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Imatinib disassembles the regulatory core of Abelson kinase by binding to its ATP site and not by binding to its myristoyl pocket

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Abstract.

It was recently reported (Xie et al. *Journal of Molecular Biology* **2022**, *434* (2), 167349) that the Abelson tyrosine kinase (Abl) ATP-site inhibitor imatinib also binds to Abl's myristoyl binding pocket, which is the target of allosteric Abl inhibitors. This was based on a crystal structure of a truncated Abl kinase domain construct in complex with imatinib bound to the allosteric site as well as some further ITC, NMR, and kinase activity data. Albeit imatinib's affinity for the allosteric site is significantly weaker (10 µM) than for the ATP site (10 nM), imatinib binding to the allosteric site may disassemble the regulatory core of Abl, thereby stimulating kinase activity, in particular for Abl mutants with reduced imatinib ATP-site affinity. It was argued that the previously observed imatinib-induced opening of the Abl regulatory core (Skora et al. *PNAS* **2013**, *110* (47), E4437–E4445, Sonti et al. *Journal of the American Chemical Society* **2018**, *140* (5), 1863–1869) may be caused by the binding of imatinib to the allosteric site and not to the ATP site. We show here that this is not the case, but that indeed imatinib binding to the ATP site induces the opening of the regulatory core at nanomolar concentrations. This agrees with findings that other type-II ATP site inhibitors (nilotinib, ponatinib) disassemble the regulatory core despite demonstrated negligible binding to the allosteric site.

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1 Introduction

Abelson tyrosine kinase (Abl) is crucial for many healthy cellular processes including proliferation, division, survival, DNA repair and migration (Van Etten, 1999; Pendergast, 2002). However, the oncogenic Philadelphia chromosomal translocation leads to the expression of the highly active fusion protein Bcr-Abl and subsequently to chronic myeloid leukemia (CML) (Rowley, 1973; Deininger et al., 2000; Braun et al., 2020). The ATP-site inhibitors imatinib (Gleevec), nilotinib (Tasigna), and dasatinib (Sprycel) constitute the front-line therapy against CML (Hantschel et al., 2012; O'Hare et al., 2009; Shah et al., 2007). The recently FDA-approved allosteric (STAMP) inhibitor asciminib (ABL001) (Wylie et al., 2017), which targets the myristoyl binding pocket, provides now additional therapeutic means, in particular to overcome emerging resistances against ATP-site inhibitors (Réa et al., 2021).

In a recent publication (P1) (Xie et al., 2022) Kalodimos and coworkers described a crystal structure of a truncated Abelson kinase domain (KD, residues 248–515, $Abl^{248–515}$, 1b numbering used throughout) in complex with dasatinib in the ATP binding pocket and imatinib in the myristoyl binding pocket. The truncated KD had half of the C-terminal αI helix deleted, which covers part of the myristoyl binding pocket (see also Figure 1C). These authors also provided ITC, NMR and kinase activity data, which are related to the binding of imatinib to the allosteric site. Intriguingly, this allosteric binding appears to promote a disassembled state of the Abl regulatory core, comprising the SH3-SH2-KD domains, and higher kinase activity in mutants with reduced imatinib affinity for the ATP site. An increased kinase activity due to core disassembly is expected, since the assembled core has significantly lower kinase activity than the KD alone or an SH2-KD construct (Sonti et al., 2018). The authors observed an imatinib affinity of 10 μM for the allosteric pocket by ITC, which is three orders of magnitude lower than the affinity for the ATP site (Agafonov et al., 2014). Unfortunately, the ITC experiments were not carried out on a full regulatory core construct, where the presence of the adjacent SH2 and SH3 domains in the assembled state is expected to influence the conformation and binding properties of the allosteric pocket. Likewise, most NMR data were obtained only on the Abl KD, but not on the full regulatory core. Nevertheless, the findings are significant, as imatinib binding to the allosteric pocket and the concomitant increase in activity may be a relevant mechanism in patients with imatinib-resistant mutations at the ATP site.

The authors of P1 now argue that the imatinib-induced opening of the Abl regulatory core (residues 83-534, Abl^{83-534}), which was previously observed by our group [publications P2 (Skora et al., 2013) and P3 (Sonti et al., 2018)], may indeed be caused by the binding to the allosteric site and not as we had suggested by binding to the ATP site. This might have been possible in our reported NMR experiments, since the NMR concentrations are in the hundred-micromolar range. However, we show in the following that the authors of P1 have ignored very significant previous evidence. We further provide clear, additional data proving that indeed imatinib opens the regulatory core by binding to the ATP pocket. A proper

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understanding of these mechanisms by taking into account all observations is crucial to make progress in this important area of rational drug development.

