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Characterization of Asciminib-Resistant Philadelphia Chromosome-Positive Cells

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Abstract

Background: Asciminib is approved for treating patients with chronic-phase chronic myeloid leukemia who were previously treated with two or more tyrosine kinase inhibitors or those with *T315I* mutation. However, the mechanisms underlying asciminib resistance remain unclear.

Methods: In this study, we established a new asciminib-resistant cell line. We examined *BCR::ABL1* gene mutation analysis and the effects of conventional chronic myelogenous leukemia inhibitors.

Results: Direct sequencing revealed *Y139D* and *T315I* mutations in asciminib-resistant cells. Ponatinib and omacetaxine were effective against asciminib-resistant cells.

Conclusions: *Y139D* and *T315I* mutations are extremely resistant to asciminib. Ponatinib and omacetaxine show potential for treating asciminib-resistant chronic myeloid leukemia.

Keywords: Chronic myeloid leukemia; Ponatinib; Omacetaxine; Asciminib; Philadelphia-positive cell

Introduction

ABL tyrosine kinase inhibitor (ABL TKI) therapies have improved the prognosis of chronic myeloid leukemia (CML) [1]. Imatinib was the first TKI approved for treating patients with chronic-phase CML (CML-CP). However, some patients with CML experience TKI intolerance or resistance. It has been estimated that more than 25% of patients diagnosed with CML will require a change in TKI therapy at some during their lifetime. This necessity arises from either intolerance or resistance to the initial TKI treatment [2]. Several mechanisms of resistance to ABL TKIs have been identified. ABL TKIs retain effectiveness against the majority of ABL1 kinase domain mutations, including those associated with resistance to ABL inhibitors such as *T315I* [2]. Clinically, the four commercially available frontline treatment options for CML TKIs are imatinib, dasatinib, nilotinib, and bosutinib [1].

Asciminib is a first-in-class drug that specifically targets the ABL myristoyl pocket (STAMP) inhibitor and suppresses *BCR::ABL1* kinase activity through allosteric binding [3]. Asciminib is indicated for the treatment of patients with CML-CP who previously received two TKIs or have a *T315I* mutation [4]. The ASCEMBL trial, a phase 3 study comparing asciminib to bosutinib in patients with CML-CP who were previously treated with at least two TKIs, demonstrated that larger number of patients in the asciminib group sustained treatment and achieved long-term benefits. These findings suggest that asciminib should be considered as a standard treatment option for patients who have been treated with at least two TKIs [5]. The mechanisms underlying asciminib resistance remain unknown. In this study, we established an asciminib-resistant Ba/F3 cell line (Ba/F3-asciminib-R: Ba/F3 asc-R) and evaluated its drug sensitivity.

Materials and Methods

Reagents

Ponatinib, dasatinib, and homoharringtonine were obtained from MedKoo Biosciences (Chapel Hill, NC, USA), LC Laboratories (Woburn, MA, USA), and MedChemExpress (Monmouth Junction, NJ, USA), respectively. Asciminib (ABL001), a selective *BCR::ABL* inhibitor, was obtained from ActiveBiochem (Maplewood, NJ, USA). Imatinib and nilotinib were supplied by Novartis Pharma AG (Basel, Switzerland). The inhibitors were prepared as stock solutions in dimethyl sulfoxide, and imatinib was dissolved in distilled water, aliquoted, and stored at -20 °C.

Cell line and cell culture

Parental Ba/F3 cells were transduced with the *BCR::ABL1* gene and better designated as *BCR::ABL*-expressing Ba/F3 cells, and Ba/F3 *BCR::ABL* cells containing the *T315I*: Ba/F3 *T315I* point mutation were described previously [6]. The cells were cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum at 37

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°C in 5% CO_2 and passaged for less than 1 month before replacement from early passage frozen stocks.

