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Mechanisms of Resistance to the BCR-ABL1 Allosteric Inhibitor Asciminib

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The development of asciminib (ABL001)[1], a low nanomolar allosteric BCR-ABL1 tyrosine kinase inhibitor (TKI), is the culmination of programs in academic and pharmaceutical industry laboratories spanning more than a decade. Currently in clinical trials for relapsed/refractory Philadelphia chromosome-positive (Ph+) leukemia patients (ClinicalTrials.gov: NCT02081378), asciminib represents a major advance from the structurally related, micromolar-potency tool compounds GNF-2 and GNF-5[2–4]. We investigated mechanisms of asciminib resistance and identified two major categories: (1) upregulation of the ABCG2 efflux pump, resulting in undetectable intracellular asciminib levels, and (2) emergence of *BCR-ABL1* mutations at the myristoyl-binding site and at a distant residue.

We generated five asciminib-resistant, BCR-ABL1-positive cell lines by adapting to increasing concentrations of asciminib : K562$^{\text{asciminib-R}}$, LAMA84$^{\text{asciminib-R}}$, KYO1$^{\text{asciminib-R}}$, Ba/F3 BCR-ABL1$^{\text{asciminib-R}}$ and KCL-22$^{\text{asciminib-R}}$ cells (Supplemental Methods). In K562$^{\text{asciminib-R}}$ cells, methanethiosulfonate (MTS)-based cell proliferation

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CONFLICT OF INTEREST

TWK has financial interests to declare: Novartis speaker's bureau, Novartis advisory board. DR receives honoraria from BMS, Novartis, Incyte and Pfizer for non-promotional scientific lectures or advisory boards and is an investigator of Novartis-sponsored CABL001X2101 phase I trial. The other authors declare no conflict of interest.

assays demonstrated a ~60-fold increase in asciminib IC_{50} compared to parental K562 cells, despite remaining sensitive to the ATP-site TKIs imatinib, nilotinib, dasatinib and ponatinib (Figure 1a; Table S1). Immunoblot analysis revealed similar results, showing marked restoration of BCR-ABL1 tyrosine kinase activity and downstream STAT5 tyrosine phosphorylation in the presence of asciminib but not ponatinib (Figure S1a). However, Next-Generation Sequencing (NGS) and Sanger sequencing of the *BCR-ABL1* kinase domain identified no mutations (Table S2)[5]. To check for the possibility of reduced intracellular drug concentrations, asciminib levels were measured following treatment using a customized liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) method[6]. Asciminib was undetectable in K562$^{asciminib\text{-}R}$ cells but present at substantial levels in parental K562 cells (Figure S1b), with an inhibitor-based screen for potential involvement of efflux pumps (Figure S1c) and analysis by qPCR and immunoblot (Figure 1b) all implicating ABCG2. Cell proliferation experiments revealed that the ABCG2 inhibitor Ko143 restored asciminib effectiveness against K562$^{asciminib\text{-}R}$ cells but had no effect on the asciminib IC_{50} for K562 cells (Figure 1c). Similar results were obtained for LAMA84$^{asciminib\text{-}R}$ (Figure S2; Table S1, S2) and KYO1$^{asciminib\text{-}R}$ cells (Figure S3; Table S1, S2). Our findings support ABCG2-mediated efflux of asciminib as the major mechanism of resistance in these cell lines, warrant its profiling among patients with asciminib resistance in the clinic, and suggest that combining asciminib with an ABCG2 inhibitor could override resistance, though development of clinical ABCG2 inhibitors is at the investigational stage[7, 8].

In contrast, mutation-based resistance mechanisms were observed in Ba/F3 BCR-ABL1$^{asciminib\text{-}R}$ and KCL-22$^{asciminib\text{-}R}$ cells. In Ba/F3 BCR-ABL1$^{asciminib\text{-}R}$ cells, a >1,000-fold increase in asciminib IC_{50} over Ba/F3 BCR-ABL1 cells was observed, despite undiminished sensitivity to ATP-site TKIs (Figure 2a; Table S1). While immunoblot analysis demonstrated restored BCR-ABL1 signaling in Ba/F3 BCR-ABL1$^{asciminib\text{-}R}$ cells treated with asciminib (Figure S4a), LC-MS/MS analysis showed similar amounts of asciminib in Ba/F3 BCR-ABL1 and Ba/F3 BCR-ABL1$^{asciminib\text{-}R}$ cells and there was no evidence of efflux pump involvement (Figure S4b–e). NGS and Sanger sequencing of the *BCR-ABL1* kinase domain identified a novel BCR-ABL1^{C464W} mutation (Table S2), which was demonstrated through computational modeling to block access of asciminib to the myristoyl-binding pocket, consistent with high-level resistance (Figure 2b). While other BCR-ABL1 mutations within or near the myristoyl-binding pocket (e.g. A337V; P465S; V468F) have been recently reported to confer asciminib resistance[1], this is the first report of BCR-ABL1^{C464W} as an asciminib-resistant mutant.

