Covalent and Non-Covalent Binding Free Energy Calculations for Peptidomimetic Inhibitors of SARS-CoV-2 Main Protease

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ABSTRACT: COVID-19, the disease caused by the newly discovered coronavirus — SARS-CoV-2, has created global health, social, and economic crisis. At the time of writing (November 12, 2020), there are over 50 million confirmed cases and more than 1 million reported deaths due to COVID-19. Currently, there are no approved vaccines, and recently Veklury (remdesivir) was approved for the treatment of COVID-19 requiring hospitalization. The main protease (M^{pro}) of the virus is an attractive target for the development of effective antiviral therapeutics because it is required for proteolytic cleavage of viral polyproteins. Furthermore, the M^{pro} has no human homologues, so drugs designed to bind to this target directly have less risk for off-target reactivity. Recently, several high-resolution crystallographic structures of the M^{pro} in complex with inhibitors have been determined — to guide drug development and to spur efforts in structure-based drug design. One of the primary objectives of modern structure-based drug design is the accurate prediction of receptor-ligand binding affinities for rational drug design and discovery. Here, we perform rigorous alchemical absolute binding free energy calculations and QM/MM calculations to give insight into the total binding energy of two recently crystallized inhibitors of SARS-CoV-2 M^{pro} , namely, N3 and α -ketoamide 13b. The total binding energy consists of both covalent and non-covalent binding components since both compounds are covalent inhibitors of the M^{pro} . Our results indicate that the covalent and non-covalent binding free energy contributions of both inhibitors to the M^{pro} target differ significantly. The N3 inhibitor has more favourable non-covalent interactions, particularly hydrogen bonding, in the binding site of the M^{pro} than the α -ketoamide inhibitor. But the Gibbs energy of reaction for the M^{pro} - α -ketoamide covalent adduct is greater than the Gibbs reaction energy for the M^{pro} -N3 covalent adduct. These differences in the covalent and non-covalent binding free energy contributions for both inhibitors could be a plausible explanation for their in vitro differences in antiviral activity. Our findings highlight the importance of both covalent and non-covalent binding free energy contributions to the absolute binding affinity of a covalent inhibitor towards its target.

Introduction

During the past two decades, a number of coronaviruses have emerged and caused outbreaks of global health concern.¹ These include severe acute respiratory syndrome (SARS-CoV) in 2003 and Middle East respiratory syndrome (MERS-CoV) in 2012. In December 2019, a novel coronavirus (SARS-CoV-2) was identified in Wuhan, Hubei province in China.^{2,3} SARS-CoV-2 is responsible for the coronavirus disease of 2019 (COVID-19). These viruses mainly attack the lower respiratory system of humans and cause major symptoms such as fever, cough, sore throat, and pneumonia.^{4,5} Among respiratory viral infections, COVID-19 has the highest infection rate and spread. As of November 12, 2020, the World Health Organization (WHO) has confirmed over 50 million cases and more than 1 million deaths due to COVID-19 in 220 countries, areas or territories across the globe.⁶

SARS-CoV-2 has several key proteins such as the main protease (M^{pro}); also known as 3CL $^{\text{pro}}$, papain-like protease (PL $^{\text{pro}}$), RNA-dependent RNA polymerase (RdRp), and spike glycoprotein. Both the M^{pro} and the PL $^{\text{pro}}$ function in the cleavage of the viral polyprotein into individual functional units.^{7,8} The RdRp is responsible for replicating viral genome.⁹ The spike glycoprotein contains a receptor-binding domain that interacts with the peptidase domain of the human angiotensin-converting enzyme 2 (h-ACE2), therefore,

mediates the fusion of the viral cell.¹⁰ It is well known that the M^{pro} is essential for most of the cleavage processes during viral polyprotein processing.^{11–14} It is critical for the processing of 12 non-structural proteins (NSP4–NSP16) including RNA replicase (NSP9), RdRp (NSP12), and helicase (NSP13).^{15–17} Moreover, the M^{pro} is considered as an attractive drug target because no human proteases with analogous cleavage processes are known. Thus, designing potent inhibitors of the M^{pro} is a safe and effective approach to inhibiting the viral activity of SARS-CoV-2, with reduced risk of off-target adverse drug reactions.

