

Halogen-aromatic π interactions modulate inhibitor residence time

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Abstract: Prolonged drug residence times may result in longer lasting drug efficacy, improved pharmacodynamic properties and “kinetic selectivity” over off-targets with fast drug dissociation rates. However, few strategies have been elaborated to rationally modulate drug residence time and thereby to integrate this key property into the drug development process. Here, we show that the interaction between a halogen moiety on an inhibitor and an aromatic residue in the target protein can significantly increase inhibitor residence time. By using the interaction of the serine/threonine kinase haspin with 5-iodotubercidin (5-iTU) derivatives as a model for an archetypal active state (type I) kinase-inhibitor binding mode, we demonstrate that inhibitor residence times markedly increase with the size and polarizability of the halogen atom. This key interaction is dependent on the interactions with an aromatic residue in the gatekeeper position and we observe this interaction in other kinases with an aromatic gatekeeper residue. We provide a detailed mechanistic characterization of the halogen-aromatic π interactions in the haspin-inhibitor complexes by means of kinetic, thermodynamic, and structural measurements along with binding energy calculations. Since halogens are frequently used in drugs and aromatic residues are often present in the binding sites of proteins, our results provide a compelling rationale for introducing aromatic-halogen interactions to prolong drug-target residence times.

The kinetics of drug binding have emerged as important parameters in drug development. A long drug residence time will result in prolonged inhibition after the free drug concentration has dropped due to *in vivo* clearance, potentially leading to improved drug efficiency and reduced off-target mediated toxicity. In cases

where slow off-rates are specific for the target, kinetic selectivity can be achieved over fast off-target dissociation despite similar binding constants^[1]. The kinetics of the interaction of a drug with its target are defined by the association rate (k_{on}) and the dissociation rate (k_{off}) constants. For bimolecular interactions, the ratio of these two parameters defines the equilibrium dissociation constant (K_D) of a drug, and hence the drug occupancy. Since on- and off-rates are coupled in simple rigid bimolecular interactions, more complicated binding modes are frequently evoked to explain fast “on” and slow “off”-rate kinetics.

Kinases are particularly dynamic proteins that provide multiple opportunities for the development of inhibitors that target induced or allosteric binding sites^[2]. One of the first kinase inhibitors for which slow dissociation rates have been described is the p38 inhibitor BIRB-796. This inhibitor binds to an inactive conformation in which the DFG motif is displaced in a so-called “DFG-out” conformation^[3]. Inhibitors that bind to this conformation are called type II inhibitors and often have prolonged residence times (τ). However, not all type II inhibitors show slow binding kinetics, suggesting that the DFG-out conformational change *per se* is not sufficient to explain the slow dissociation rates of BIRB-796^[4]. Indeed, more recent studies attributed the slow binding kinetics to efficient hydrophobic contacts rather than the kinetic dissociation barrier introduced by the DFG-out transition^[5]. However, conformational change also contributes to the slow off-rate of the breast cancer drug lapatinib, a type I inhibitor of the epidermal growth factor receptor^[6].

In addition to protein conformational changes, the rearrangement of water molecules has been discussed as a potential mechanism influencing inhibitor residence time^[7]. An example for the influence of water molecules on ligand binding kinetics is the type I CDK inhibitor rofeciclib whose slow off-rate is the result of changes in the hydration network coupled to conformational adaptation of the DFG motif^[8]. In some cases, the presence of water-shielded hydrogen bonds can also lead to slow dissociation behavior^[9]. In addition, reversible covalent inhibitors provide an interesting strategy for prolonging target engagement by transient covalent bond formation^[10]. However, the design of reversible covalent interactions requires the presence of cysteine residues in the drug binding site. Many drug receptors, including a large number of kinases, contain cysteines in close proximity to their active sites^[11], but the development of covalent inhibitors may not be feasible for all drug targets.

