Ibrutinib is the first covalent inhibitor of Bruton’s tyrosine kinase (BTK) to be used in the treatment of B-cell cancers. Understanding the mechanism of covalent inhibition is crucial for the design of safer and more selective covalent inhibitors that target BTK. There are questions surrounding the precise mechanism of covalent bond formation in BTK as there is no appropriate active site residue that can act as a base to deprotonate the cysteine thiol prior to covalent bond formation. To address this, we have investigated several mechanistic pathways of covalent modification of C481 in BTK by ibrutinib using QM/MM reaction simulations. The lowest energy pathway we identified involves a direct proton transfer from C481 to the acrylamide warhead in ibrutinib, followed by covalent bond formation to form an enol intermediate. There is a subsequent rate-limiting keto-enol tautomerisation step ($\Delta G^\ddagger = 10.5$ kcal mol$^{-1}$) to reach the inactivated BTK/ibrutinib complex. Our results represent the first mechanistic study of BTK inactivation by ibrutinib to consider multiple mechanistic pathways. These findings should aid in the design of covalent drugs that target BTK and related proteins.
Mechanism of Covalent Binding of Ibrutinib to Bruton’s Tyrosine Kinase revealed by QM/MM Calculations

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Abstract

Ibrutinib is the first covalent inhibitor of Bruton’s tyrosine kinase (BTK) to be used in the treatment of B-cell cancers. Understanding the mechanism of covalent inhibition is crucial for the design of safer and more selective covalent inhibitors that target BTK. There are questions surrounding the precise mechanism of covalent bond formation in BTK as there is no appropriate active site residue that can act as a base to deprotonate the cysteine thiol prior to covalent bond formation. To address this, we have investigated several mechanistic pathways of covalent modification of C481 in BTK by ibrutinib using QM/MM reaction simulations. The lowest energy pathway we identified involves a direct proton transfer from C481 to the acrylamide warhead in ibrutinib, followed by covalent bond formation to form an enol intermediate. There is a subsequent rate-limiting keto-enol tautomerisation step (ΔG‡=10.5 kcal mol⁻¹) to reach the inactivated BTK/ibrutinib complex. Our results represent the first mechanistic study of BTK inactivation by ibrutinib to consider multiple mechanistic pathways. These findings should aid in the design of covalent drugs that target BTK and related proteins.

Introduction

Covalent inhibitor drug discovery has re-emerged because of advantages compared with conventional non-covalent reversible binding that can include complete target blockage, increased selectivity and
duration of action.\textsuperscript{1–3} Recent years have seen the approval of several new marketed covalent drugs targeting protein kinases.\textsuperscript{4,5} In particular, inhibition of Bruton’s tyrosine kinase (BTK) is an attractive target for blood cancers and autoimmune diseases due to its function in signal transduction in the B-cell antigen receptor (BCR) pathway.\textsuperscript{6,7} BTK inhibitors have also been explored as treatments in the fight against SARS-CoV-2 coronavirus, the cause of a global pandemic that started in 2019.\textsuperscript{8} Ibrutinib and acalabrutinib are two BTK inhibitors that are approved for the treatment of B-cell cancers including mantle cell lymphoma (MCL) and chronic lymphocytic leukaemia (CLL).\textsuperscript{9} Both drugs contain electrophilic Michael acceptor warheads that covalently modify a cysteine residue (C481) in the kinase domain of BTK. Utilising warheads of this type to target poorly conserved cysteine residues is a common technique to developing covalent inhibitors in drug discovery.\textsuperscript{10} Despite the massive investments made to discover and develop these drugs, the detailed mechanism of covalent binding to BTK is unknown. Understanding the precise mechanism will help in the design of improved covalent inhibitors targeting BTK, and also other covalent drug targets. The ability to rationally tune covalent reactivity should lead to safer covalent drugs that have fewer side effects.\textsuperscript{11,12}

The addition of a Michael acceptor warhead such as an acrylamide group to a thiol side chain is typically modelled in 3 steps. First, deprotonation of the cysteine thiol occurs to form a thiolate anion, followed by nucleophilic attack of the thiolate on to the electrophile to form an enolate intermediate, and finally re-protonation of the enolate to form a covalent thiol-adduct.\textsuperscript{13} Sulfur reactivity in proteins has been studied previously using combined quantum mechanics/molecular mechanics (QM/MM) approaches. Effort has focused on cysteine proteases and protein kinases, given their roles in some disease processes. For example, covalent nitrile inhibitors of the cysteine protease rhodesain have been investigated using QM/MM reaction simulations at the semi-empirical PM6 level.\textsuperscript{14} The protocol was found to be a useful predictor of reversible covalent binding affinity, in good agreement with experimental data. The mechanism of covalent modification of C797 by an acrylamide warhead in the
protein kinase EGFR has been elucidated by QM/MM modelling at the self-consistent-charge density-functional-based tight-binding (SCC-DFTB) level. These results identified a neighbouring aspartate residue, D800 in the i+3 position relative to C797, that acts as a catalytic base to deprotonate the cysteine thiol.