KCL-22$^{asciminib\text{-}R}$ cells exhibited two mutations at similar allelic frequencies[9]: BCR-ABL1^{M244V} near the ATP site and BCR-ABL1^{A337V} in the myristoyl-binding pocket (Table S2), and single-cell sorting followed by clonal sequencing revealed a BCR-ABL1$^{M244V/A337V}$ compound mutation[10, 11]. KCL-22$^{asciminib\text{-}R}$ cells were completely insensitive to asciminib (IC_{50}: 10,000 nM as compared to 2.2 nM for parental KCL-22 cells), but remained sensitive to ATP-site TKIs (Figure 2c; Figure S5a; Table S1). LC/MS-MS showed comparable intracellular asciminib levels in KCL-22$^{asciminib\text{-}R}$ and KCL-22 cells, and there was no evidence of either altered effectiveness of asciminib against KCL-22$^{asciminib\text{-}R}$ cells by inclusion of an efflux pump inhibitor or upregulation of an efflux

pump by qPCR or ABCG2 immunoblot (Figure S5b–e). Sequencing of increasingly asciminib-resistant cells collected in the process of generating the final KCL-22$^{asciminib\text{-}R}$ cell line revealed a progression from BCR-ABL1^{A337V} to BCR-ABL1$^{M244V/A337V}$ (Figure 2d). The reported asciminib IC_{50} value of 702 nM for KCL-22^{A337V} cells[1] is consistent with the inability of this mutation alone to confer the high-level resistance observed in KCL-22$^{asciminib\text{-}R}$ cells. The BCR-ABL1$^{M244V/A337V}$ compound mutant confers high-level asciminib resistance but inclusion of clinically achievable concentrations of imatinib lead to outgrowth of BCR-ABL1$^{S229P/T315I}$ at the expense of BCR-ABL1$^{M244V/A337V}$ (Figure S6), highlighting a potential complication of addressing asciminib resistance by switching to or including an ATP-site TKI.

