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BCR-ABL1 tyrosine kinase inhibitor K0706 exhibits preclinical activity in Philadelphia chromosome-positive leukemia

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BCR-ABL1 tyrosine kinase inhibitors (TKIs) are the cornerstone of treatment in chronic myeloid leukemia. Although there are now four TKIs approved for use in the front-line setting, acquired TKI resistance via secondary kinase domain mutations remains a problem for patients. K0706 is a novel BCR-ABL1 TKI currently under clinical investigation with structural elements similar to those of ponatinib and dasatinib. In this article, we functionally characterize the anti-leukemic activity of K0706 using cell proliferation assays in conjunction with drug resistance screening. We provide details from molecular modeling to support our in vitro findings and additionally describe our limited clinical experience with this drug in two patients treated on trial. We demonstrate that although K0706 retains efficacy against a large spectrum of clinically relevant mutations, it does not appear to have activity against BCR-ABL1^{T315I}. Early trial experience suggests excellent tolerability, which may positively affect the place of K0706 within the ever-expanding chronic myeloid leukemia treatment paradigm.

Patients with chronic-phase CML (CP-CML) are effectively managed with BCR-ABL1 tyrosine kinase inhibitors (TKIs), and their survival approaches that of age-matched controls [1]. K0706 was designed as a BCR-ABL1 TKI to provide an option for patients experiencing resistance or intolerance to the first-line TKIs imatinib, nilotinib, dasatinib, and bosutinib [2,3]. Structural elements that overlap with dasatinib and ponatinib are noted (Figure 1A).

Methods

See Supplemental Information (online only, available at www.exphem.org).

Results and discussion

Cellular proliferation assays established that K0706 is a potent inhibitor of BCR-ABL1 (IC_{50}: 7 nmol/L) and exhibits activity against most clinically important BCR-ABL1 point mutants. The only BCR-ABL1 point mutants with an IC_{50} >100 nmol/L were: BCR-ABL1^{L248R} (IC_{50}: 167 nmol/L), BCR-ABL1^{Y253H} (IC_{50}: 154 nmol/L), BCR-ABL1^{E255V} (IC_{50}: 165 nmol/L), and BCR-ABL1^{T315I} (IC_{50}: 1967 nmol/L) (Figure 1B). K0706 was compared with the BCR-ABL1 TKIs bosutinib, dasatinib, nilotinib, and ponatinib across a comprehensive panel of cell lines expressing TKI-resistant mutants of BCR-ABL1 (Supplementary Figure E1, online only, available at www.exphem.org). Immunoblot analysis demonstrated direct, potent inhibition of BCR-ABL1 tyrosine autophosphorylation by K0706, as well as inhibition of BCR-ABL1^{L248R}, BCR-ABL1^{Y253H}, and BCR-ABL1^{E255V} at high concentrations of inhibitor. BCR-ABL1^{T315I} was not inhibited to a level reaching the IC_{50} at any of the concentrations of K0706 tested (Figure 1C).

To establish the point mutation-based resistance profile of K0706, an ENU-based resistance screen was performed