Here, we present data that suggest that interactions mediated by halogens, that are common in drugs, and aromatic residues, that are also typically found in drug binding sites on proteins^[12], can be utilized to design ligands with slow off-rates. We used 5-iodotubercidin (5-iTU), a close analogue of ATP, as a model inhibitor for a canonical active state kinase binding mode (type I). Screening against more than 100 diverse kinases showed that an aromatic gatekeeper residue that interacts with the halogen moiety of this inhibitor is required for high affinity binding. We chose haspin, a serine/threonine kinase with known three-dimensional structure^[13], as a model system. Analysis of ligand binding kinetics surprisingly showed that 5-iTU had slow binding kinetics. Mutation of the gatekeeper residue as well as removal or substitution of the iodide with other halogens showed that the slow inhibitor off-rates are due to a π -stacking interaction of the halogen with the aromatic gatekeeper. Here, we present

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structural, biophysical and computational data that strongly suggest that halogen interactions with aromatic residues can be exploited for the development of inhibitors with slow off-rates.

Comparative analysis of the high resolution haspin structures revealed high conservation of the binding modes of both 5-ITU and the nucleoside adenosine (Figure 1A-B). In contrast to adenosine or ATP, which binds with a K_D of about $180 \mu\text{M}$ ^[13b], 5-ITU showed high affinity for haspin and an unexpectedly long target engagement time. We then further assessed the thermodynamics and kinetics of the binding with ITC (Isothermal Titration Calorimetry), BLI (BioLayer Interferometry) and SPR (Surface Plasmon Resonance) experiments, which consistently confirmed the tight binding with a relatively slow binding kinetics, as measured for instance by BLI (Figure 1C). Comparing the binding mode of 5-ITU with that of adenosine, the most striking structural difference between these highly similar molecules was the presence of the iodide moiety which was positioned in close proximity to the F605 gatekeeper forming a halogen-aromatic π interaction (Figure 1D). We therefore hypothesized that this interaction might contribute most of the increase in binding free energy (ΔG) and be responsible for the slow dissociation rate of 5-ITU from haspin.

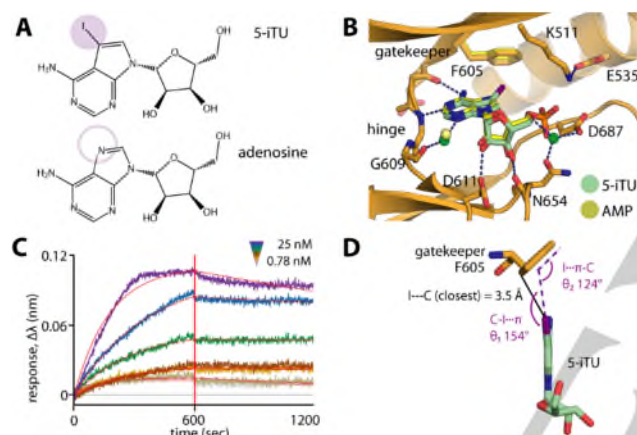


FIGURE 1. The 5-iodotubercidin inhibitor (5-ITU) exhibits tight binding with slow dissociation kinetics from haspin. A) Chemical structures of 5-ITU and adenosine. B) Superimposition of haspin-5ITU and AMP (PDB ID: 4ouc) reveals similar binding modes of the two compounds. C) BLI sensorgram suggests slow kinetic behavior of the 5-ITU-haspin interaction. D) The iodide and the benzene moieties of 5-ITU and F605, respectively, are located in close proximity with a favorable geometry for a halogen- π bond.

To address our hypothesis, we screened 5-ITU against 137 diverse kinases using temperature shift assays^[14] and observed unexpected selectivity of the inhibitor with only 10 kinases exhibiting ΔT_m of $>5^\circ\text{C}$ (Figure 2A and Supplemental Table S1). Interestingly, analyses of the gatekeeper residues of kinase targets that showed significant temperature shifts and therefore high affinity for 5-ITU, revealed a strong preference for kinases harboring a phenylalanine (Phe) at this position whereas kinases that showed weak interaction (ΔT_m $2\text{--}5^\circ\text{C}$) revealed no preference for a certain residue. This analysis supported our hypothesis that the aromatic gatekeeper is important for high affinity binding (Figure 2B). To confirm these results, we determined the structure of 5-ITU bound to another high affinity target that is structurally diverse from haspin, CLK1 (ΔT_m of 8.6°C). As expected, the interaction of 5-ITU with CLK1 remarkably resembled that observed in haspin, including the conserved interaction geometry of the iodide with the CLK1 gatekeeper F241. 5-ITU bound CLK1 with high affinity (K_d of $\sim 7 \text{ nM}$ by ITC) and slow off-rates estimated to be $\sim 50 \text{ mins}$ by BLI (Figure 2C-E and Supplemental Figure S1).