Much effort has been spent on developing effective therapeutics in the fight against COVID-19. As of November 12, 2020, there are 48 candidate vaccines in clinical evaluation (phase I, II, and III) and about 164 candidate vaccines in preclinical evaluation as reported by the WHO.¹⁸ Further, a plethora of drugs are being investigated for repurposing in the treatment of the disease.¹⁹ These drugs are also the subject of several clinical trials. Despite these efforts, to date, there are no approved vaccines for the treatment of COVID-19. For this reason, the pursuit of safe and effective therapeutics against the emerging SARS-CoV-2 virus is critically important and urgent in the global fight against COVID-19.

In silico approaches play a crucial role in accelerating the research and development processes of new potential pharmaceutical drugs.²⁰

The M^{pro} structure-based drug design approach^{21,22} is one of the promising strategies for developing effective broad-spectrum antivirals against coronaviruses because inhibiting M^{pro} blocks a key piece of the cell-replication machinery of the virus. Molecular docking, structure-activity relationship studies, and molecular dynamics (MD) simulations have been implemented for the discovery of potential inhibitors of the viral M^{pro}. For instance, computational studies have explored drug repurposing or repositioning of a wide array of chemical compounds, including FDA approved drugs,^{16,23} antiviral compounds,^{24–27} protease inhibitors,²⁸ antiviral phytochemicals,²⁹ and natural products from medicinal plants,^{28,30,31} — with the sole aim of disrupting the viral function of the M^{pro}.

Recently, a number of high-resolution inhibitor-bound structures of the M^{pro} have been determined.^{32–35} The availability of these structures have enabled structure-based modeling approaches in an effort to understand key inhibitor–receptor interactions, conformational dynamics, and structural stability of the binding domain of the M^{pro}.^{36–44} A principal goal of computational drug design is the ability to accurately and reliably predict the binding mode, selectivity, and affinity of a drug candidate towards a target. Alchemical absolute binding free energy (ABFE) calculations are suitable in this regard and provide a theoretically rigorous way of computing ligand binding energies of disease targets.⁴⁵ ABFE calculations have been shown to provide accurate estimates of the binding affinity and selectivity of drug-like molecules to therapeutically relevant enzyme targets.^{46–51} We employ this approach to compute the ligand binding energies of two recently crystallized peptidomimetic inhibitors of SARS-CoV-2 M^{pro}, namely, N3³³ and α -ketoamide 13b³⁴ (**Figure 1**). Both inhibitors are targeted covalent inhibitors of SARS-CoV-2 M^{pro}. They initially bind to the M^{pro} by forming a non-covalent protein–ligand complex, after which a covalent bond is formed between the electrophilic reactive group of the inhibitor (commonly referred to as “warhead”) and cysteine-145 (“Cys145”) of the M^{pro}. Given that these inhibitors are covalent modifiers, modeling their binding steps would require calculation of both the non-covalent and covalent binding energy contributions.^{51–53}

Figure 1. Structure of SARS-CoV-2 M^{pro} in complex with peptidomimetic inhibitors N3 (**a**), and α -ketoamide 13b (**b**). The chemical structures of the inhibitors are highlighted. Crystal structures of the M^{pro} in complex with N3 and α -ketoamide 13b inhibitors were derived from PDB accession codes 6LU7 and 6Y2G, respectively.

Here, we perform alchemical ABFE calculations to predict the binding free energy of N3 and α -ketoamide 13b (“ α -ketoamide”) to SARS-CoV-2 M^{pro}. This approach computes the absolute non-covalent binding energy of the inhibitors to the M^{pro} target and includes protein dynamics, conformational and orientational entropy of the inhibitors, in addition to non-covalent binding interaction energies. The covalent binding energy contribution of the chemical reaction was estimated using quantum mechanics/molecular mechanics (QM/MM) calculations within the ONIOM⁵⁴ framework. We also computed the conformational Gibbs energy of the inhibitors upon binding to the M^{pro} using umbrella sampling/replica-exchange molecular dynamics method. To determine the preferred protonation states of key active site residues in the binding pocket of the protein, we performed constant-pH molecular dynamics simulations on the model systems, prior to running the binding free energy calculations. This combined computational approach provides insight into understanding key molecular interactions and free energy contributions that drive the binding processes of the inhibitors to SARS-CoV-2 M^{pro}. The information from this work will guide lead optimization studies and help with ongoing drug design efforts towards the development of effective antivirals to combat COVID-19.

