Leukemia (2015), 1–4

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LETTER TO THE EDITOR

Novel TKI-resistant BCR-ABL1 gatekeeper residue mutations retain *in vitro* sensitivity to axitinib

Leukemia advance online publication, 20 November 2015; doi:10.1038/leu.2015.303

Tyrosine kinase inhibitor (TKI) resistance due to acquired secondary kinase domain (KD) mutations in BCR-ABL1 represents a common clinically encountered problem in patients with chronic myeloid leukemia (CML). The prototypic ABL1 TKI imatinib is vulnerable to a large number of resistance-conferring secondary KD mutations.[1] Clinical management of imatinib-resistant disease has been successfully guided by *in vitro* studies of mutant sensitivities to alternative TKIs, which are largely predictive of clinical responsiveness.[2]

Kinase 'gatekeeper' residues (T315 in BCR-ABL1) restrict access to a deeper hydrophobic pocket within the KD and mutations at this residue are problematic for numerous inhibitors of pathologically activated kinases (KIT, FIP1L1-PDGFRB, EGFR, EML4-ALK and FLT3-ITD). These substitutions can impact ATP affinity, affect the conformation of the kinase activation loop and alter substrate preference. In BCR-ABL1, an isoleucine substitution has been the most commonly reported and problematic gatekeeper mutation (T315I). Crystal structures have demonstrated that imatinib[3] and all approved second-generation ABL1 TKIs (dasatinib,[4] nilotinib[5] and bosutinib[6]) make critical contact with the T315 residue, providing structural rationale for the vulnerability of these TKIs to the T315I mutation. The third-generation ABL1 TKI ponatinib was rationally designed to retain activity against the T315I substitution[7] and represents the only approved TKI option for BCR-ABL1/T315I mutant CML. Although highly active in this setting, ponatinib is associated with a substantial risk of thrombotic events, and alternative strategies appear necessary for safer long-term management of T315I-associated CML. Ponatinib is considered a 'pan-BCR-ABL' inhibitor because of its invulnerability to mutations that may arise as a consequence of single-nucleotide substitutions. However, select 'compound' mutations (two or more amino-acid substitutions or a single-amino-acid substitution if both nucleotide changes occur within the same codon) can confer ponatinib resistance *in vitro* and clinically.[8] With the increasing prevalence of CML worldwide, the extensive use of sequential ABL1 TKI therapy and the ability to more effectively suppress mutants that arise from a single-nucleotide change, compound mutations will likely be increasingly encountered in the clinical management of CML.

In vitro mutagenesis screens typically generate simple single-nucleotide substitutions, and potentially problematic compound mutations are not easily generated by these assays. The resistance profile of such substitutions remains largely unknown. Here we profiled the seven BCR-ABL1 gatekeeper mutations that have been clinically described to date against all approved ABL1 TKIs (imatinib, dasatinib, nilotinib, bosutinib and ponatinib) as well as the approved vascular endothelial growth factor receptor (VEGFR) TKI axitinib, which has been reported to have higher affinity for the ABL1/T315I mutant than wild-type ABL1[(ref. 9)] and has shown signs of clinical activity in a single case of a BCR-ABL1/T315I mutant CML patient briefly treated with this agent.[10] Using structural models, we rationalized the impact of the clinically observed mutations against ponatinib and utilized them to predict TKI resistance of uncharacterized mutations.

A 59-year-old male patient presented to the University of California, San Francisco 2 weeks after initiating 140 mg dasatinib daily monotherapy for *de novo* T-lymphoid blast crisis CML. The patient initially responded to dasatinib clinically, but relapsed after 6 weeks of treatment. Sequencing of the *BCR-ABL1* KD revealed two substitutions at the T315 codon that resulted in the genesis of both T315I and T315L mutations (Figure 1a, middle panel). Whereas the T315I mutation, the result of a single-nucleotide substitution (ACT → ATT), was readily detectable, T315L, which requires a two-nucleotide substitution (ACT → CTT), represented the predominant isoform. Although the clinical sensitivity of BCR-ABL1/T315L to ABL1 TKIs had not been determined, the patient was treated with 45 mg ponatinib daily, but had no objective response. After 4 weeks of ponatinib treatment, re-evaluation of the *BCR-ABL1* KD revealed persistence of only the T315L mutation (Figure 1a, right panel). The disappearance of the T315I mutation while on ponatinib and enrichment of the T315L allele strongly suggest clinical resistance of BCR-ABL1/T315L to ponatinib.