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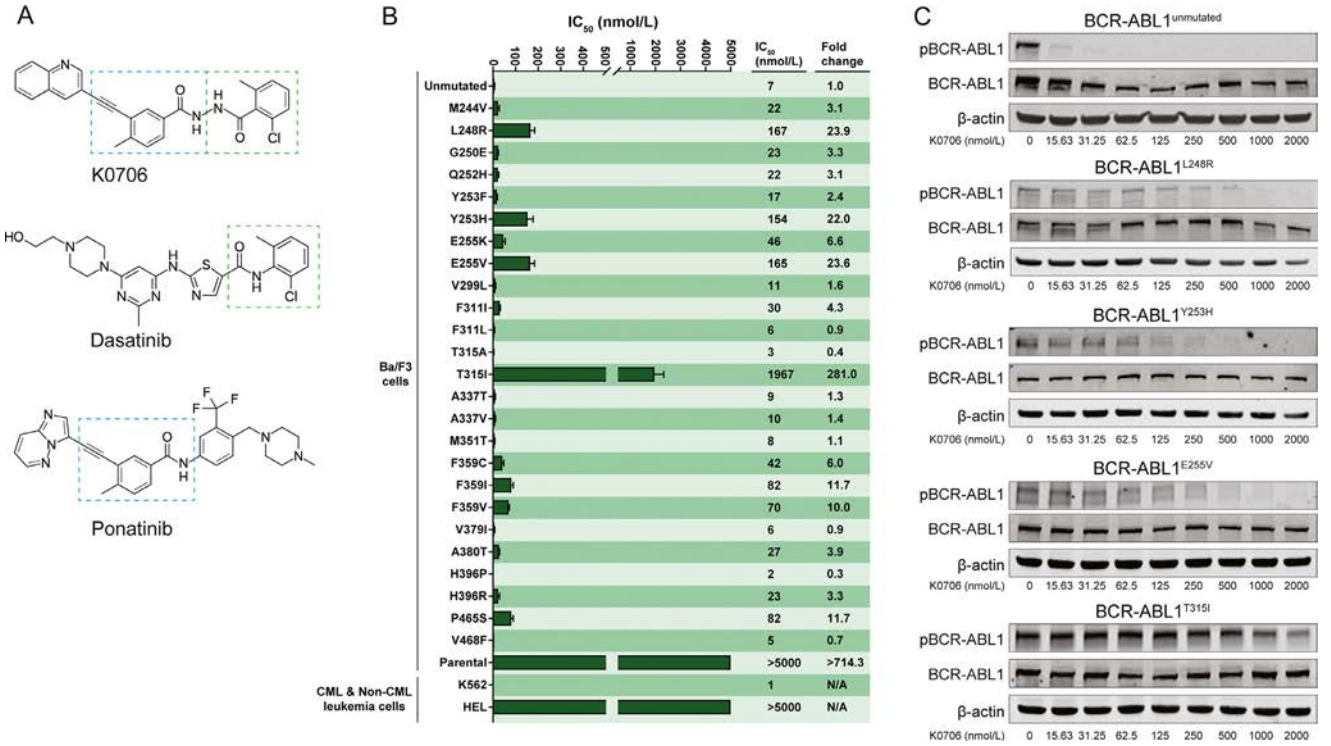

Figure 1. K0706 inhibits native and mutant BCR-ABL1. **(A)** Structure of K0706. The *boxed areas* indicate substructure elements shared with ponatinib (*blue box*) and dasatinib (*green box*), which are shown for comparison. **(B)** Cellular IC_{50} values for K0706 in BCR-ABL1-positive and −negative cell lines. In Supplementary Table E1, K0706 is compared with the BCR-ABL1 TKIs bosutinib, nilotinib, dasatinib, and ponatinib. **(C)** Immunoblot analysis of phosphorylation of BCR-ABL1 (pBCR-ABL1) in Ba/F3 cells expressing BCR-ABL1, BCR-ABL1^{L248V}, BCR-ABL1^{Y253H}, BCR-ABL1^{E255V}, or BCR-ABL^{T315I} following exposure to K0706.

using Ba/F3 BCR-ABL1 cells. K0706 exhibited a narrow scope of BCR-ABL1 mutations (Figure 2A; Table E1, online only, available at www.exphem.org), and the resistance profile narrowed exclusively to BCR-ABL1^{T315I} at concentrations >800 nmol/L. A dose-finding study is current ongoing, and achievable plasma drug concentrations are not yet known. Based on our in vitro results, we speculate that BCR-ABL1^{T315I} is beyond the reach of K0706 and that three additional mutants (BCR-ABL1^{L248R}; BCR-ABL1^{Y253H}; BCR-ABL1^{E255V}) confer resistance to this investigational TKI. All other tested BCR-ABL1 point mutants are expected to be sensitive to K0706. Molecular modeling provides a structural basis for the reduced potency of K0706 against BCR-ABL1^{L248R}, BCR-ABL1^{Y253H}, BCR-ABL1^{E255V}, and BCR-ABL1^{T315I}. The binding mode is predicted to be similar to the type II inactive binding mode accessed by ponatinib (Figure 2B), and the two structurally related inhibitors bind in a strikingly similar conformation (Figure 2C). As in the case of ponatinib, the Y253H and E255V mutations cause local re-orientations within the phosphate-binding loop that result in lowered inhibitor affinity (Figure 2D) [4,5]. The resistance profile of K0706 demonstrated high-level resistance to the L248R mutant, in contrast to the resistance profile of ponatinib. BCR-ABL1^{L248R} has been reported clinically, and was found to be highly resistant against imatinib, bosutinib, dasatinib, and nilotinib and intermediately resistant against ponatinib (IC_{50}: 13 nmol/L) [6]. In our experiments, the IC_{50} values were 2.8 nmol/L (ponatinib) and 167 nmol/L (K0706). P-Loop residues interact favorably with the aromatic ring of the type II inhibitor, and any mutation in this region can cause local perturbation and potential steric clashes with the inhibitor, thereby altering its binding affinity significantly. Leu248 (which is part of the P-loop) interacts with the quinoline ring of K0706, which is larger than the imidazopyridazine ring system of ponatinib. Based on the structural alignment, we hypothesize that upon introduction of the K-to-R substitution at residue 248, a steric clash with K0706 significantly reduces binding affinity (Figure 2D). Although ponatinib binding is also adversely affected, the magnitude is not as large. The most important mutational liability for K0706 is BCR-ABL1^{T315I}. Minor differences in the alignment of K0706 as compared with ponatinib within the binding pocket bring the inhibitor into direct contact with the side chain of I315 and carbonyl group of K0706, an interaction that is incompatible with high-potency binding (Figure 2D).