Formation of a halogen- π interaction ($\text{C-X}\cdots\pi$) is driven by the directional positive polarization along the halogen σ -bond with the π molecular orbital of the aromatic system^[15]. However, the main focus of halogen-protein interactions has been on halogen

carbonyl/sulphonyl interaction ($\text{C-X}\cdots\text{O,S}$ bonds) with few examples on the analysis of halogen-aromatic interactions^[16].

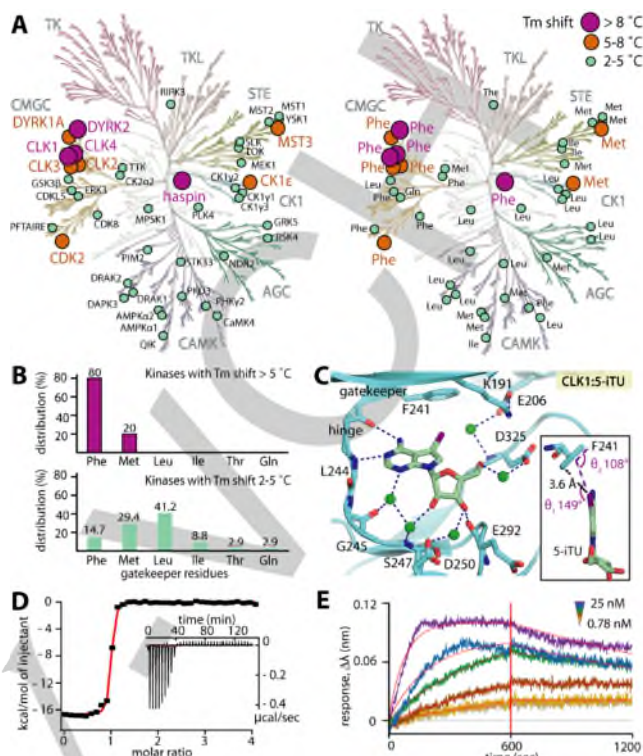


FIGURE 2. Interaction of 5-ITU with various human kinases. A) Temperature shift assays show high ΔT_m preferentially for kinases harboring an aromatic gatekeeper. B) Distribution of gatekeeper residues reveals that the majority of strong ΔT_m hits harbor a phenylalanine gatekeeper. C) The binding mode of 5-ITU in the off-target CLK1, including the iodide gatekeeper interaction, is highly conserved. CLK1 has a high affinity for 5-ITU as measured by ITC (D) and a slow off-rate (E) as assessed by BLI.

Because the partial positive charge along the halogen σ -bond diminishes with the size of the halogen, we next substituted the iodide by smaller halogens and characterized the affinities and binding kinetics of these 5-ITU derivatives. Indeed, ITC experiments showed that the affinities of 5-ITU halogen derivatives were reduced with decreasing size of the halogen (Figure 3 and Supplemental Table S2).

Removal of the halogen led to a 42-fold decrease in the potency of tubercidin (TU) when compared to that of 5-ITU, and similarly an 8-fold decrease was measured for 5-fluorotubercidin (5-fTU). Analyses of the binding kinetics of these five synthesized 5-tubercidin halogen derivatives with haspin were performed using three independent techniques: kinetic probe competition assays (kPCA)^[17], BLI and SPR. Binding affinities determined by these three independent methods correlated well with each other and also with the binding constants determined in solution using ITC (Figure 3B). Dissociation rate constants from all experiments revealed the same behavior with 5-ITU displaying the slowest off-rate. The off-rates increased with decreasing halogen size from 5-iodo to 5-fluoro substituted tubercidin and the unsubstituted tubercidin showed the fastest off-rates of binding (Figure 3C-D). However, the absolute values differed somewhat between the different experimental methods used with the residence time ranging from 60 mins (BLI) to 7 mins (SPR) for 5-ITU (Supplemental Table S2 and S3). We also observed slower on rates using BLI but not in the SPR experiments. While the general trends were the same in both technologies, the differences in on- and off-rates that have been observed might be due to differences in protein immobilization. As SPR is the more established technology, we used SPR kinetic data for quantitative analysis. The substitution from hydrogen to iodide at the 5 position of TU

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led to a 48-fold increase in the on-rates and 274-fold decrease in the off-rates (Figure 3D and Supplemental Table S3).