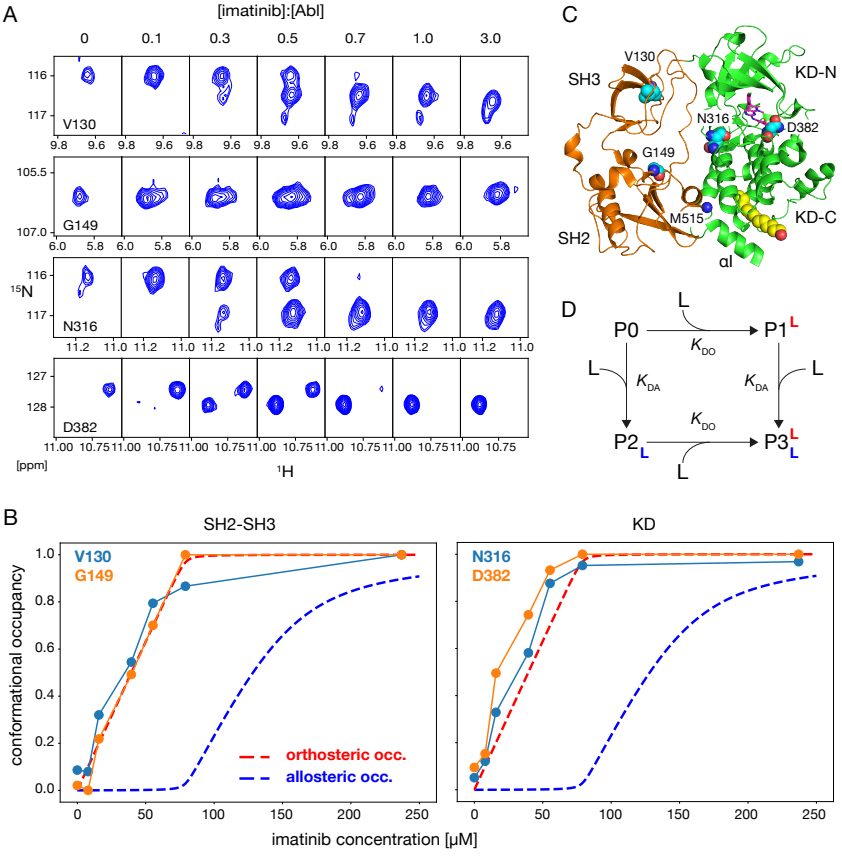

Figure 1. Correlation between imatinib binding to the Abl ATP site and imatinib-induced Abl core disassembly observed by NMR. (A) Individual ^{1}H-^{15}N TROSY resonances of selected residues of the Abl^{83-534} core, which show characteristic shifts for the apo and imatinib-bound state, as a function of imatinib concentration ([Abl^{83-534}] = 79 µM). Molar ratios of imatinib vs. Abl^{83-534} are given above the panels. (B) Occupancies of Abl^{83-534} states as a function of imatinib concentration derived from resonance intensities in (A). Predicted imatinib occupancies of orthosteric (red) and allosteric pockets (blue) according to the equilibrium model (D) are shown as dashed lines for parameters [Abl^{83-534}] = 79 µM, K_{DO} = 10 nM, and K_{DA} = 10 µM. (C) Crystal structure of Abl regulatory core (PDB ID: 2FO0) in complex with ATP-site inhibitor PD166326 (magenta sticks) and myristic acid (yellow spheres). Abl SH3, SH2 domains, and SH2-kinase linker (all orange), as well as KD N- and C-lobe (KD-N, KD-C, green) are shown as cartoon. Residues V130, G149, N316, and D382 are shown as cyan spheres, and M515 (end of $Abl^{248–515}$ construct used for crystal structure in P1) as a single blue sphere, respectively. (D) Chemical equilibrium model describing the binding of the imatinib ligand (L) to Abl's allosteric (dissociation constant K_{DA}) and orthosteric (dissociation constant K_{DO}) binding sites. P0-P3 present Abl apo and various imatinib-bound states.

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2 Experimental procedures

2.1 Protein expression and purification

The Abl regulatory core fragment Abl^{83-534} was expressed in ^{15}N-labeled form in *E. coli* strain BL21(DE3) and purified as described previously in P3 (Sonti et al., 2018).