Given the lack of response to ponatinib and the unknown resistance profile of T315L to other ABL1 TKIs, we sought to characterize the activity of all approved ABL1 TKIs against the BCR-ABL1 gatekeeper mutations that have been identified clinically to date (T315A[11]/F(Smith manuscript submitted)/I[12]/L[13]/M[8]/N[14]/V[15]) in Ba/F3 cells. BCR-ABL1/T315A demonstrated the greatest relative sensitivity ($<$ 10-fold resistance compared with native BCR-ABL1) to imatinib, nilotinib and bosutinib (Supplementary Figure 1; Supplementary Table 1). Ponatinib was the most generally active agent, with all of the T315A/I/N/V substitutions retaining sensitivity (Supplementary Figure 1; Supplementary Table 1). However, the T315F/L/M mutants conferred a high degree of *in vitro* resistance to ponatinib and all other ABL1 TKIs (Supplementary Figure 1; Supplementary Table 1).

Given the reported activity of axitinib against BCR-ABL1/T315I,[9,10] we sought to determine its activity against other T315 variants. Encouragingly, BCR-ABL1/T315L/N/V mutants were more sensitive to axitinib than BCR-ABL1/T315I (Figure 1b; Supplementary Table 1) whereas T315F and T315M mutants both conferred a significant degree of cross-resistance to axitinib (Figure 1b). Consistent with the IC_{50} data, a clinically achievable concentration of axitinib decreased BCR-ABL1 phosphorylation at both the activation loop residue Y412 and the SH2-KD linker residue Y245 as well as STAT5 phosphorylation (Y694), a downstream substrate of BCR-ABL1, in the T315F/I/L/N/V mutant cell lines (Figure 1c). These data suggest that axitinib inhibits the kinase activity of select T315 mutants and might be clinically active in BCR-ABL1/T315L/N/V-associated cases.

To understand the structural basis of resistance toward ponatinib, we modeled its binding to the seven T315 mutants. The impact of these mutations can be discussed in three categories: first, mutations T315A and T315V introduce small, hydrophobic side chains and are most sensitive to ponatinib (Figure 2a). Like threonine, these side chains are predicted to avoid steric clash with ponatinib and to make favorable van der Waals interactions. Because the alanine side chain is smaller than