K0706 is currently in clinical evaluation for several indications. A multicenter phase 1/2 trial of K0706 in adult patients with chronic, accelerated, or blast-phase

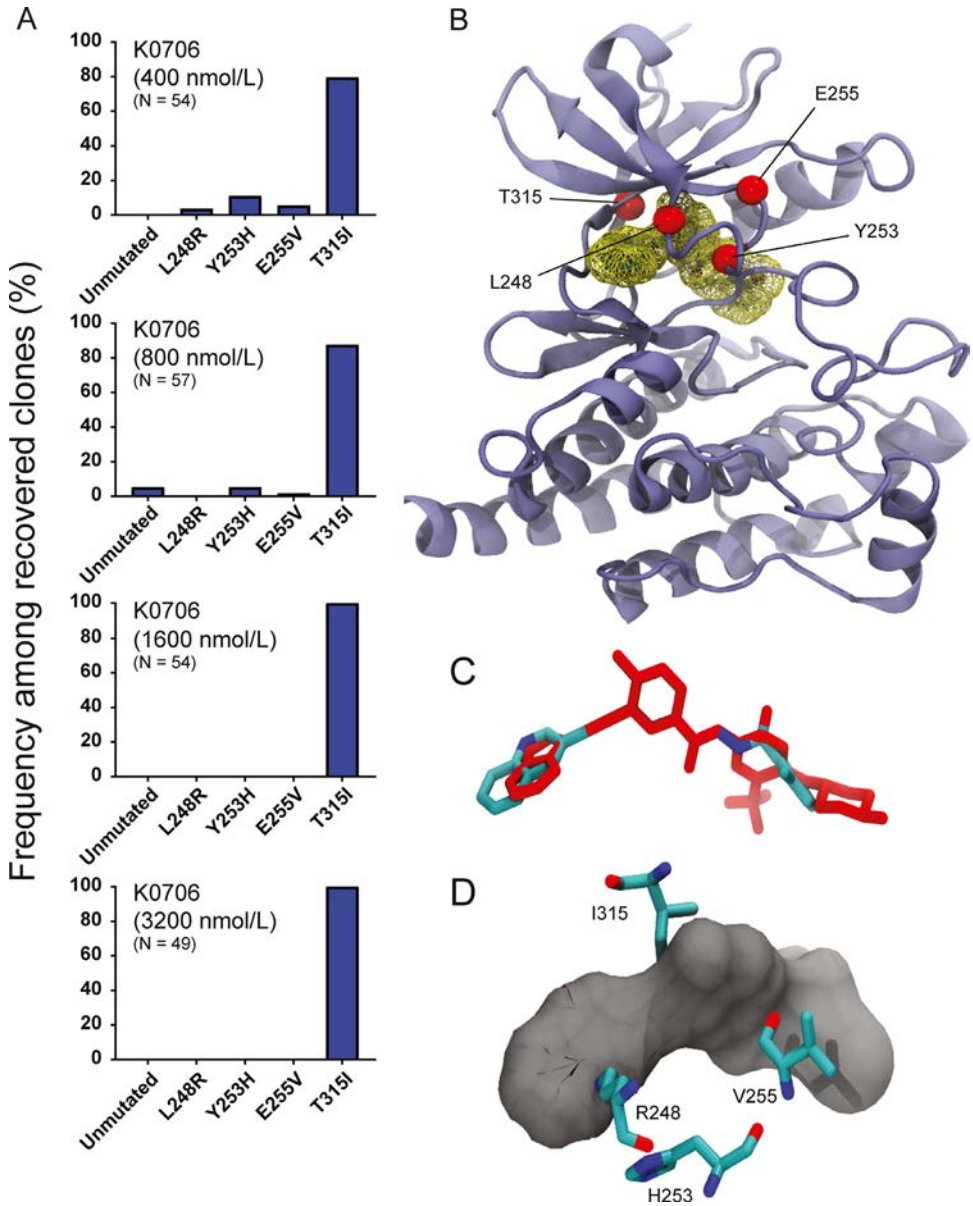

Figure 2. K0706 exhibits few BCR-ABL1 mutational vulnerabilities. **(A)** BCR-ABL1 mutants recovered from cell-based resistance screens for K0706, starting from Ba/F3 BCR-ABL1 cells. ENU-mutagenized cells were plated with inhibitor, monitored for outgrowth, expanded, and sequenced for mutations. *Bars* represent frequency of a given mutant among all recovered clones at a given inhibitor concentration; the percentage of wells demonstrating outgrowth and number of clones sequenced is indicated. **(B)** K0706 in ABL1 binding site, with residues identified in the resistance screen highlighted. **(C)** K0706 (*cyan*) superimposed on ponatinib (*red*) based on x-ray structure. **(D)** Close-in view of K0706 in ABL1 binding site, with mutated residues identified in the resistance screen shown in ball-and-stick representation.

CML or Ph+ acute lymphoblastic leukemia refractory to or intolerant of at least three TKIs is enrolling patients in its two-part dose escalation and open label arms (SPARC: NCT02629692). K0706 is also currently being studied in a multisite, phase 2 randomized, placebo-controlled trial (PROSEEK: NCT03655236) in patients with early Parkinson's disease. The basis for this study is preclinical data indicating that c-ABL inhibition reverses neurodegeneration related to α-synuclein accumulation and early clinical data suggesting cognitive improvement with nilotinib treatment [7,8]. A concomitant Sun Pharma-sponsored study is enrolling healthy volunteers to evaluate the pharmacokinetics of K0706 in cerebrospinal fluid (NCT03445338).