BCR::ABL1 **kinase domain mutation analysis**

Semi-nested reverse transcription polymerase chain reaction (PCR) was performed to amplify *BCR::ABL1* fusion transcripts, and then the transcripts of the *ABL1* kinase domain were amplified in a second round of PCR. Total RNA was extracted from the cells using a QIAamp RNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Semi-nested PCR was performed using Platinum Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) and PrimeScript RT Master Mix (Perfect Real Time) from Takara Bio (Shiga, Japan) for DNA amplification. The following PCR primers were used in semi-nested PCR and sequencing reactions: NM_minor forward 5′-GGAGTACCAGCCCTACCA-GA-3′ and reverse 5′-AGAAGGCGCTCATCTTCATT-3′; NM_ABL_3-6 forward 5′-ACTTTGAGCCTCAGGGTCTG-3′ and reverse 5′-ACTTTGAGCCTCAGGGTCTG-3′, NM_ABL_6-9 forward 5′-ACACCATGGAGGTGGAAGAG-3′ and reverse 5′-AGAAGGCGCTCATCTTCATT-3′; NM2_ABL1_253-359 forward 5′-TACGACAAGTGGGAGATGGA-3′ and reverse 5′-CAATACTCCAAATGCCCAGA-3′. Sequencing was performed using a BigDye Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific), and DNA was sequenced on an ABI PRISM 3130xl DNA Analyzer (Thermo Fisher Scientific). The optimal PCR conditions were not disclosed by SRL Medisearch. We compared these sequences to the wild-type ABL sequence of a normal control human sample (GenBank accession number X16416.1).

Cell proliferation assay

CML cells were treated with the indicated concentrations of chemicals for 72 h, after which cell proliferation was analyzed using Cell Counting Kit-8 (Dojindo Laboratories, Mashiki-machi, Kumamoto, Japan). Absorbance was measured at 450 nm using an EnSpire Multimode Plate Reader (PerkinElmer, Waltham, MA, USA).

Caspase 3/7 activity

To examine caspase activity, we utilized a Caspase Glo® 3/7 assay kit from Promega (Madison, WI, USA), following the manufacturer's instructions. The luminescence of each sample was quantified using an EnSpire Multimode Plate Reader after 48-h incubation with the specified concentrations of chemicals.

Cytotoxicity assay

Cytotoxicity towards CML cells exposed to the specified concentrations of chemicals for 48 h was assessed. Lactate dehydrogenase (LDH) release from cells was used as an indicator for cytotoxicity evaluation, utilizing the Cytotoxicity LDH Assay Kit-WST (Dojindo Laboratories). The EnSpire Multimode Plate Reader was used to measure the absorbance at 490 nm, which represented the quantity of LDH released from dead cells.

Statistical analyses

GraphPad Prism version 10 software (GraphPad, Inc., San Diego, CA, USA) was used to analyze all data. Two-tailed Student's *t*-tests were used to evaluate statistical significance. If one of the groups in the study is considered the control group, data were analyzed using Dunnett's test as the *post-hoc* test following analysis of variance. When comparing three or more samples, data were examined using one-way analysis of variance with Turkey *post-hoc* comparison tests with alpha = 0.05 ($n \geq 3$). Significance was expressed as *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

Results

Establishment of asciminib-resistant cells and sequence of *BCR::ABL1*

We generated cells resistant to asciminib to examine the resistance mechanism. Ba/F3 *T315I* mutant cells were cultured in medium containing low concentrations of asciminib, which was changed twice per week while adjusting the concentration of asciminib every 2 weeks. Asciminib concentrations were higher than 1 µM and we established resistant strains after 2 months. We established the asciminib-resistant Ba/F3 cell line (Ba/F3 asc-R). Direct sequencing is commonly performed to identify point mutations in oncogenic *BCR::ABL1*. Direct sequencing of *BCR::ABL1* revealed *Y139D* and *T315I* mutations in Ba/F3 asc-R cells (Fig. 1). Cells with *Y139D* and *T315I* mutations were resistant to asciminib.

Drug sensitivity of asciminib-resistant cells

Ba/F3 asc-R cells were resistant to high doses of asciminib, with an IC_{50} of up to 10 µM (Fig. 2a). In contrast, Ba/F3 *T315I* cells were sensitive to lower concentrations of asciminib. Ba/F3 *T315I* cells were sensitive to asciminib, indicating that this cell line is a single asciminib-resistant clone. Ponatinib is used to treat specific types of leukemia, such as CML and Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ ALL) [7]. As a third-generation kinase inhibitor, ponatinib was developed to overcome the gatekeeper *T315I* mutation [8]. Ponatinib was effective against Ba/F3 *T315I* cells, and a high dose of ponatinib was effective against Ba/F3 asc-R cells (Fig. 2a).