Accepted article preview online 29 October 2015

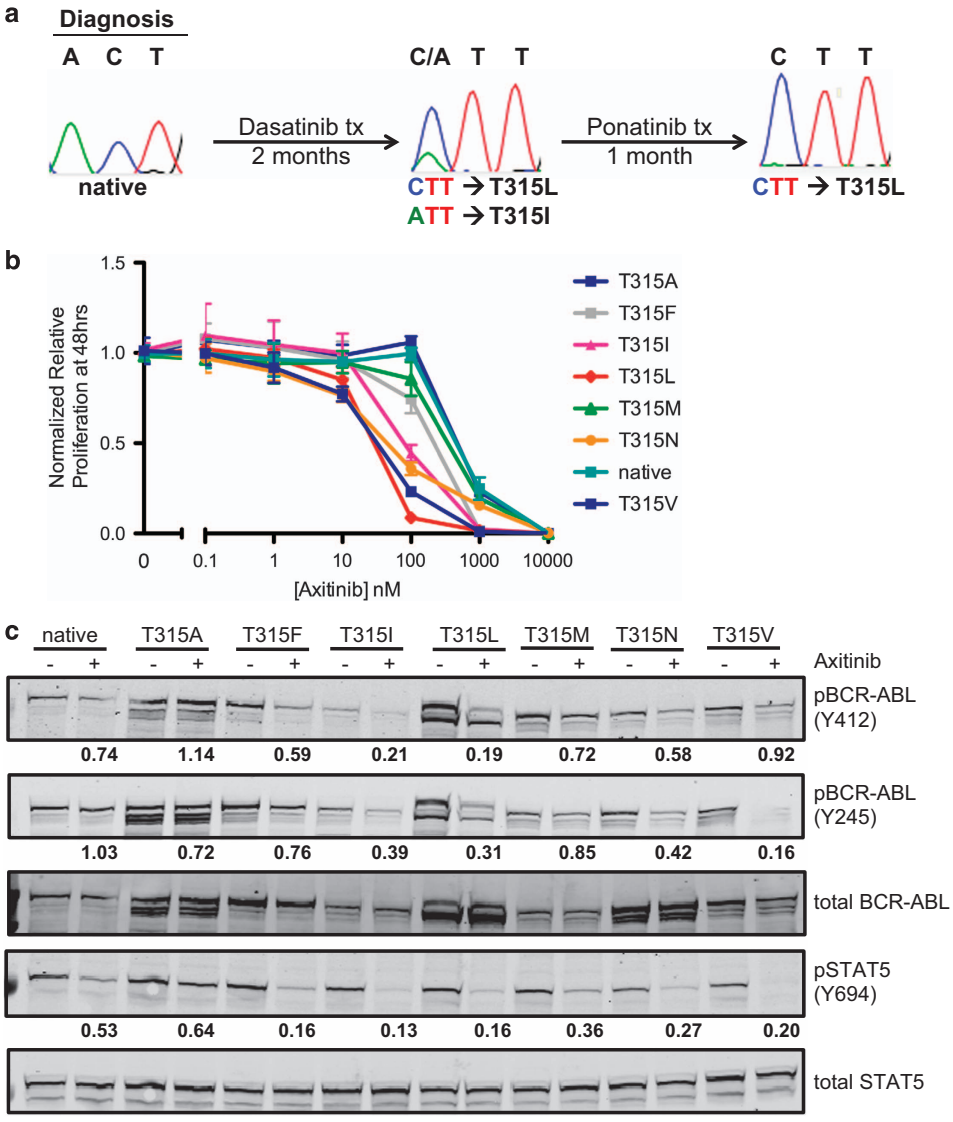

Figure 1. Acquisition of BCR-ABL1/T315L mutation in a patient following dasatinib monotherapy and characterization of clinical mutants against ABL TKIs. (**a**) Sequencing traces of the T315 codon at diagnosis (left panel), following relapse on dasatinib treatment (middle panel) and following 4 weeks of subsequent treatment with ponatinib (right panel). ACT = T; ATT = I; CTT = L. (**b**) Normalized relative proliferation curves for the BCR-ABL1 T315 mutations described clinically. Cells were treated with increasing concentrations of axitinib for 48 h (error bars represent s.d. of three independent experiments). For each concentration the values were normalized to the median of the untreated cells for that cell line. (**c**) Western blot analysis using anti-phospho-ABL, anti-ABL, anti-phospho-STAT5 and anti-STAT5 on lysates from BCR-ABL1 mutant cell lines treated for 3 h with 75 nM axitinib. Normalized phosphorylation of the mutant treated with axitinib compared with untreated cells for that mutant is indicated under the blot. The data are representative of multiple independent experiments.

threonine, it creates a larger binding area that is potentially more accessible to ponatinib. Second, T315I represents the mild resistance category of ponatinib. The added methyl group of the isoleucine side chain compared with valine leads to a minor steric clash with the A ring of ponatinib and requires a subtle change in the binding conformation of ponatinib to alleviate the strain (Figure 2a).[16] Third, mutations T315F/L/M represent the high-resistance category of ponatinib. The bulky hydrophobic side chains of these residues are sterically incompatible with the binding mode of ponatinib (Figure 2a). The T315N mutant was excluded from the training set because structural changes necessary to accommodate the ligand are larger than those allowed by the conservative assumptions of homology modeling.

The maximum statistically organized atomic potential (SOAP) scores for each of the T315 mutants correlated well with the experimental ponatinib IC_{50} values (R-squared = 0.81; Figure 2b). This correlation indicated that the SOAP-protein score is a sensitive metric able to capture the physicochemical compatibility between the mutant and the ligand, and may serve as a predictive metric for uncharacterized mutations. The relative uncertainty in the modeling of axitinib interactions with BCR-ABL1 mutants precludes an equivalent analysis for axitinib. Crystal structures have demonstrated that wild-type ABL1 binds axitinib in the inactive DFG-out conformation, whereas the T315I ABL1 mutant binds axitinib in the active DFG-in conformation.[10] The differences between the two structures make it difficult to model other mutants because it is unknown whether these mutations are stabilizing toward the active kinase conformation.