Theory and Methods

System Preparation: The initial structures of the peptide-like inhibitors (N3 and α -ketoamide) complexed with the SARS-CoV-2 M^{pro} were derived from PDB entries 6LU7³³ and 6Y2G,³⁴ respectively. Model systems of the protein–ligand complexes of these structures were prepared in explicit solvent. All crystallographically resolved water molecules were retained in the model system. The parameters for the protein and ligand model structures were obtained from the CHARMM36 protein force field⁵⁵ and the CHARMM General Force Field (CGENFF),⁵⁶ respectively. The TIP3P water model⁵⁷ was chosen to describe water molecules in a cubic box. Molecular dynamics simulations were performed on the model system using NAMD 2.13.⁵⁸ Simulations were performed under periodic boundary conditions at a constant temperature of 298.15 K and pressure of 1 atm using Langevin dynamics and Langevin piston method, respectively. A timestep of 2 fs and a Langevin damping coefficient of 1 ps was used for the MD simulations. The Particle Mesh Ewald (PME) method^{59,60} was used to describe long-range interactions and a cut-off distance of 12 Å was used for the Lennard-Jones interactions. Covalent bonds involving hydrogen atoms were constrained using the SHAKE algorithm.⁶¹ Energy minimization calculations were performed on the protein–ligand model complexes, after which a series of equilibration runs lasting 50 ns were performed on the model structures first with restraints on the α -carbon of the protein backbone, and then without restraints on the model system.

Constant-pH Molecular Dynamics Simulations: Following equilibration of the protein–ligand model complexes, constant-pH molecular dynamics simulations were performed to determine the protonation states of key amino acid residues, particularly, Cys145 and His41 which constitute the catalytic dyad of the SARS-CoV-2 M^{pro}. This was done in order to account for the variation in protonation states of titratable active site residues relevant for the absolute binding free energy calculations, as well as pH-induced conformational changes and inter-residue coupling effects within the model system. Recently, constant-pH molecular dynamics simulations was used to investigate the protonation states and proton-coupled conformational dynamics of catalytic residues in coronavirus PL^{pro}s.⁶²

The constant-pH MD simulations were performed using the hybrid non-equilibrium molecular dynamics/Monte Carlo method⁶³ implemented in NAMD 2.13.⁵⁸ Simulations were performed in explicit solvent for the monomeric apo form (i.e., no inhibitor bound, PDB ID: 6M03) and holo forms (i.e., inhibitor bound, PDB IDs: 6LU7 and 6Y2G) of the coronavirus M^{pro} model structures. The CHARMM36 protein force field was used for generating structural models for the apo and holo forms of the model system. Our previous studies have shown that for titratable residue pK_a calculations, particularly for cysteines, the CHARMM36 protein force field gives accurate cysteine pK_a's^{64,65} and correctly represents the hydration structure of model thiolates.⁶⁶ All ionizable residue side chains in the model system were allowed to titrate. The equilibrated model system structures were used as the starting point for the constant-pH MD simulations. The constant-pH simulations were initially minimized for 200 steps within the constant-pH molecular dynamics framework, and this was followed by a 100 ns run for each pH window within intervals of 1.0–14.0. All simulations attempted protonation state moves of 10 ps, with switching times of 15 ps. The titration curve was plotted from the simulation output by fitting the deprotonated fraction (f_{deprot}) and pH for each replica window to the Hill equation, Eqn. (1), where n is the Hill coefficient.

$$f_{deprot} = \frac{1}{1 + 10^{n(pH - pK_a)}} \quad (1)$$

Absolute Binding Free Energy Calculation: The absolute binding free energy of N3 and α -ketoamide inhibitors were calculated using alchemical free energy calculations following the protocol reported by Aldeghi *et al.*⁴⁹ This approach follows a nonphysical thermodynamic cycle depicted in **Figure 2** to determine the binding free energy of a ligand to a protein target.

Figure 2. Scheme depicting the alchemical thermodynamic cycle used for computing the absolute binding free energies of the peptidomimetic inhibitors to SARS-CoV-2 M^{pro}, following the protocol of Aldeghi *et al.*⁴⁹ $\Delta G^{solv}_{elec+vdw}$ represent the electrostatic and van der Waals interaction

energy following decoupling of the fully interacting ligand in solution (**A**) into a non-interacting ligand (**B**). ΔG^{solv}_{restr} represents the free energy cost of imposing restraints on the non-interacting ligand in solution (**C**); state **C** (non-interacting ligand in solution) and state **D** (non-interacting ligand in protein) are equivalent to each other. $\Delta G^{prot}_{elec+vdw}$ represent the electrostatic and van der Waals interaction energy of the fully interacting but restrained ligand in the protein (**E**). ΔG^{prot}_{restr} represent the free energy cost associated with removal of restraints on the ligand to yield a fully interacting ligand in the protein binding site (**F**).