Taken together, our findings suggest that mechanisms of acquired resistance to the allosteric BCR-ABL1 inhibitor asciminib involve restoration of BCR-ABL1 signaling, due to either asciminib efflux or select *BCR-ABL1* mutations. It may be possible to override ABCG2-mediated drug efflux by dose escalation of asciminib. The achievable clinical dose is estimated to be at least 1 μM[12]. Another possibility to circumvent this resistance mechanism is to design a next-generation allosteric inhibitor that is not a substrate for efflux pump(s). While development of a next-generation allosteric inhibitor[13] is possible, the potential for cross-resistant myristoyl-binding pocket mutations may be high, as those observed for asciminib to date completely block inhibitor access to the binding pocket. Alternatively, myristoyl-binding site mutation-based resistance to asciminib could be countered with a TKI that binds at the ATP site, though such a mutation occurring in tandem with an additional kinase domain mutation could result in resistance to both types of inhibitor. The strategy of simultaneously treating with asciminib and an ATP-site TKI (imatinib, nilotinib or dasatinib) to minimize opportunity for resistance is under clinical investigation. Ponatinib, which has activity against the T315I mutant and reported potency as an ABCG2 inhibitor[14, 15], may also warrant consideration in this setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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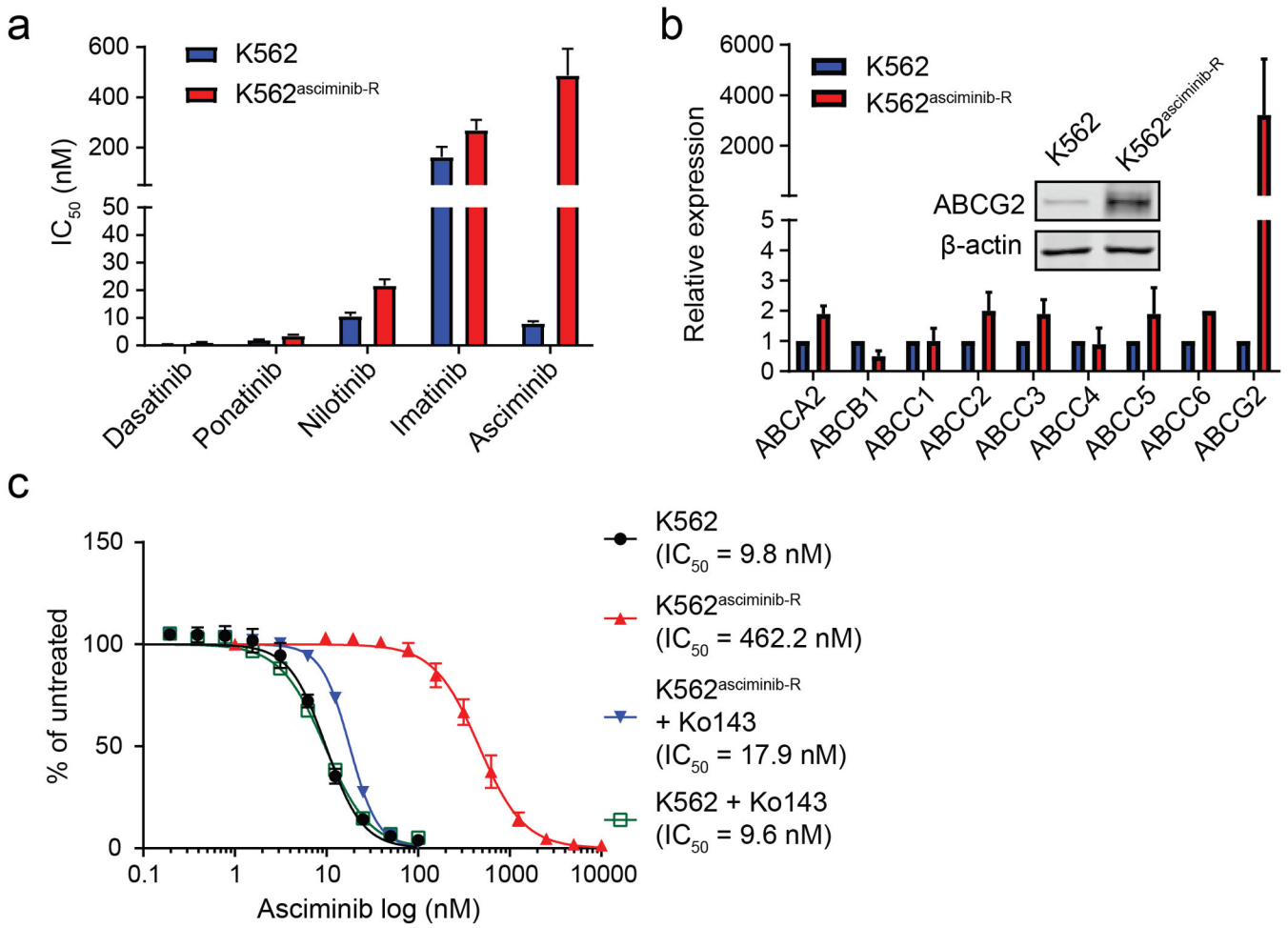

Figure 1.

Upregulation of the ABCG2 efflux pump eliminates asciminib from K562$^{\text{asciminib-R}}$ cells and confers high-level resistance to asciminib. (**a**) K562$^{\text{asciminib-R}}$ cells retain sensitivity to TKIs that target the ATP site. (**b**) qPCR of candidate efflux pumps and (*inset*) ABCG2 immunoblot confirm ABCG2 upregulation in K562$^{\text{asciminib-R}}$ cells. (**c**) Inclusion of the ABCG2 inhibitor, Ko143, restores asciminib sensitivity to K562$^{\text{asciminib-R}}$ cells.