Omacetaxine mepesuccinate (homoharringtonine) has been approved for the treatment of adult patients with CML-CP and accelerated-phase CML who are resistant to two or more TKIs such as imatinib, dasatinib, or nilotinib [9]. At lower doses, omacetaxine inhibited the development of Ba/F3 asc-R cells (Fig. 2a). Other ABL TKIs (imatinib, nilotinib, and dasatinib) had no

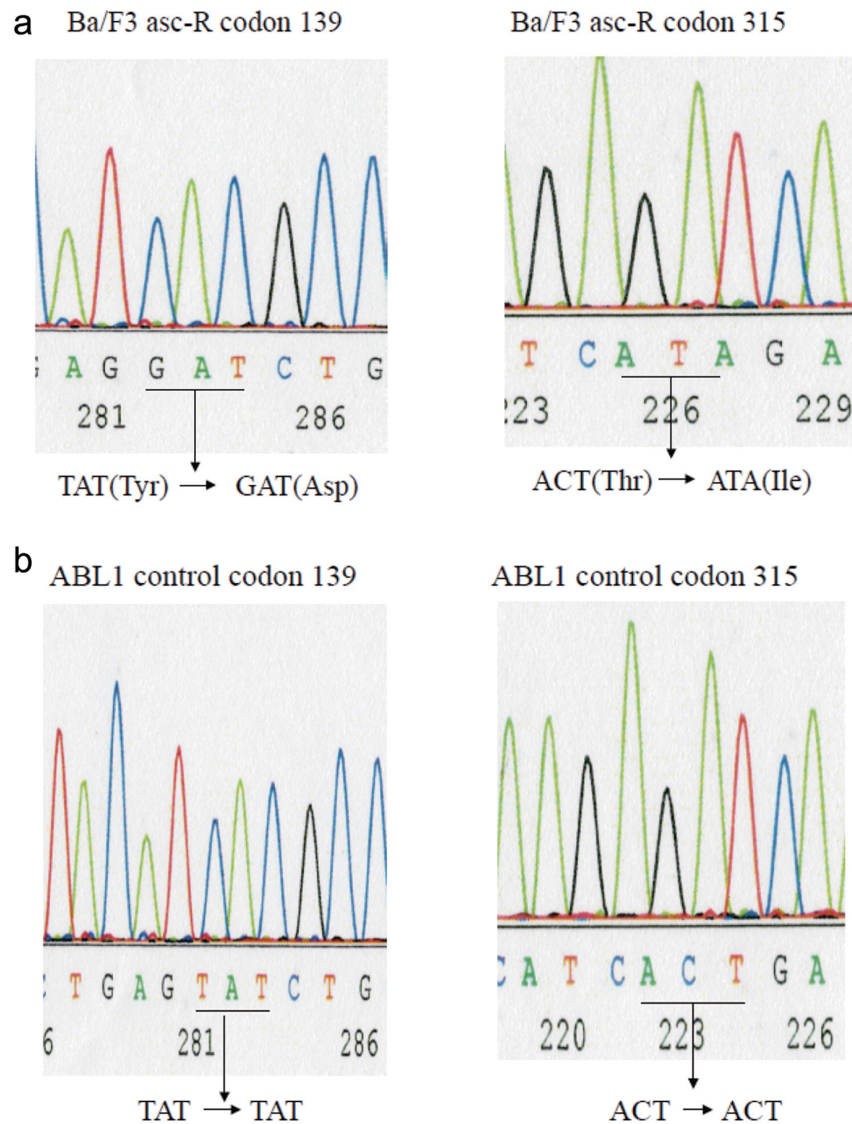

Figure 1. *BCR-ABL* kinase domain mutation analysis. Mutation screening via sequence analysis was performed to identify the ABL kinase domain in Ba/F3-asciminib-R (Ba/3 asc-R) cells (a) and normal control samples (b). Direct sequencing to detect mutations in the ABL kinase domain in the cell lines revealed *Y139D* and *T315I* mutations in Ba/F3 asc-R cells.