The computed binding free energy, which corresponds to the absolute non-covalent binding energy of the ligand to the target, is computed by following a series of alchemical transformations that characterize the binding event, Figure 2. The resulting ligand binding energy is simply derived from the sum of the multiple steps where the ligand is desolvated from solution and then introduced into the binding site of the target protein. The calculations are performed for the ligand in bulk solution (i.e., unbound state) and in the protein binding site (i.e., bound state), and correspond to the forces and intermolecular interactions of the ligand accompanying the binding process.

The simulations were performed using the GROMACS molecular dynamics software package (version 2019.4).⁶⁷ The AMBER99SB-ILDNP⁶⁸ and GAFF⁶⁹ force fields were used to generate parameters for the protein and ligands, respectively. For the ABFE calculations, two sets of simulations were performed; where the ligand was decoupled from bulk solution (i.e., unbound state) and from the complex (i.e., bound state). The van der Waals and coulombic interactions of the ligand in both bulk and complex environments were slowly decoupled during the simulation via a linear alchemical pathway with $\Delta\lambda = 0.05$ and $\Delta\lambda = 0.1$, respectively. A total of 31 λ windows were employed for decoupling the ligand in bulk solution and 42 λ windows were employed for the complex simulation (see Supporting Information). For each λ window, energy minimization, NVT and NPT equilibrations, and production runs were performed. The energy minimization was carried out using the steepest descent algorithm for a maximum of 100,000 steps. This was followed by a 1 ns equilibration under canonical ensemble conditions (NVT) with harmonic position restraints applied to ligand heavy atoms with a force constant of 1000 kcal mol⁻¹ nm⁻². Simulation temperature was coupled using Langevin dynamics⁷⁰ with reference temperature set to 298.15 K. Following the NVT equilibration runs, a 1 ns position restrained simulation under isothermal-isobaric (NPT) ensemble conditions was performed using the Berendsen algorithm.⁷¹ Subsequently, a 12 ns unrestrained production run was performed using Hamiltonian replica exchange (HREX) for each λ window under NPT conditions using the Parrinello–Rahman pressure coupling scheme.⁷² For the HREX simulations, 1 million global replica exchange swaps were attempted every 1000 time steps. The average acceptance ratio between replicas ranged from 0.2 to 0.3. The simulations were performed in triplicate for both the ligand in bulk solution and in the protein, yielding an aggregate total of ≈ 1.1 μ s and ≈ 1.5 μ s simulation time for the bulk and protein simulations, respectively. The results were analyzed using the alchemical_analysis.py python tool.⁷³ The reported free energies are the averages of the triplicate runs, and were calculated using multistate Bennet acceptance ratio (MBAR),⁷⁴ after discarding the first 2 ns of each window as equilibration. The timestep used for all simulations was 2 fs and the PME algorithm was used to treat long-range electrostatic

interactions with a cut-off of 12 Å. The LINCS constraint algorithm was used to constrain H-bonds.

The relative position and orientation of the bound ligand in the complex was described using six internal coordinates; one distance, two angles, and three dihedrals (see Supporting Information). These restraints help to keep the ligand in its bound pose in the binding pocket of the enzyme during the decoupling steps of the free energy calculations. The force constant used for the distance restraint was 1000 kcal mol⁻¹ nm⁻², and a force constant of 10 kcal mol⁻¹ rad⁻² was used for both angle and dihedral harmonic potentials. For the simulations involving the decoupling of the ligand from bulk solution, the restraint energy term ($\Delta G_{restr.}^{solv}$) is computed analytically using the method proposed by Boresch *et al.*⁷⁵ The decoupling simulations of the ligand in complex with the protein ($\Delta G_{elec+vdw+restr.}^{prot}$) on the other hand, is evaluated numerically from the free energy calculations. The resulting absolute binding free energy of ligand to enzyme target, given by the expression in Eqn. (2), simply represents the interaction energy following the dissociation of the ligand from bulk solution and its association in the binding site of the protein.

$$\Delta G_{binding}^o = \Delta G_{elec+vdw+restr.}^{prot} + \Delta G_{elec+vdw}^{solv} + \Delta G_{restr.on}^{solv} \quad (2)$$