Thus, the fast dissociation kinetics observed for 5-ITU coincided with the lack of a pronounced σ -hole in smaller halogens, and hence an inability to form a polar halogen- π interaction with the aromatic gatekeeper. The increase in enthalpically favorable polar interactions of larger halogens was also evident in the calorimetric data that showed a steadily decreasing (more negative) binding enthalpy change from tubercidin to the larger halogens ($H < F < Cl < Br < I$). This effect of the halogen moieties was supported by the crystal structures showing that despite the highly conserved binding mode of all derivatives, the presence of an additional water in the tubercidin complex within the space adjacent to the gatekeeper created by the removal of larger halogen substituents and the longer distance to the fluoro group, presented suboptimal geometry for direct contacts with F605 in tubercidin and 5-FTU, respectively (Figure 3E and Supplemental Figure S2).

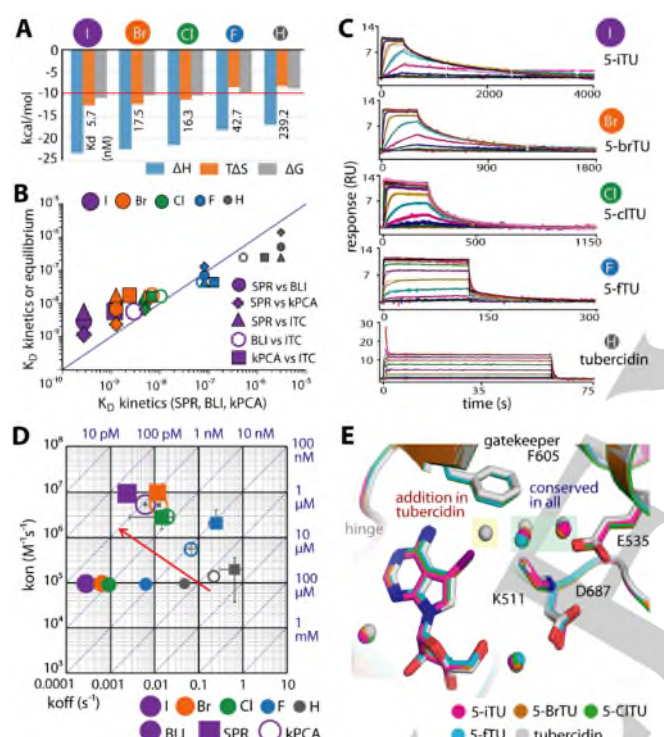


FIGURE 3 Binding kinetics of haspin with five tubercidin derivatives harboring halogen substituents at the 5-position. A) ITC thermodynamic binding parameters. B) Comparison of dissociation constants (K_D) measured by ITC, BLI, SPR and kPCA shows good correlation of the measured equilibrium data. C) SPR sensorgrams demonstrating increasingly slow dissociation rates with increasing size of the halogens. D) Rate plot with Isoaffinity Diagonal (RaPID) of k_{on} and k_{off} constants measured by BLI, SPR and kPCA. The red arrow indicates the trend to increasing k_{on} and decreasing k_{off} upon increasing the atomic radii of the halogens. E) Crystal structures reveal conserved binding modes of all five tubercidin derivatives, albeit with an additional water molecule adjacent to the inhibitor and F605 gatekeeper in tubercidin.

We next investigated the contributions of the Phe gatekeeper to the binding affinity and the observed slow dissociation kinetics of 5-ITU. Six haspin gatekeeper mutants using amino acids commonly found in kinases were generated, and their affinities against 5-ITU and its halogen-derivatives were analyzed using DSF assays (Figure 4A). As expected, the potency of 5-ITU with the aromatic tyrosine mutant was comparable to wild-type haspin.