When comparing kinases that contain cysteine at the same position as C481 in BTK, different amino acids are to be found at the i+3 position, with the majority being either aspartate (Asp) or asparagine (Asn). BTK contains an Asn residue, N484, rather than an Asp or equivalent proton acceptor in the i+3 position (Figure 1). It is understood that residues in the surrounding microenvironment can effectively modulate cysteine pKₐ, depending on their properties. Although there is no experimentally determined C481 pKₐ available, the pKₐ of a free cysteine thiol in solution is 8.6. A nearby more acidic i+3 Asp sidechain leads to an upshift in cysteine pKₐ, calculated for EGFR in a recent study to be 11.1. Due to its hydrogen bonding capability and neutral sidechain the i+3 Asn in BTK results in a slightly more acidic cysteine pKₐ of 10.4 in the same study. Thus, a pKₐ of around 10 would result in a 1:1000 ratio of ionised to neutral cysteine at pH 7. This makes C481 majority neutral at physiological pH and suggests a nearby proton acceptor is required for deprotonation of the thiol group either prior to or concurrent with the reaction with electrophiles. Although asparagine performs catalytic roles in enzymes, for example in GTPase activation and in some protein splicing reactions, the amide side chain of Asn is unlikely to accept a proton and perform a similar role to the Asp in EGFR.
Figure 1. Binding mode of ibrutinib in the active site of BTK, that represents a reactive conformation observed in classical MD simulations. The acrylamide warhead is positioned in close proximity to C481, with an asparagine in the i+3 position.

Ruling out a nearby amino acid acting as acid or base leaves several other possible mechanistic pathways for covalent inhibition of C481 in BTK by Michael acceptor warheads. These include pathways that proceed via direct addition between C481 and a Michael acceptor (Figure 2), such as a direct transfer of the thiol proton to the α-carbon of the acrylamide warhead to afford the covalently bound keto adduct in a single step. Alternatively, the thiol proton could transfer to the carbonyl oxygen atom of the acrylamide inhibitor to form an enol intermediate followed by a tautomerization step to form the covalently bound keto product. There is also the possibility that water could be involved in the reaction, assisting in the proton transfer between the cysteine thiol and acrylamide warhead. Here, we assess each of these pathways using a QM/MM umbrella sampling approach. The results identify the probable mechanism of covalent binding between the acrylamide warhead in ibrutinib and BTK.
Methods

A QM/MM umbrella sampling protocol was used to explore each mechanistic pathway in the kinase domain of BTK. The QM region includes all of the ibrutinib ligand and the side chain of C481. For the water mediated pathways, an adjacent crystallographic water molecule hydrogen bonded to the carbonyl oxygen of the acrylamide was also included. The umbrella sampling protocol consisted of generating a free energy surface (FES) using the density-functional tight-binding (DFTB3) method with 20ps of sampling per reaction coordinate (RC) window to get an approximate minimum energy pathway (MEP) for the reaction. This was followed by running an additional 10 ps of sampling along the minimum energy path at the same level of theory. Previous studies have compared using DFTB, PM3 and PM6 methods for modelling the reactivity of thiol compounds. Our extensive benchmarking showed that PM3/6/7 performed poorly for modelling thiol addition mechanisms, whereas DFTB3 gave geometries in good agreement with higher-level electron-correlation (MP2) and DFT methods. In all cases the umbrella sampling FESs showed good energy convergence with respect to simulation time and good overlap between neighboring simulation windows.

Results/Discussion

Our QM/MM umbrella sampling simulations suggest that the lowest energy pathway of C481 modification by ibrutinib proceeds by a direct proton transfer (PT) from the C481 thiol group to the carbonyl oxygen atom of ibrutinib, resulting in a Cys-S/C=OH+ ion pair (E-I1, Figure 2). This is followed by C-S bond formation to form a covalent enol complex (E-I2, Figure 2). Finally, a solvent assisted keto-enol tautomerization step forms the covalent keto product (E-P, Figure 2). This pathway was lower in energy than the three alternative pathways we investigated, including a solvent assisted PT resulting in enol formation, a direct 1,2-olefin addition pathway and a solvent assisted variant of the 1,2-olefin addition pathway.
Figure 2. Lowest energy pathway of BTK inhibition by covalent inhibitor ibrutinib from QM/MM umbrella sampling simulations at the DFTB3 level.