Calculation of Ligand Conformational Degrees of Freedom:

Upon ligand binding to target protein, there is a free energy cost associated with the loss of conformational degrees of freedom when the ligand adopts a particular conformation in the binding pocket of the target protein. The conformational free energy term accompanying ligand binding to the target site (i.e., ΔG_{conf}) was computed using umbrella sampling/replica-exchange molecular dynamics simulations. The simulations were performed for the ligand in bulk solution ($\Delta G_{conf,solv}$) and in the protein binding site ($\Delta G_{conf,site}$), and consisted of 12 replicas centered on RMSD offsets increasing from 0.0–5.5 in increments of 0.50 Å. The force constant for the RMSD restraints was 50 kcal mol⁻¹ Å⁻². The simulation length for each replica window was 20 ns, resulting in an aggregate simulation time of 240 ns for a single run. For each system, three independent runs were performed, yielding a cumulative total of 720 ns simulation time. Weighted histogram analysis method⁷⁶ (WHAM) was used to compute the potential of mean force (PMF) as a function of the root-mean square deviation (RMSD) relative to a reference bound state conformation of the ligand. The average conformation of the bound-state ligand from the equilibration run was used as the reference structure.

ONIOM(QM:MM) Methodology: The QM/MM calculations performed in our study employed the ONIOM⁵⁴ approach as implemented in Gaussian 16.⁷⁷ The final coordinates of the binding free energy calculations were used to generate the ONIOM (QM:MM) models. In these models, explicit solvent water molecules and ions were removed. The system was partitioned into two layers: (1) High-level QM region, which consists of the thiol side chain of Cys145 of the M^{pro} with a molecular scaffold of the inhibitor that includes the electrophilic warhead, **Figure 3**; and (2) Low-level molecular mechanical region, which includes all the remaining residues of the M^{pro}. The high-level QM region was treated using the M06-2X functional with the def2-TZVP basis set. The M06-2X functional has been shown to perform very well for thermochemistry of main group

elements⁷⁸ and yields accurate reaction energies for covalent modification of thiols.⁵³ The Amber molecular mechanics force field was used to treat the low-level MM region, with parameters and charges the same as those used for the absolute binding free energy calculations. The chemical bonds at the QM/MM boundary interface were capped with hydrogen link atoms.

Figure 3. High-level QM region defined in our ONIOM (QM:MM) model to estimate the covalent bonding reaction energy of the inhibitors following chemical addition to Cys145 of the M^{pro}.

For the M^{pro}-N3 ONIOM(QM:MM) model, the high-level layer consisted of 47 atoms and the low-level layer consisted of 4732 atoms. Similarly, for the M^{pro}- α -ketoamide ONIOM(QM:MM) model, the high-level layer consisted of 46 atoms and the low-level layer consisted of 4667 atoms. All chemical structures of the model systems were optimized using the ONIOM (M06-2X/def2-TZVP:AMBER) method within a mechanical embedding formalism. The optimized structures on the free energy surfaces (FESs) were validated by analysis of the corresponding Hessian matrices. The covalent and non-covalent M^{pro}-inhibitor complexes associated with the transition states were analyzed using intrinsic reaction coordinate calculations.^{79,80} It should be noted that the transition states were based on the bimolecular concerted reaction mechanism between Cys145 of the M^{pro} and the electrophilic warhead of the inhibitors. Single point energy calculations were also performed on the optimized structures using ONIOM (M06-2X/def2-TZVP:AMBER) method within an electrostatic embedding formalism.

Results and Discussion

The binding free energies of two recently crystallized peptidomimetic inhibitors (i.e., N3 and α -ketoamide) of SARS-CoV-2 M^{pro} were computed using rigorous alchemical absolute binding free energy calculations and hybrid QM/MM methods. Given that these inhibitors are covalent modifiers of the M^{pro} target, their binding modes consists of both covalent and non-covalent binding steps. As a result, the total binding free energy of the inhibitors involved the calculation of both covalent and non-covalent binding free energies. The non-covalent binding free energy of the inhibitors to the M^{pro} target was calculated using alchemical ABFE calculations, while the covalent binding free energy was estimated using the ONIOM QM/MM method.

