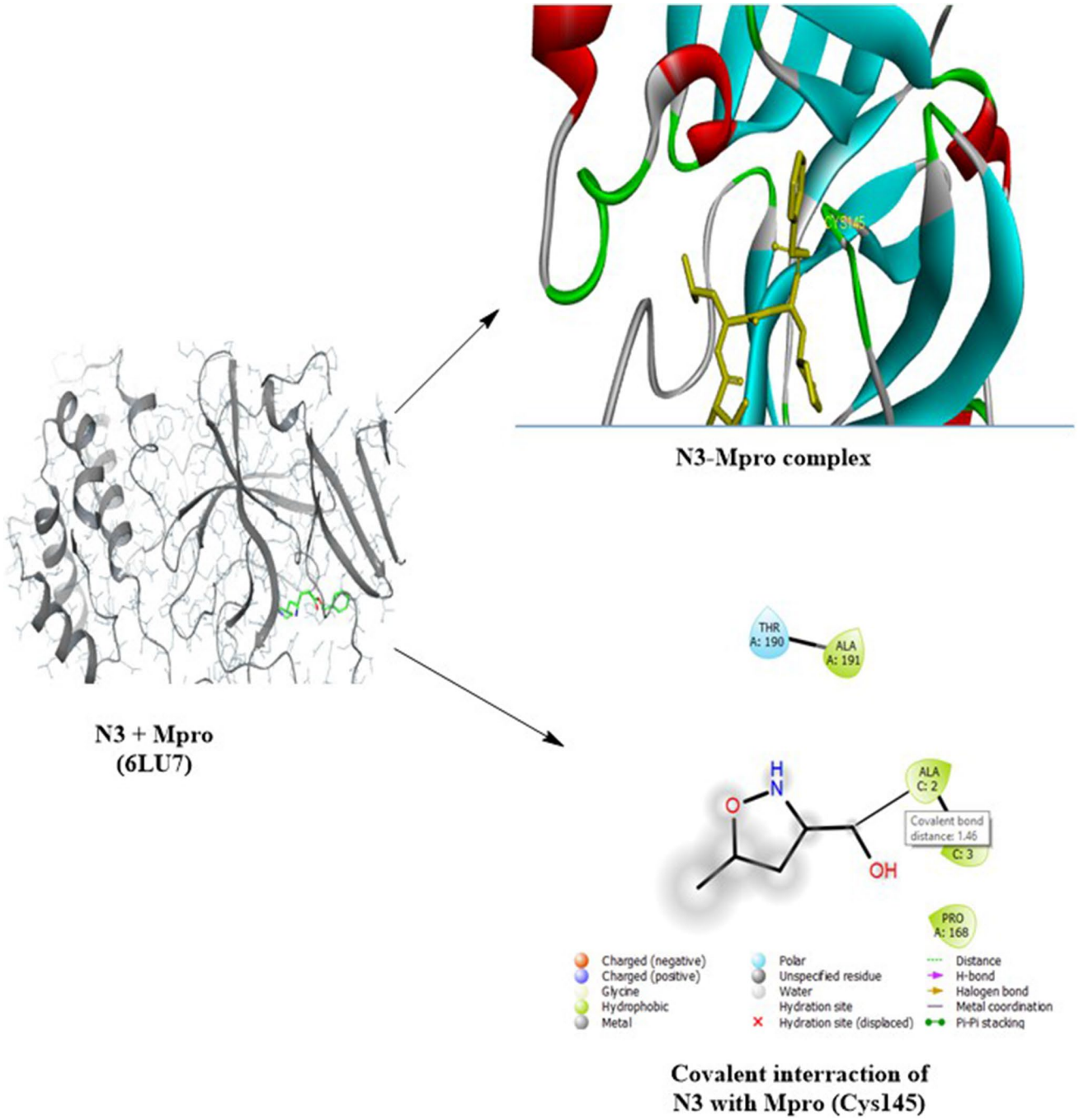


Fig. 6 Covalent binding of N3 with Cys145 residue of Mpro

docking research that considers N3 as a standard for validating their docking protocols prior to a virtual-screening campaign should make use of one of the two approaches to docking covalent systems. Unfortunately, this has not been the case for several retrospective computational studies where noncovalent interactions of N3 with the Mpro target (6LU7) have been reported [107–116].