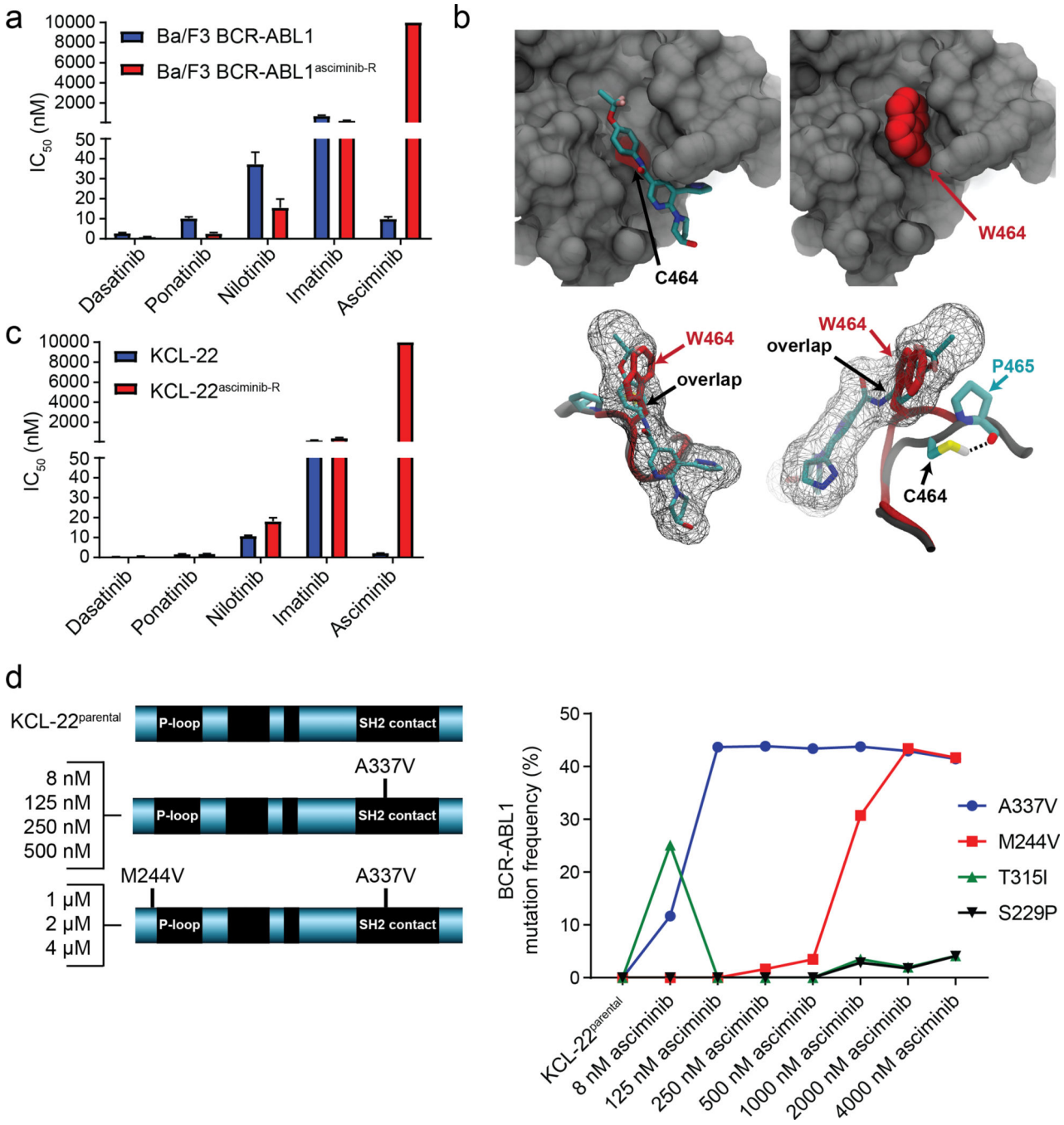

Figure 2.

BCR-ABL1 mutations in the myristoyl-binding pocket and at a remote site confer asciminib resistance. (**a**) Myristoyl-binding site mutation BCR-ABL1^{C464W} confers high-level resistance to asciminib but not to ATP-site TKIs. (**b**) Structural analysis of the C464W mutation in the allosteric myristoyl-binding pocket. (*upper left*) Close-up view of the asciminib-bound allosteric pocket. For visualization of the deeper pocket some residues are not shown. The C^{α} position of the C464 residue is highlighted in red, while the sidechain is partially buried inside the pocket. (*upper right*) C464W mutation occupies the deep

hydrophobic cleft due to its bulky sidechain, making critical interaction with the αI helix, while blocking access of asciminib. The mutated residue is shown as a space-filling model (highlighted in red). (*lower*) Two sideviews of the asciminib-binding pocket show the native and C464W mutant sidechain alignments with respect to asciminib binding. The first image is oriented to match the preceding images in this panel. The second image is rotated to allow visualization of hydrogen-bonding interaction. The sidechain of C464 is buried in the interior of the protein and participates in thiol-mediated hydrogen bonding with the P465 residue. However, on mutation to W464, the tryptophan sidechain is sterically incompatible with P465 and is forced to occupy the myristoyl-binding pocket, interfering with asciminib binding. (**c**) KCL-22$^{\text{asciminib-R}}$ cells exhibit high-level resistance to asciminib but not to ATP-site TKIs. (**d**) NGS sequencing of KCL-22 cells, increasingly asciminib-resistant cells collected in the process of generating the final resistant line and the KCL-22$^{\text{asciminib-R}}$ cell line reveals a progression from BCR-ABL1 through BCR-ABL1$^{\text{A337V}}$ to BCR-ABL1$^{\text{M244V/A337V}}$.