effect on Ba/F3 *T315I* or Ba/F3 asc-R cells (Fig. 2b). Asciminib was cytotoxic towards Ba/F3 *T315I* cells because not Ba/F3 asc-R cells (Fig. 2C). Additionally, ponatinib and omacetaxine induced dose-dependent cytotoxicity in Ba/F3 *T315I* and Ba/F3 asc-R cells (Fig. 2c). Asciminib increased caspase 3/7 activity in Ba/F3 *T315I* cells but not in Ba/F3 asc-R cells (Fig. 2d). High concentrations of ponatinib enhanced caspase 3/7 activity in Ba/F3 asc-R cells (Fig. 2d). Omacetaxine also enhanced caspase 3/7 activity in Ba/F3 *T315I* and Ba/F3 asc-R cells (Fig. 2d).

Discussion

Asciminib is an investigational *BCR::ABL1* TKI that is currently being studied for its potential for treating CML. This drug was designed to target *BCR::ABL1* protein in a unique manner compared with traditional TKIs such as imatinib, dasatinib, nilotinib, or ponatinib by specifically targeting the myristoyl pocket of ABL1 [10]. Protein kinase activity is regulated by various molecular pathways, and its disruption is a typical cause of oncogenesis. ABL inhibitors that bind to regulatory regions reduce kinase activity [3]. As compared with ATP-competitive inhibitors, allosteric inhibitors are highly selective for ABL kinases. Asciminib has high affinity for myristoyl-binding sites [3]. The NH_2 terminus of ABL contains three SRC homology domains (SH1-SH3) [11]. The SH1 domain functions as a tyrosine kinase, whereas the SH2 and SH3 domains enable protein interactions. The inactive and