There have also been studies that have noncovalently docked covalent entities whose X-ray crystallography structure with the targeted receptor has never been elucidated [117–124]. A typical example is the docking and drug repurposing of remdesivir (a clinically approved anti-Ebola and anti-COVID-19 drug) against SARS-CoV2 main protease (Mpro). The nucleotide analog prodrug, which has a broad spectrum of activity against viruses of different families, was also found to be potent against SARS-CoV2 and subsequently got wide approval for the emergency treatment of COVID-19 in 2020 [125]. At the molecular level, remdesivir was shown to inhibit the SARS-CoV2 RdRp enzyme (RNA-dependent RNA polymerase), while x-ray crystallographic experiment revealed a covalent mechanism of inhibition of the drug with the protein target [126]. On the other hand, no structural basis for inhibition of remdesivir has been proposed with the SARS-CoV2 main protease till date. Hence, computational drug discovery studies that have focused on repurposing this drug against the nucleophilic cysteine-like protease without careful consideration of the nucleotide analog (remdesivir) as a covalent agent and consequently proposing only a noncovalent mechanism of inhibition (with the Cys145 crucial residue of Mpro) after their docking experiments may be irrational and erroneous.

In another study with different covalent targets (other than Mpro), Kaliamurthi et al. aimed to repurpose known BTK covalent inhibitors such as ibrutinib and zanubritinib against SARS-CoV2 [127]. However, the group failed to consider the potential covalent inhibitory strategy of the warhead group (α,β-Unsaturated carbonyl) of these covalent agents in their computational study as this was pivotal in their potency against the Cys481 residue of the BTK receptor (PDB ID: 5P9J and 6J6M). Hence, they went ahead to noncovalently dock the compounds against SARS-COV2 proteins such as Mpro, which could be covalently explored. Surprisingly and disappointingly, the authors also noncovalently docked the inhibitors against their known therapeutic target (BTK) and yet failed to report the covalent and even noncovalent binding of these covalent agents with BTK's Cys481. Apparently, this study represents an irrational drug discovery approach involving covalent targets and could be classified as erroneous.

Finally, Ezat and colleagues proposed analogs of boceprevir as potential inhibitors of both the wild-type HCV (Hepatitis-C virus) NS3 protease and 19-mutated HCV NS3 proteases using molecular docking screening [128]. It is important to mention that boceprevir is a clinically approved drug against the HCV NS3 target and its α-ketoamide warhead group is known to form a covalent bond with Ser139 of the protease (PDB ID: 3LOX). In an attempt to compare the potency of boceprevir's modified compounds, the authors noncovalently docked the clinically approved boceprevir to the binding pocket of HCV NS3 protease. The group of researchers' docking experiments also failed to capture the binding interaction (covalent and noncovalent) of boceprevir (including its analogs) with Ser139 reactive residue of HCV protein, thus, representing another irrational and erroneous drug discovery practice involving covalent targets.

Conclusion/future perspectives

Improved potency is one of the major advantages that covalent inhibitors have over reversible inhibitors. This property is presumed to have clinical application in terms of the reduction of the drug dosage. Although there are still reservations as to the usage of this class of drug but the recent trend in the field such as the approval of sotorasib for KRAS G12C is changing the paradigm and writing a good story in favor of this drug class. The search for more covalent agents for the treatment of diseases is a raging pursuit and the use of computational methodologies like molecular docking is promising in that regard. With the increasing report of covalent docking software and tools, it is believed that the percentage of true positives would increase from virtual-screening studies if these tools are utilized when docking covalent targets. However, deficiency in scoring function is a major concern with these softwares after high computing time. While the latter is a general problem to all docking software class, software developers are already in the pursuit of reporting scoring function that would perfectly model covalent systems, the former could be addressed by introducing covalent docking cloud-computing facilities which will be of great help to scientists in the developing part of the world. It is important to state that although covalent docking could be achieved using conventional-docking programs by taking cognizance of the distance between the ligand electrophilic warhead and the target nucleophilic center. It is, however, logical to dock a covalent target using the covalent-bond-recognizing tools. With the growing amount of irrational and erroneous computational studies on targeting covalent receptors, we hope our comprehensive review do not only help reduce the rate of false-positive results but also aid the identification of ideal covalent-docking programs for virtual drug discovery campaigns.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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