The free energy surface for the lowest energy pathway is flat in nature, with low free energy barriers of 3.1 kcal mol\(^{-1}\), 2.6 kcal mol\(^{-1}\), and 10.5 kcal mol\(^{-1}\) for the initial PT step, S-C formation and solvent assisted tautomerization steps respectively. Invoking a water molecule to assist in the initial proton transfer step is entropically unfavorable, resulting in a higher barrier than a direct PT (8.4 kcal mol\(^{-1}\) vs 3.1 kcal mol\(^{-1}\)). An equivalent solvent assisted PT and enol formation has been modelled for the modification of cysteine residues by microcystins, where a high barrier of 21.9 kcal mol\(^{-1}\) was calculated for the reaction pathway involving water.\(^{23}\) The 1,2-addition pathway, consisting of a direct PT from the thiol to the \(\alpha\)-carbon of the acrylamide warhead and simultaneous S-C formation has been reported as a high energy pathway by Rowley et al. who calculated the TS barrier of the 1,2-\(\sigma\) addition of methylvinyl ketone and methyl thiolate to be 65.2 kcal mol\(^{-1}\) at the CCSD(T)//\(\omega\)B97X-D level.\(^{24}\) QM/MM simulations of 1,2-addition in BTK suggested a very high barrier (approximately 47.7 kcal mol\(^{-1}\)), and we subsequently discounted it as a feasible mechanism for covalent C481 modification based on our simulations and findings from Rowley et al. We also investigated the solvent assisted variant of this pathway, but the free energy surface did not represent a plausible reaction pathway as
we observed the occurrence of additional PTs that indicated a strong preference for the reaction to proceed via an enol intermediate.

**Figure 3.** (A) Free energy profile of BTK inhibition by covalent inhibitor ibrutinib at the DFTB3/MM level. The lowest energy pathway consists of a direct proton transfer step, followed by S-C formation. (B) The final step is a rate limiting solvent assisted keto-enol tautomerization with a free energy barrier of 10.5 kcal mol\(^{-1}\).

The importance of the i+3 asparagine residue was revealed in our QM/MM umbrella sampling simulations. Although the N484 residue does not directly participate as a proton acceptor in the reaction (unlike the i+3 aspartate residue in EGFR), it has an essential role in providing stabilization to the TS structures and intermediates along the reaction pathway in BTK (Figure 4). This is particularly evident in the initial proton transfer step, where the asparagine interacts strongly with the developing negative charge on the C481 sulfur atom as the proton transfer occurs. Without the N484/C481-S\(^-\) interaction no stable intermediate was predicted for E-I1. Concerned that the protonated acrylamide group (E-I1, Figure 2) might be favored by the umbrella sampling restraints, 25ps of unrestrained QM/MM MD was run on the E-I1 structure at the DFTB3 level. Over the course of the
trajectory no proton transfers occurred, and the structure did not collapse indicating it is a stable intermediate. The stabilizing N484/C481-S⁻ interaction continues until TS2 is reached, at which point the N484/C481-S⁻ interaction breaks as the S-C bond begins to form between the thiol group and the inhibitor (Figure 3). The stabilization of the thiolate by Asn and water molecules helps to explain why the barriers for the PT step and S-C formation steps are so low. In EGFR kinase, a low reaction barrier of 8.6 kcal mol⁻¹ was predicted for the S-C formation step between C797 and a covalent acrylamide inhibitor at the DFTB3 level. In the EGFR study, desolvation of the thiolate anion prior to nucleophilic attack was found to be an important reactivity determinant. In BTK, the negatively charged thiolate is stabilized by only two water molecules compared to three in EGFR, possibly contributing to the lower reaction barrier in BTK.

**Figure 4.** Approximate transition state structures corresponding to TS1, TS2 and TS3 along the free energy pathway of BTK inhibition by ibrutinib at the DFTB3/MM level. TS1 shows an important C481-S⁻ interaction, that is crucial in stabilizing TS1 and the Cys-S⁻/C=OH⁺ ion pair (E-I1). Distances reported in Å.

Keto-enol tautomerization steps have been previously reported to be high energy pathways and thus unlikely to occur in thio-Michael addition reactions in protein active sites.²⁵ However, our QM/MM simulations show that this is a feasible reaction step in this BTK covalent inhibition mechanism due
to the low free energy barrier and the solvent exposed nature of the edge of the ATP binding pocket. Both of the crystal structures available for BTK complexed with ibrutinib (PDB: 5P9I and 5P9J) contain a water molecule positioned above the carbonyl oxygen atom (d[O_{ibrutinib}−O_{wat}] = 2.9 Å) and the α-carbon (d[O_{wat}−Cα_{ibrutinib}] = 3.6 Å) that are ideally placed for a solvent assisted tautomerization step. Our QM/MM umbrella sampling simulations also indicate that the enol intermediate E-I2 is well solvated. The FES produced at the DFTB3 level for this step (Figure 3) shows a reaction barrier of 10.5 kcal mol$^{-1}$ and the covalent keto adduct lies 36.8 kcal mol$^{-1}$ lower in energy than the enol intermediate. This is in good agreement with a previous study that investigated the energetics of solvent assisted keto-enol tautomerization in a substituted triazolone compound, and found a reaction barrier of 10.6 kcal mol$^{-1}$ at the B3LYP level.$^{26}$