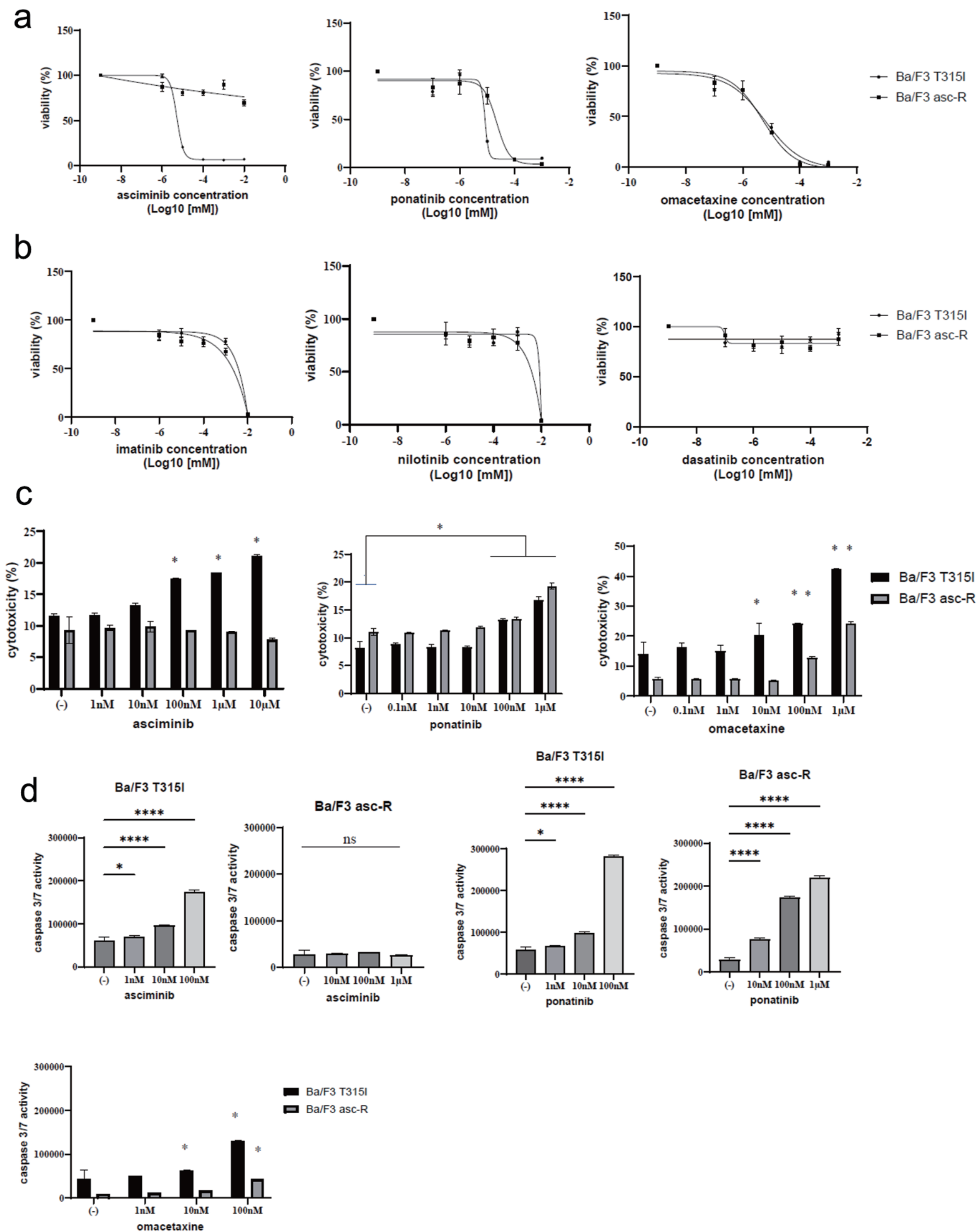

Figure 2. Activity of ABL inhibitors in Ba/F3-asciminib-R (Ba/F3 asc-R) and Ba/F3 *T315I* cells. (a) Ba/F3 asc-R and Ba/F3 *T315I* cell lines were cultured with the indicated concentrations of asciminib, ponatinib, or omacetaxine for 72 h. Cell growth was evaluated using Cell Counting Kit-8. (b) Ba/F3 asc-R and Ba/F3 *T315I* cell lines were cultured with the indicated concentrations of imatinib, nilotinib, or dasatinib for 72 h. Cell growth was evaluated using Cell Counting Kit-8. (c) Ba/F3 asc-R and Ba/F3 *T315I* cell lines were cultured with the indicated concentrations of asciminib, ponatinib, or omacetaxine for 72 h. Cell death was evaluated using a Cytotoxicity LDH Assay Kit. *P < 0.05 compared to control. (d) Ba/F3 asc-R and Ba/F3 *T315I* cell lines were cultured with the indicated concentrations of asciminib, ponatinib, or omacetaxine for 48 h. Caspase 3/7 activity was evaluated. *P < 0.05, **P < 0.01, ****P < 0.0001 compared to control. ns: not significant.

active forms of ABL kinases are reportedly regulated by dynamic intramolecular interactions that modulate ABL kinase activity [12]. The SH3 domain binds to the linker sequence that connects the SH2 and kinase (SH1) domains, and the SH2 domain interacts with the C-terminal lobe of the kinase domain to form an SH3-SH2 clamp structure that locks the kinase in an inactive state [13]. Myristoyl pocket mutations have either been clinically documented (A337T, P465S, and V468F) or predicted using *in vitro* models (A344P) [10]. Several ABL-activating mutations (*PP*, *K51A*, *W99A*, and *Y139D*) failed to confer imatinib resistance to *BCR::ABL*. The SH2 domain mutation, *Y139*, activates ABL1 by disrupting the SH2 kinase domain connection, which may be the basis for myristoyl-mediated autoinhibition. Previous research showed that *BCR::ABL1* compound mutations (*Y253H*/*T315I*) are resistant to asciminib [14]. Other *BCR::ABL1* mutations within or near the myristoyl-binding pocket confer asciminib resistance (e.g., *A337V*, *P465S*, and *V468F*) [15]. The *T315I* mutation confers resistance to all currently approved TKIS except for ponatinib, which is a third-generation drug [16].