pK_a Calculations of His41 and Cys145 residues of the M^{pro}

Prior to running the binding free energy calculations, we performed constant-pH MD simulations on the model systems to determine the preferred protonation states of key binding site residues in the target M^{pro}, particularly the catalytic His41/Cys145 dyad. Constant-pH MD allows multiple protonation states to be modeled simultaneously. Given that changes in the protonation states of amino acid residues can induce protein conformational dynamics, we performed constant-pH MD simulations to explore variations in residue protonation states and the dynamics of ionization equilibria in the model systems. The constant-pH MD simulations were performed for both apo and holo forms of the M^{pro} model structures. Figures 4 and 5 depict the percentage of time that Cys145 and His41 are deprotonated as a function of pH in the apo and holo forms of the M^{pro}. For apo form of the M^{pro} (PDB ID: 6M03), the pK_a's of His41 and Cys145 were predicted to be 5.8 and 9.4, respectively (Figure 4). This suggests that in the ligand-free form of the M^{pro}, the preferred protonation state of both Cys145 and His41 is uncharged; where the thiol group of Cys145 is protonated and the imidazole functional group of His41 is neutral. Similarly, the pK_a's of His41 and Cys145 in both N3 and α -ketoamide bound complexes of the M^{pro} also suggest that both residues are in their neutral form (Figure 5). This result is in agreement with recent multiscale simulation studies on the proteolysis mechanism of SARS-CoV-2 M^{pro}.^{81,82} Additionally, experimental studies performed on the catalytic mechanism of SARS-CoV M^{pro} concluded that Cys145 and His41 are in their neutral form.⁸³ Both SARS-CoV and SARS-CoV-2 share very high structural and sequence identity (96 %) for their M^{pro}'s.⁸⁴ Therefore, in our binding free energy simulations both Cys145 and His41 were treated as neutral uncharged residues. In addition, the tautomeric states of His41–His164 pair for the α -ketoamide and N3-bound M^{pro} structures were chosen based on molecular dynamics simulation results reported by Gumbart and coworkers⁴¹ on the structural stability of the M^{pro} as a function of protonation state assignment.

Figure 4. Titration curves of His41 and Cys145 in the apo form of SARS-CoV-2 M^{pro} (PDB ID: 6M03). The predicted pK_a's of His41 and Cys145 of the M^{pro} are 5.8 and 9.4, respectively.

Figure 5. Titration curves of His41 and Cys145 in the holo form of SARS-CoV-2 M^{pro}. (a) For the N3 bound complex of the M^{pro} (PDB ID: 6LU7), the predicted pK_a's of His41 and Cys145 are 5.3 and 9.7, respectively. (b) For the α -ketoamide bound complex of the M^{pro} (PDB ID: 6Y2G), the predicted pK_a's of His41 and Cys145 are 5.5 and 10.0, respectively.

Non-covalent Binding Free Energy Contribution

Using the nonphysical thermodynamic cycle depicted in Figure 2, the binding free energies of N3 and α -ketoamide to SARS-CoV-2 M^{pro} were predicted to be -6.3 ± 0.6 kcal mol⁻¹ and -2.5 ± 0.2 kcal mol⁻¹, respectively. These values represent the non-covalent interaction energies between the inhibitors and the M^{pro} and provide a good estimate of their binding affinities. In particular, the binding free energy results of N3 to SARS-CoV-2 M^{pro} is in good agreement with experimental binding affinity measurements of N3 binding to SARS-CoV M^{pro}. The experimental inhibitory constant (K_i) of N3 to SARS-CoV M^{pro} is $9.0 \mu\text{M}$,⁸⁵ corresponding to a $\Delta G^\circ_{\text{exptl}}$ value of -6.9 kcal mol⁻¹. Both SARS-CoV and SARS-CoV-2 M^{pro}'s are highly similar—sharing ~96% sequence identity—so we can expect that the

binding affinity of N3 is similar for both targets. This makes us confident in our predicted binding free energy results.

The computed binding energy suggests that N3 inhibitor enjoys slightly more favourable non-covalent interactions in the binding site of the M^{pro} than the α -ketoamide inhibitor. Analysis of the ligand interaction diagram of the equilibrated protein–ligand complexes of both inhibitors with the M^{pro} (**Figure 6**) indicates that N3 inhibitor indeed forms more hydrogen bonding interactions with key amino acid residues in the binding site of the M^{pro} than the α -ketoamide inhibitor. **Figure 6(a)** shows that N3 interacts with residues Thr190, Gln189, Glu166, His163, His164, and Gly143 of the M^{pro} through hydrogen bonding. The α -ketoamide inhibitor on the other hand, interacts through hydrogen bonding with residues Glu166, Cys145, His41, and His164 of the M^{pro} , **Figure 6(b)**. Both inhibitors form hydrogen bonding interactions with residues Glu166 and His164. This result indicates that hydrogen bonding interactions play a significant role in the non-covalent binding interaction energy of N3 and α -ketoamide inhibitors to the M^{pro} . In addition, the relatively large size of N3 in comparison with the α -ketoamide inhibitor could also contribute to enhancing the non-covalent binding interactions since this could lead to more dispersive interactions in the binding site of the target — favouring ligand binding.