For comparison, we analyzed the binding characteristics of 5-ITU with a representative mutant (F605T) in detail. Structural superimposition between the wild-type and the mutant structures (F605Y and F605T) showed that the gatekeeper mutation did not affect the binding mode of 5-ITU, yet led to slight variation of the environment in the pocket. The substitution of the bulky aromatic

residue F605 with the small threonine resulted in extension of a water network that filled the expanded binding site in the mutant (Figure 4B and Supplemental Figure S3). No direct contact was observed between the threonine side chain and the iodide of 5-ITU, although interactions might be mediated through a water bridge. The absence of any strong contact was in agreement with the fast kinetics with ~16-fold lower affinity as demonstrated by SPR (Figure 4C-D). In comparison to the wild-type, the loss of the halogen- π contact in the F605T mutant led to a 6-fold decrease and 8-fold increase in association and dissociation rates, respectively, with the estimated residence time of 5-ITU dramatically dropping to less than a minute (Figure 4E and Supplemental Table S3).

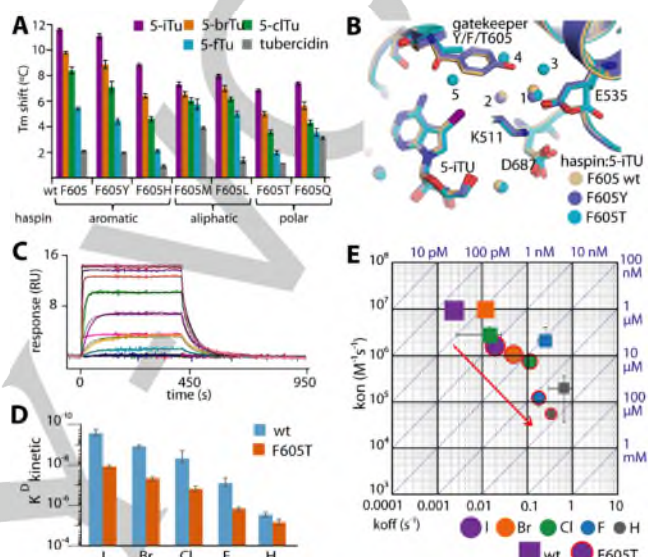


FIGURE 4 Effect of gatekeeper mutation on the binding kinetics of haspin with tubercidin derivatives. A) Tm shifts of six haspin mutants against five tubercidin derivatives. B) Superimposition of 5-ITU-complexed crystal structures of wild-type, F605Y and F605T haspin reveals conserved binding mode of the inhibitor, yet differences in bound water molecules within the binding site. C) SPR sensorgram demonstrates fast binding kinetics for the interaction between 5-ITU and the F605T mutant, and this is accompanied by a significant decrease in K_D (D) and decreased k_{on} and generally increased k_{off} constants (E).

In order to assess the energetic contributions of the halogen-aromatic gatekeeper interaction, we calculated the energy of this interaction using *ab initio* quantum mechanics and classical methods. The second order Møller-Plesset interaction energies (E_{MP2}) between the inhibitor and the gatekeeper residue correlate well with dissociation rate constants and equilibrium dissociation constants determined experimentally (Figure 5A and Supplemental Figure S5 and Tables S6-S9). Partitioning of E_{MP2} into its constituent energy components using a many-body interaction energy decomposition scheme shows that the major contribution to E_{MP2} is the correlation energy (E_{CORR}) which describes second-order intermolecular dispersion interactions and the correlation corrections to the Hartree-Fock energy. E_{CORR} increases in magnitude with increasing size of the halogen, corresponding to the decreasing rate of dissociation measured experimentally (Figure 5B), and indicating the importance of the halogen interaction with the aromatic gatekeeper for the prolongation of residence times as halogen size increases. The computed *ab initio* energies also correlate for the interaction of 5-ITU with the F605Y mutant but the magnitude of the interaction energy of 5-ITU with the threonine mutant was underestimated. To account for the complete protein structure in the computation of the binding free energies of the haspin-ligand complexes, we used the classical MMGBSA approach with an implicit solvent model. The computed energies correlate well with calorimetric data measured by ITC, consistent with the increasingly favorable enthalpic contribution to binding as halogen size increases (Figure 5C-D and Supplemental Table S10).