Studies of BTK inhibition kinetics are available that estimate the inactivation rates ($k_{\text{inact}}$) of several covalent BTK inhibitors.$^{27}$ These are summarized in Table 1, along with the corresponding $\Delta G^\ddagger$ values, calculated using the Eyring equation. The similarity of $k_{\text{inact}}$ for inhibitors with different chemical scaffolds and reactive warheads suggests little dependence on the ligand structure. For ibrutinib, the inactivation rate corresponds to a $\Delta G^\ddagger$ value of 19.6 kcal mol$^{-1}$. This suggests the barrier heights from our simulations at the DFTB3 level underestimates the experimental barrier. However, the experimental $\Delta G^\ddagger$ value will account for any free energy penalty the system must undergo in order to adopt a reactive conformation so that the reaction can proceed, in addition to the free energy barrier to reaction. The semi-empirical DFTB3 Hamiltonian can predict inaccurate reaction energetics,$^{28-30}$ and our validation of QM methods confirmed that DFTB3 may underestimate the reaction barrier for C-S bond formation compared to higher levels of theory such as ω-B97-XD. However, DFTB3 accurately described the energetics of the rate limiting solvent assisted keto-enol tautomerization step and predicted similar reaction pathways to higher level methods in model IRC calculations. The balance of speed and accuracy afforded by DFTB3 therefore make it an appropriate method for performing a
complete assessment of the several possible reaction pathways based on full relative free energy barrier heights, whilst maintaining a relatively large QM region.

Table 1. Inactivation rates of 5 covalent BTK inhibitors taken from ref 27. The corresponding free energy of activation $\Delta G^\ddagger$ values calculated using transition state theory are shown for comparison.

| Inhibitor      | BTK inactivation rate, $k_{\text{inact}}$ (s$^{-1}$) | Free energy of inactivation $\Delta G^\ddagger$ (kcal mol$^{-1}$) |
|----------------|-----------------------------------------------------|-----------------------------------------------------------------|
| Ibrutinib      | 2.66 x 10$^{-2}$                                    | 19.6                                                            |
| Acalabrutinib  | 5.59 x 10$^{-3}$                                    | 20.5                                                            |
| Zanabrutinib   | 3.33 x 10$^{-2}$                                    | 19.5                                                            |
| Spebrutinib    | 1.36 x 10$^{-2}$                                    | 20.0                                                            |
| Tirabrutinib   | 9.72 x 10$^{-3}$                                    | 20.2                                                            |

Summary/Conclusions

Our results indicate that the most probable mechanism for BTK inhibition by ibrutinib occurs in three steps. An initial proton transfer occurs from the thiol group to the carbonyl oxygen atom of the acrylamide group in ibrutinib ($\Delta G^\ddagger = 3.1$ kcal mol$^{-1}$). This is followed by S-C bond formation to form an enol intermediate ($\Delta G^\ddagger = 2.6$ kcal mol$^{-1}$). A rate determining solvent assisted tautomerization step then occurs to form the covalently bound BTK/ibrutinib complex ($\Delta G^\ddagger = 10.5$ kcal mol$^{-1}$). This pathway was lower in energy than all the other pathways that were investigated.

Understanding the precise mechanism by which C481 in BTK is covalently modified by ibrutinib is essential for the design of safer, and more selective covalent drugs. To our knowledge, there are currently no other studies that have investigated the covalent mechanism of action of ibrutinib at the atomistic level, and insights from this work can help rationally tune the covalent reactivity of
acrylamide (and potentially other) covalent inhibitors of BTK. Our simulations highlight the importance of inhibitor conformation, thiol reactivity, and the hydration of the binding site. Strategies common to drug discovery could be employed to enhance or attenuate covalent reactivity. These include the use of substituted acrylamides with different electronic properties, alternative linker groups to attach the electrophilic warhead to the main drug scaffold, or even using different covalent reactive groups. However, the kinetic data for five covalent BTK inhibitors shown in Table 1 for five covalent BTK inhibitors suggest that even changes in the linker and/or different warheads have virtually no effect on the reported inactivation rates. This raises the possibility that reactivity is influenced by the protein itself, in particular the orientation and pK_a of the cysteine residue. Designing new inhibitors that can effectively modulate cysteine pK_a could therefore be useful for tuning covalent reactivity.

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