2.2 NMR spectroscopy and data analysis

Similar to our previous studies, the isotope-labeled Abl^{83-534} was concentrated to 79 µM and 300 µl volume in 10 mM Tris·HCl, 100 mM NaCl, 2 mM EDTA, 2 mM TCEP, 0.02% NaN_3, pH 8.0 ("NMR buffer"). Imatinib was purchased from Selleck Chemicals GmbH and used without further purification. An imatinib stock 50-mM solution in DMSO was prepared by weighing in the required amount of dry imatinib. This stock was then diluted with NMR buffer to 0.5 mM imatinib, from which it was added to Abl^{83-534} at molar ratios of 0, 1:10, 3:10, 5:10, 7:10, 1:1, and 3:1 (imatinib:Abl^{83-534}). The concentrations of the imatinib stock solutions and of Abl^{83-534} in the NMR sample were confirmed by absorbance measurements at 255 nm for imatinib [$\varepsilon_{250} = 3.3338 \cdot 10^4$ $M^{-1}cm^{-1}$ (Haque et al., 2016)] and at 280 nm for Abl^{83-534} [$\varepsilon_{280} = 9.5230*10^4$ $M^{-1}cm^{-1}$ derived by ExPASy (Gasteiger et al., 2003) from UNIPROT entry P00519 (isoform 1b, residues 83-534], respectively, using a Nanodrop 2000 spectrometer (Thermo Scientific).

All NMR experiments were performed at 303 K on a Bruker Ascend 600-MHz spectrometer equipped with a TCI triple resonance cryoprobe. ^1H-^{15}N TROSY experiments were recorded with 140 (^{15}N) × 1024 (^1H) complex points and acquisition times of 42 ms (^{15}N) and 48 ms (^1H). Data were processed with the NMRPipe software package (Delaglio et al., 1995) and analyzed with SPARKY (Goddard and Kneller, 2008). For quantitative analysis, resonances of the entire titration data set were fitted with the program NLINLS contained in NMRPipe.

Theoretical Abl imatinib binding curves were calculated by solving the respective mass action law equations for a four-state model (see also Figure 1D) using Mathematica (Wolfram Research, Inc.). These equations are: $P0*L == K_{DO}*P1$, $P0*L == K_{DA}*P2$, $P1*L == K_{DA}*P3$, $P2*L == K_{DO}*P3$, $P0 + P1 + P2 + P3 == Pt$, $L + P1 + P2 + 2*P3 == Lt$. P0, P1, P2, P3, L, Pt, Lt present the concentrations of apo Abl, Abl with imatinib bound to ATP site, Abl with imatinib bound to allosteric site, Abl with imatinib bound to both sites, free imatinib, total Abl, total imatinib, respectively. K_{DO}, K_{DA} are the dissociation constants for binding to the ATP site (orthosteric site) and allosteric site, respectively.

3 Results and discussion

3.1 Previous data on ATP-site inhibitor-induced opening of Abl's regulatory core

Kalodimos and colleagues argue that we had not stated the imatinib concentrations in our work. While this is true for publication P2 (Skora et al., 2013), where we had only stated that Abl^{83-534} had formed a

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complex with imatinib at concentrations of 150–200 µM for the NMR experiments, publication P3 (Sonti et al., 2018) clearly indicated a 3:1 ligand:protein ratio for SH3-SH2-KD Abl$^{83-534}$ concentrations of ~100 µM. These concentrations are in the range where effects of allosteric imatinib binding at a K_D of 10 µM would become appreciable and the regulatory core could open due to imatinib binding to the allosteric site. However, for the reasons given in the next paragraphs, this argumentation is not correct.

P2 (Skora et al., 2013) clearly showed that not only imatinib, but also nilotinib disassembles the regulatory core. The evidence was given by ^{1}H-^{15}N chemical shifts within the SH2-SH3 domains as well as by SAXS data, which are identical for the nilotinib- and imatinib-bound SH3-SH2-KD Abl$^{83-534}$. However, no binding of nilotinib to the allosteric pocket was observed in P1. P2 showed that the imatinib-induced disassembled state of the regulatory core is highly dynamic with the SH2 and SH3 domains moving on the nanosecond time scale relative to the KD. Cellular experiments on Bcr-Abl, reported in P2, give evidence that at nanomolar concentrations both imatinib and nilotinib lead to phosphorylation of residue Y245, which resides in the linker between the SH3 and KD domains. The linker is only accessible in the disassembled state of the core, but buried in the assembled state. This observation was explained by an imatinib- resp. nilotinib-induced opening of the core according to their nanomolar affinity for the ATP pocket.