To prospectively predict the sensitivity of other gatekeeper substitutions that have yet to be clinically encountered, we modeled ponatinib binding of the 12 remaining T315 mutations. On the basis of SOAP-protein score, we calculated the predicted ponatinib IC_{50} value for each mutant using the correlation derived from the clinical T315 mutant dataset (Supplementary Figure 2)

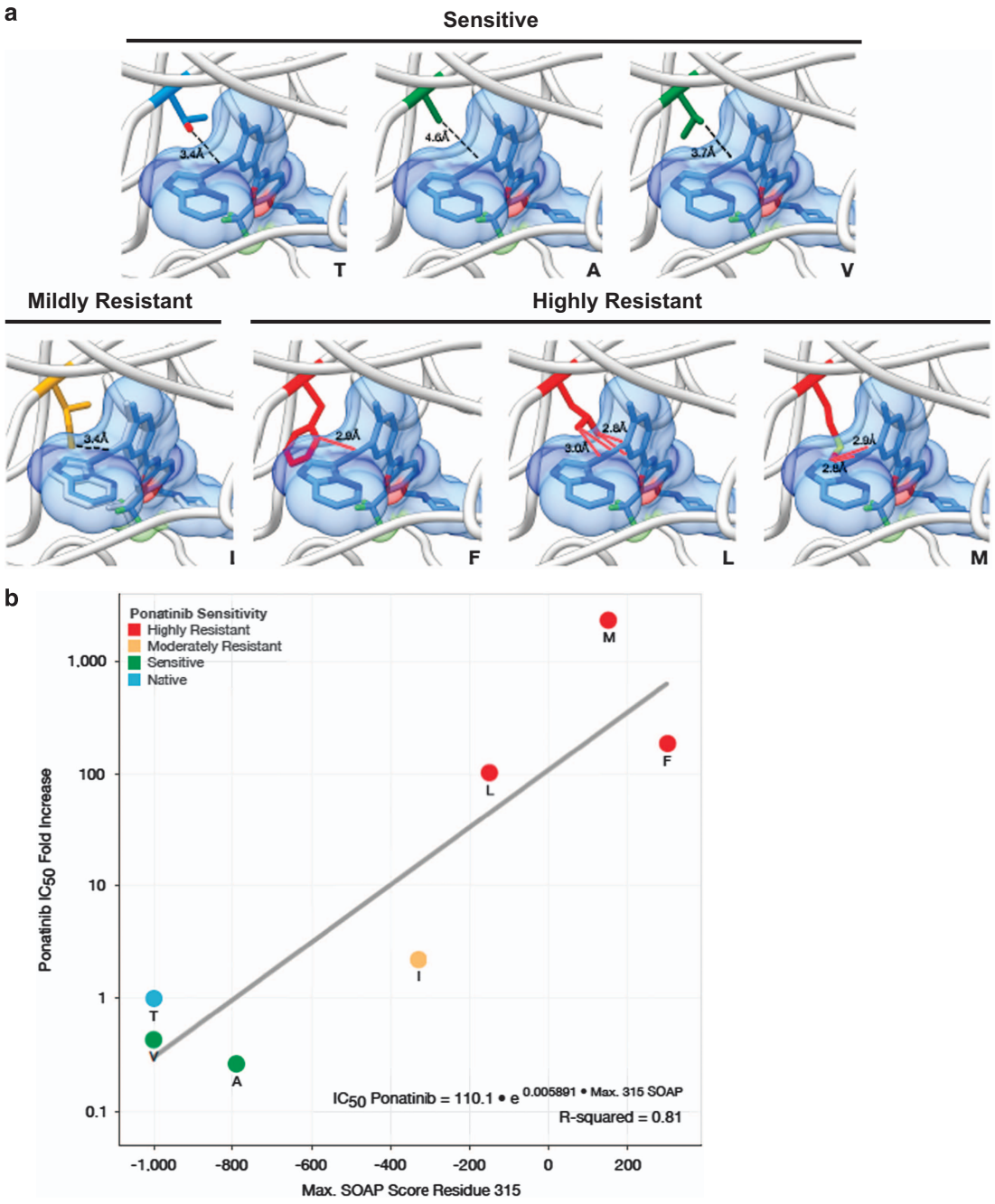

Figure 2. Crystal structures and comparative models of the ABL1 kinase domain bound to inhibitors. (**a**) Cartoon representation of the crystal structure of ponatinib bound to native ABL1 (PDB ID 3OXZ), the model of ponatinib bound to native ABL1 (T), ABL1 T315A and ABL1 T315V (sensitive group), the crystal structure of ponatinib bound to ABL1 T315I (PDB ID 3IK3; mildly resistant group), the model of ponatinib bound to ABL1 T315F, ABL1 T315L, and ABL1 T315M (highly resistant group). Black lines indicate the closest position between the side chain and ponatinib. The red lines indicate steric clashes between the side-chain atoms and ponatinib. In the T315I panel, the pose of ponatinib from the native ABL1 crystal structure is overlaid in white, to indicate the slight change in the binding conformation. (**b**) Maximum SOAP-protein score of residue 315 for each of the clinically observed mutants extracted from the comparative structural models. The best-fit exponential line is shown in gray and its equation and coefficient of determination are shown in the bottom right corner. The points are colored according to the experimentally determined ponatinib sensitivity.

and compared these values with experimentally determined IC_{50} values of ponatinib in T315-mutant Ba/F3 cells (Supplementary Table 1). The models successfully predicted the category of ponatinib sensitivity in 7 of 12 mutants (three mutants were less than one order of magnitude from the experimentally determined value; Supplementary Figure 2). The models failed to predict the sensitivity of the T315W/D mutants (Supplementary Figure 2; W mutant not shown). As with T315N, the models of these mutants are relatively inaccurate because the structural changes are larger than allowed by template-based homology modeling by MODELLER.