A phase 1 study enrolled 141 patients with CML-CP and nine patients with accelerated-phase CML who had developed resistance to or experienced unacceptable side effects from at least two previous ATP-competitive TKIs. Among the patients, 12 (28%) achieved or maintained a major molecular response by the 12-month mark, with five patients showing a *T315I* mutation at the start of the study. Patients with the *T315I* mutation achieved complete cytogenetic and major molecular responses, with most receiving asciminib doses of more than 150 mg twice daily, which was higher than the required dose for patients without the *T315I* mutation [17]. Asciminib was effective in patients with CML who had not responded to ponatinib and those with the *T315I* mutation [17]. Another study showed that 42% of the 45 patients with the *T315I* mutation achieved a major molecular response by 24 weeks of asciminib treatment [18]. In ABL TKI resistance, the H-RAS T81C polymorphism was found to be associated with CML risk and prognosis [19].

Ponatinib is a third-generation kinase inhibitor developed for treating patients with the *T315I* gatekeeper mutation, which is a common resistance mutation in cancer cells. This drug targets specific kinases involved in the growth and proliferation of cancer cells and has shown promising results in clinical trials for treating various types of cancer. Ponatinib exhibits inhibitory activity against native *BCR::ABL1* kinase and several ABL1 mutations [7, 8]. Ponatinib is currently approved for treating CML in patients who are resistant and/or intolerant to dasatinib and nilotinib, as well as in those who can no longer take imatinib or have the *T315I* mutation [7, 8]. Omacetaxine is a distinct inhibitor of protein synthesis that does not overlap with kinase inhibition. Numerous studies demonstrated that omacetaxine can yield responses in patients who have been extensively treated for either CML-CP or accelerated-phase CML, regardless of whether they possess tyrosine kinase domain mutations [9]. Ponatinib and omacetaxine mepesuccinate may be effective against asciminib-resistant strains, resulting in clinical improvement. To the best of our knowledge, this is the first study to show that *BCR::ABL1 Y139D* and *T315I* is an asciminib-resistant mutant. Because we were unable to obtain cells bearing the *BCR::ABL1 Y139D* mutation, a *Y139D* mutant cell line must be generated to explore the impact of asciminib. Asciminib is currently used in clinical practice; however, resistance to asciminib may develop. In such cases, alternative medications such as ponatinib or omacetaxine may be effective for treating patients with asciminib resistance. We determined the mechanisms of ABL TKI resistance and demonstrated the clinical potential of ponatinib and omacetaxine mepesuccinate for treating patients with asciminib-resistant CML.

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Conflict of Interest

AG received research funding from Eisai Co., Ltd., Ono Pharmaceutical Co., Ltd., Taiho Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., Nippon Shinyaku Co., Ltd., Chugai Pharmaceutical Co., Ltd., MSD K.K., Otsuka Pharmaceutical Co., Ltd., Sumitomo Pharma Co., Ltd., Nippon Shinyaku Co., Ltd., Bayer Yakuhin, Ltd., Daiichi Sankyo Co., Ltd., and Nihon Pharmaceutical Co., Ltd. AG received honoraria from Novartis Pharma K.K., Alexion Pharmaceuticals, Inc., Eisai Co., Ltd., Ono Pharmaceutical Co., Ltd., Taiho Pharmaceutical Co., Ltd., Takeda Pharmaceutical Co., Ltd., Nippon Shinyaku Co., Ltd., Chugai Pharmaceutical Co., Ltd., Otsuka Pharmaceutical Co., Ltd., Sumitomo Pharma Co., Ltd., Daiichi Sankyo Co., Ltd., Nihon Pharmaceutical Co., Ltd., Kyowa Kirin

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Informed Consent

Not applicable.

Author Contributions

SO and MM designed the study. SO and AG wrote the manuscript. SO performed the experiments and assisted in creating figures. The final manuscript was read and approved by all authors.

Data Availability

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

Abbreviations

ABL TKI: ABL tyrosine kinase inhibitor; CML: chronic myeloid leukemia; CML-CP: chronic-phase CML; PCR: polymerase chain reaction; SH: SRC homology domains; STAMP: specifically targets the ABL myristoyl pocket

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