The more recent publication P3 (Sonti et al., 2018) extended these findings in a systematic way to 14 ATP-site ligands, comprising all FDA-approved Bcr-Abl inhibiting drugs. Compelling evidence by ^{1}H-^{15}N chemical shifts from ~100 residues within the SH2-SH3 domain showed that all type-II ATP-site inhibitors, which induce an “inactive” (closed, DFG out) conformation of the activation loop (A-loop), disassemble the Abl regulatory core. In contrast, type-I ATP-site inhibitors, which lead to an “active” conformation of the A-loop, leave the regulatory core in the assembled state. The type-II inhibitor-induced opening of the regulatory core was explained by a force from the inhibitor onto the A-loop and subsequently P-loop, which leads to a slight rotation (observed in crystal structures) of the kinase N-lobe towards the SH3 domain breaking the delicate force balance of the assembled core. Importantly, the type-II inhibitor-induced disassembly was observed in an identical manner for all investigated type-II inhibitors, i.e. imatinib, nilotinib, ponatinib, rebastinib, and bafetinib. Of these, imatinib, nilotinib, and ponatinib were tested in P1 for allosteric pocket binding, with imatinib having micromolar affinity, ponatinib much weaker affinity than imatinib, and nilotinib no observable binding. The observed core disassembly by all investigated type-II inhibitors in P2 and P3 contradicts the hypothesis of P1 that the disassembly is caused by a binding to the allosteric pocket.

It is doubtful whether the imatinib K_D value of 10 µM for the allosteric pocket determined on the KD construct is relevant in the context of the entire Abl regulatory core. Indeed well before the publication of P1 Skora and Jahnke (Skora and Jahnke, 2017) had already assayed both ATP and allosteric site binding of imatinib to Abl$^{83-534}$ by ^{19}F-labeled competitive binders to the ATP and allosteric sites. No displacement of the ^{19}F-labeled reporter to the allosteric site (K_D = 43 µM) was detectable for an Abl$^{83-534}$

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concentration of 4 µM and concentrations of reporter ligand and imatinib of 25 µM each. This indicates that the imatinib affinity to the allosteric site of Abl's regulatory core must be in the high double-digit micromolar range or even weaker, which disagrees with the value of 10 µM reported for KD binding in P1. Very likely, the additional coordination by the SH2 and SH3 domains and a subsequent rearrangement of the αI helix reduce the affinity in the context of the entire Abl regulatory core. Albeit highly relevant, the work by Skora and Jahnke is not cited in P1.

In P1, Kalodimos and colleagues argue that our explanation of an imatinib-induced push via the closed A-loop towards the SH3 domain leading to core disassembly is not valid, since they observed a closed A-loop in the assembled state in recent work on an apo SH3-SH2-KD fragment, which they compared to obtained solution NMR structures of the KD alone (Xie et al., 2020). However, the apo conformation of the A-loop in SH3-SH2-KD is irrelevant, since imatinib fills the ATP pocket and exerts additional forces. Moreover, the A-loop in the apo form is certainly not in a single conformation but in dynamical exchange. We have observed a dynamical equilibrium of the A-loop already in Vajpai et al. (Vajpai et al., 2008) even in the presence of inhibitors as well as in P2 (Figure S2) where many resonances of the A-loop were bleached out due to conformational exchange for both the apo form and the GNF5 complex. In contrast, binding of imatinib rigidifies the A-loop to the 'closed' conformation of the crystal structure (Vajpai et al., 2008). It also needs to be indicated that the obtained KD solution structures (Xie et al., 2020), on which the argument is based, are of low definition having at most 3 NOE and 2 dihedral (the origin of which is not documented) constraints per residue. Hence, a well-defined A-loop conformation cannot be postulated without additional evidence.

3.2 Imatinib titration to Abl's regulatory core followed by TROSY

To further characterize the binding of imatinib to Abl, we have carried out a titration of imatinib to the Abl regulatory core construct (SH3-SH2-KD, Abl^{83-534}, 79 µM) used in our previous studies and followed the response of ^{1}H-^{15}N resonances in a TROSY experiment (Figure 1). Well separated resonances are observed (Figure 1A) e.g. for residues V130, G149 in the SH3 and SH2 domains as well as for N316 and D382 in the KD close to the ATP site (Figure 1C). The addition of imatinib from 0 µM to 240 µM leads to the appearance of a second set of resonances for all residues, which become dominant at high concentrations. This phenomenon indicates slow exchange between apo and imatinib-bound forms. Based on the chemical shift separations, the exchange rate must be slower than $2\pi \cdot 0.6$ ppm·60 MHz ≈ 200 s^{-1}, as expected for an affinity in the nanomolar range. The change in chemical shifts of V130 and G149 upon imatinib binding had been shown before in P2 and P3 to be indicative of the core disassembly.