IC_{50} values of these 12 T315-mutant cell lines were also determined for imatinib, dasatinib, bosutinib, nilotinib and axitinib (Supplementary Table 1; Supplementary Figure 3). Of the ABL1 TKIs, ponatinib maintained the broadest activity, with 9/19 mutants having an IC_{50} value less than native BCR-ABL1

(Supplementary Figure 3; Supplementary Table 1). Importantly, axitinib demonstrated increased potency against T315L/N/V/Y mutations compared with T315I (Supplementary Figure 3; Supplementary Table 1). Altogether, axitinib was relatively more active *in vitro* against six gatekeeper mutants (T315F/K/L/M/Q/Y) when compared with the approved ABL1 TKIs.

Here we provide evidence that implicates T315L in clinical resistance to ponatinib. In all, we identified nine BCR-ABL1 T315 mutations (E/F/H/K/L/M/Q/R/Y) that conferred >10-fold resistance to all approved ABL1 TKIs relative to native BCR-ABL1 (Supplementary Table 1). Of these, T315L and T315Y showed greater sensitivity to axitinib than T315I. The *in vitro* activity of axitinib against T315L/Y warrants clinical investigation, particularly in light of the lack of effective alternatives. Like ponatinib, axitinib is also a multi-kinase inhibitor and therefore the benefit that arises from use of this compound will have to be balanced against the clinical risks. The T315E/H/K/M/Q/R substitutions, which are highly pan-resistant to all approved ABL1 TKIs, also conferred substantial *in vitro* resistance to axitinib. For patients whose disease evolves these substitutions, prompt consideration of allogeneic stem cell transplantation seems prudent. Multiple T315 mutations demonstrated resistance to all ABL1 inhibitors, including several that have been described clinically (T315F/L/M), and investigation of novel strategies, such as alternative TKIs or allosteric inhibitors, to treat these vulnerabilities therefore is warranted.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

NPS and CCS were supported by the Leukemia & Lymphoma Society, and wish to acknowledge the generosity of Arthur Kern and the Edward S Ageno family. NPS has received funding for the conduct of research from Bristol–Myers Squibb, ARIAD and Pfizer.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)