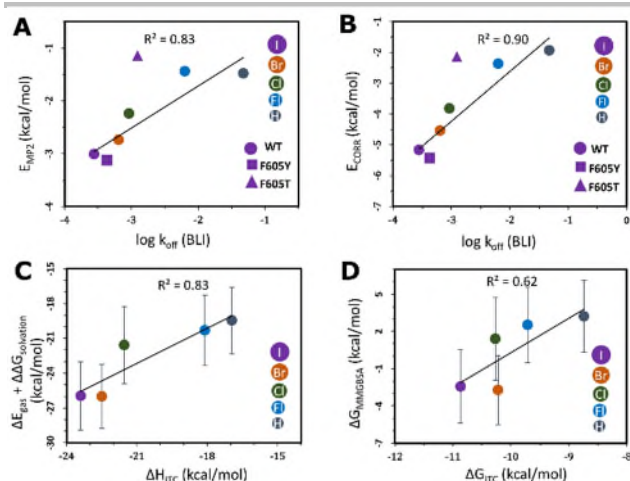


FIGURE 5 Correlation of calculated binding free energies with experimental parameters for the halogen-gatekeeper interaction. A) Second-order Møller–Plesset interaction energy (E_{MP2}) and B) second-order correlation correction energy term (E_{CORR}) between the TU derivatives and the gatekeeper residues vs BLI k_{off} values. The linear fits and correlation coefficients (R^2) were computed omitting the outlier F605T mutant. The experimental error bars are smaller than the size of the data plots. Comparison of C) MMGBSA internal and solvation contributions ($\Delta E_{gas} + \Delta \Delta G_{solvation}$) vs the ITC enthalpies (ΔH_{ITC}) and D) MMGBSA binding free energies (ΔG_{MMGBSA}) vs the ITC binding free energies (ΔG_{ITC}) of the interactions between haspin and TU derivatives. Some ΔG_{MMGBSA} values are positive as they only include translational and rotational entropic terms and do not include vibrational and conformational entropy contributions.

Halogens are frequently found in approved drugs [12]. A recent survey of the PDB for halogen-protein interactions reported that 33% of all non-bonded interactions (excluding C-X...H contacts) of the heavier halogens in the protein database form aromatic stacking interactions of the C-X... π type [18]. It is tempting to speculate that the presence of halogens may lead to longer lasting drug-protein interactions. Examples for contribution of halogens to prolonged off rates in drug candidates have been demonstrated for the CDK inhibitor roniciclib^[8] and it is likely that halogens also contribute to the very slow off rates observed for the ERK inhibitor SCH772984 and VTX-11e^[2b]. For the selected case of protein kinases, aromatic gatekeeper residues are the most frequently found residue type. Our proposed strategy of incorporating heavier halogens into inhibitors with the goal of increasing target residence time by designing interactions with aromatic residues is a general approach that may lead to active compounds with improved pharmacological properties. The biophysical, structural and computational data presented here on 5-halogen substituted tubercidin derivatives provides a good basis for future studies on this exciting topic.

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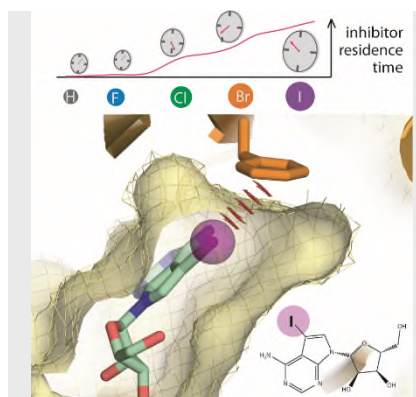
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Halogens are frequently used in medicinal chemistry. Here we show that halogen interactions with aromatic gatekeeper residues leads to prolonged inhibitor off-rates in protein kinases which are dependent on the size and polarizability of the halogen moiety.



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