Notably the ratio of the resonance intensities of the apo (I_a) and imatinib-bound (I_i) forms of the individual residues in the SH3 and SH2 domains and close to the ATP site changes identically with imatinib concentration. This is evident from a plot of the intensity ratios $I_i/(I_i + I_a)$, which equals the relative population p_i of the imatinib-bound form, versus the added imatinib concentration (Figure 1B). For the

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SH3/SH2 residues V130, G149 as well as for the ATP-site residues N316 and D382, the dependence of p_i on the imatinib concentration is identical and its increase almost quantitative with added imatinib up to the concentration of Abl SH3-SH2-KD. For comparison, Figure 1B also depicts the theoretical binding curves of a two-site binding model with K_{DO} (orthosteric ATP site) = 10 nM, K_{DA} (allosteric site) = 10 µM. Clearly both the SH3/SH2 and the ATP-site residues exhibit a dependence on the imatinib concentration that is expected for the high-affinity orthosteric ATP site binding (red dashed lines) and not for the low-affinity allosteric site binding (blue dashed lines). These data unequivocally show that the imatinib-induced opening of Abl's regulatory core is caused by imatinib binding to the ATP site and not to the allosteric site.

3.3 Additional problematic points in P1

Publication P1 contains a number of further problematic points that weaken the evidence of the described experiments and make their results hard to reproduce. We are surprised that these problems escaped the refereeing and editorial process. A request to JMB to address this criticism in an editorial correspondence between us and the authors of P1 remained unheeded.

1. It often is not specified which Abl constructs were used for the measurements. E.g. there is no indication of the amino acid sequence for the NMR experiments on the Abl KD construct. Thus, it often remains unclear whether the entire αI helix was present or whether the truncation of the construct for the structure had been used. The same applies to the ITC experiments. In particular, it would have been interesting to compare a construct harboring the entire αI helix with the truncated construct used for the crystal structure. More importantly, both NMR and ITC experiments should have been carried out on full regulatory core constructs, and not only on Abl KD, in order to understand the effects of the adjacent SH2 and SH3 domains onto the imatinib binding.

2. The conclusions of P1 heavily depend on the ITC data. However, syringe concentrations of imatinib, other ATP-site blocking inhibitors, myristic peptide, GNF5, and of the Abl constructs themselves are not properly indicated for the crucial ITC titrations shown in Figures 1, S1, S2, S3, S5, S8. Thus, it is impossible for others to reproduce these data.

3. Figure 4B shows a methyl spectrum of the Abl regulatory core construct (termed Abl^{FK} by the authors) in complex with imatinib. The imatinib concentration is not indicated. It would have been crucial for the conclusions of P1 to vary the imatinib concentration in this experiment.

4. Figure 5 shows a titration of imatinib added to Abl KD constructs monitored by methyl resonances. Again, neither imatinib nor Abl concentrations are indicated. Intriguingly, the left spectrum for V357 (panel A, row 2), which is presumably the apo form according to the concentration arrow at the top of the figure, does not agree with the blue spectrum on the right, which is annotated as apo Abl by the color legend at the top.

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5. The authors of P1 argue that they developed a new method to differentiate Abl allosteric inhibitors from activators by monitoring resonances of αI helix residues. However, a highly similar method (using ^{1}H-^{15}N instead of ^{1}H-^{13}C observations) had already been described and used successfully by the Novartis group many years ago (Jahnke et al., 2010). This work is not properly acknowledged.

6. In addition, P1 contains many typos and other inconsistencies, which make the findings extremely hard to follow. Such problems are e.g. a wrong labeling of amide $^{1}H^{N}$ resonances as ^{13}C in Figure 3B, a wrong labeling of the statistical significances in Figure 6, a definition of k_{ex} as $-k_{on}$[imatinib] + k_{off} and a 'lore ipsum' statement in Figure 6A.

4 Conclusion

In summary, previous data and the new evidence presented here unequivocally show that binding of imatinib to Abl's myristoyl pocket does not cause the observed disassembly of its regulatory core. Rather the disassembly must be caused by imatinib binding to the ATP site and forces transmitted from there to the interface between the KD and the SH3-SH2 domains as explained in P3. Careful documentation of experimental procedures and taking into account all observations pertinent to a system as complicated as Abl is the only way forward to an in-depth understanding of its function and towards the goal of rational intervention.

Data availability

NMR raw and processed data in NMRPipe format have been deposited in the Zenodo repository under DOI 10.5281/zenodo.6368036.

Author contributions

J.P. and S.G. conceived the study. J.P. recorded the NMR experiments. J.P., J.H., and S.G. analyzed data. S.G., J.P. and R.S. wrote the manuscript.

Competing interests

The authors declare no competing interests.

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