Figure 6. 2D Ligand interaction map showing active site residues of the M^{pro} that engage in hydrogen bonding interactions with N3 (a) and α -ketoamide (b) inhibitors. Only residues that are within 2.5 Å of the inhibitors are shown. N3 and α -ketoamide inhibitors in complex with the M^{pro} were derived from PDB accession codes 6LU7 and 6Y2G, respectively.

Covalent Binding Free Energy Contribution

Both N3 and α -ketoamide are covalent inhibitors of the M^{pro} ,^{33,34} as such their total binding energy consists of both non-covalent and covalent binding energy terms. The covalent binding energy results from the covalent adduct formation between Cys145 of the M^{pro} and the electrophilic warhead of the inhibitors (**Scheme 1**). To calculate the binding free energy associated with the covalent bond formation, we performed ONIOM QM/MM calculations to estimate the relative Gibbs energy difference between the non-covalent inhibitor-bound complex and the final covalent adduct.

Scheme 1: Reaction scheme depicting covalent bond formation between Cys145 residue of the M^{pro} with the electrophilic warheads of N3 (a), and α -ketoamide (b). Only sections of the protein and ligand included in the QM region of our QM/MM model are shown.

From our ONIOM (QM:MM) calculations, the free energy difference between the non-covalent complex and the covalently-bound M^{pro} –N3 adduct is $-3.5 \text{ kcal mol}^{-1}$. For the α -ketoamide inhibitor complexes, the computed free energy difference between the non-covalent and covalent adduct is $-7.9 \text{ kcal mol}^{-1}$. This result indicates that the free energy leading to the formation of the inhibitor-bound covalent complexes represents an exergonic process. In addition, the results suggest that the covalent binding energy of the α -ketoamide inhibitor to the M^{pro} is more than two times the covalent energy contribution of N3 to the M^{pro} . Overall, our binding free energy results indicate that α -ketoamide is a more potent inhibitor of the M^{pro} than N3, based on the total binding free energy (**Table 1**). This result is corroborated by experimental findings which report that α -ketoamide inhibitor displays slightly better in vitro antiviral activity than N3 in SARS-CoV-2 cell-based assays ($4\text{--}5 \text{ }\mu\text{M}$ for α -ketoamide vs. $\sim 16.8 \text{ }\mu\text{M}$ for N3).^{33,34} This indicates that both covalent and non-covalent binding free energy terms are important in the total binding affinity of a covalent inhibitor towards its target.

Table 1. Summary of Binding Free Energy Results for the M^{pro} Inhibitors Studied in this Work.

Inhibitor	$\Delta G_{non-covalent}$ (kcal mol ⁻¹)	$\Delta G_{covalent}$ (kcal mol ⁻¹)	ΔG_{total} (kcal mol ⁻¹)
N3	-6.3 ± 0.6	-3.5	-9.8 ± 0.6
α -ketoamide	-2.5 ± 0.2	-7.9	-10.4 ± 0.2

Conformational Energies. The conformational degrees of freedom accompanying protein–ligand binding is a significant component of the binding free energy. Typically, upon ligand binding to a target protein, there is a free energy cost associated with the loss of conformational freedom as a result of the ligand taking up a specific conformation in the binding site of the protein. This conformational free energy cost can be evaluated by calculating the PMF (in bulk solvent and in the protein binding site) as a function of the RMSD of the ligand relative to a reference bound state conformation.⁸⁶ **Figure 7** shows the PMF of the inhibitors in bulk solution and in the protein binding site of the M^{pro}. From this figure, the PMF of the inhibitors in bulk solution is generally broader than in the binding pocket of the protein. This suggests that a range of stable conformational states are accessible to the inhibitors in bulk solution than in the protein binding site. It is also evident from this figure that there is a significant difference between the low energy conformational states of the inhibitor in the protein-binding site and in bulk solution, **Figure 8**.

Figure 7. Potential of mean force (PMF) for the peptidomimetic inhibitors N3 (**a**), and α -ketoamide (**b**) as a function of the root-mean-square deviation (RMSD) relative to an average reference bound state conformation in bulk solution and in the M^{pro} binding site.

Figure 8. Low energy conformational states of the inhibitors in bulk water and in the M^{pro} binding site. The red and blue structures represent the lowest free energy conformational states adopted by the inhibitors in bulk solution and in the protein binding site, respectively.

The conformational free energy ($\Delta\Delta G_{conf}$) of the N3 and α -ketoamide inhibitors upon binding to the M^{pro} is predicted to be 5.4 ± 0.6 kcal mol⁻¹ and 2.9 ± 0.6 kcal mol⁻¹, respectively (**Table 2**). This difference in conformational energies can be attributed to the relative size and flexibility of the inhibitors. Between both inhibitors, N3 has the larger size and molecular weight. Large and flexible ligands tend to have higher conformational energies upon binding than small and rigid ligands.⁸⁷ The free energy cost arising from the conformational free energies suggest that there is a greater penalty for N3 than for α -ketoamide binding to the M^{pro}. In general, these observations provide insights into the strain energy experienced by the peptidomimetic inhibitors in the binding pocket of the M^{pro}.

Table 2. Conformational Energies of Peptidomimetic Inhibitors upon Binding to the SARS-CoV-2 M^{pro}.

Inhibitor	$\Delta\Delta G_{conf}$ (kcal mol ⁻¹)
N3	5.4 ± 0.6
α -ketoamide	2.9 ± 0.6

Conclusion

In this work, we performed rigorous alchemical ABFE calculations and ONIOM QM/MM calculations to compute the total binding energy of two recently crystallized peptidomimetic inhibitors (N3 and α -ketoamide) of SARS-CoV-2 M^{pro}. The total binding energy consists of both covalent and non-covalent binding energy terms, since these compounds are covalent inhibitors of the M^{pro} target. Our results corroborate experimental findings that both N3 and α -ketoamide are potent inhibitors of SARS-CoV-2 M^{pro}.^{33,34} We find that the covalent and non-covalent binding free energies of both inhibitors to the M^{pro} differ significantly. N3 enjoys more favourable non-covalent interactions than α -ketoamide in the binding site of the M^{pro}. Hydrogen bonding interactions and dispersion forces are significant to the non-covalent binding energy contributions of the inhibitors to the M^{pro} target. The covalent binding free energy of the M^{pro}– α -ketoamide covalent adduct is greater than the energy

contribution resulting from the formation of the M^{pro} -N3 covalent adduct. This greatly favours the total binding free energy of the α -ketoamide inhibitor to the M^{pro} . The differences in the covalent and non-covalent binding energies of the inhibitors could be a plausible explanation for their slight differences in antiviral inhibition activity. We also computed the conformational Gibbs energy of the inhibitors upon binding to the M^{pro} and found that N3 experiences a greater free energy penalty upon binding to M^{pro} than the α -ketoamide inhibitor. Our constant-pH molecular dynamics simulations of the M^{pro} indicate that the His41–Cys145 catalytic dyad of the M^{pro} are uncharged, supporting recent multiscale MD studies.^{81,82} Overall, our results provide insight into the various free energy contributions that drive the binding processes of two potent covalent inhibitors of SARS-CoV-2 M^{pro} . This information could prove useful in the rational design and assessment of new inhibitors as potential drug leads against the emerging coronavirus disease and future infectious disease outbreaks.

ASSOCIATED CONTENT

Supporting Information. Details of molecular dynamics simulations and parameters, constant-pH MD simulations and parameters, absolute binding free energy simulations and breakdown of binding free energy components. The molecular topology and input files for the binding free energy calculations are also provided.

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Author Contributions

E.A-W was the primary contributor to writing the manuscript. A.A.A.-S was a secondary contributor to writing the manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

ABFE, absolute binding free energy; COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; M^{pro} , main protease; 3CL pro , 3C-like protease; PL pro , papain-like protease; MD, molecular dynamics; QM/MM, quantum mechanics/molecular mechanics; ONIOM, Our own N-layered Integrated molecular Orbital and molecular Mechanics; CpHMD, constant-pH molecular dynamics; PMF, potential of mean force; RMSD, root-mean square deviation.

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