Two CML patients at our institution have received K0706 in the clinical trial setting (Part B: NCT02629692). Patient 1 is a 29-year-old man diagnosed with CP-CML in 2002 and initially treated with imatinib (400 mg daily) who lost molecular and cytogenetic response in 2006. Mutation analysis following imatinib failure did not reveal

a BCR-ABL1 kinase domain mutation, and he was switched to dasatinib (140 mg daily), which was subsequently dose-reduced to 50 mg because of joint pain. He maintained a deep molecular response until March 2016, when his BCR-ABL1 transcript levels rose to 0.92% on the international scale (IS). The patient was switched to ponatinib (15 mg daily) but tolerated it poorly because of gastrointestinal toxicity, grade 4 lipase elevation, and headaches. Subsequently, bosutinib was prescribed, and although the patient had grade 4 lipase elevation requiring dose reduction, he achieved sustained molecular response approaching major molecular response (MMR) on substandard doses until January 2018, when his BCR-ABL1 transcript levels rose to 7% IS. Mutation analysis was negative, and the patient was switched back to ponatinib (15 mg every other day), on which he experienced a rise in BCR-ABL1 transcript level to 34% IS in July 2018 and was found to have accelerated-phase CML based on clonal evolution (karyotype 46,XY,inv(3)(q21q26.2),t(9;22)(q34;q11.2)[17]/46,XY[3]). He enrolled in the SPARC study and began treatment with K0706 in July 2018 (90 mg daily). Cytogenetics following 3 months of K0706 revealed a complete cytogenetic response, and the patient achieved MMR at 5 months with maintenance of MMR ongoing through March 2019. This dose has been well tolerated with grade 1 fatigue and hyperbilirubinemia and grade 2 lipase elevation that resolved without treatment interruption. Of note, use of approved second- and third-generation BCR-ABL1 TKIs to treat this patient was majorly limited by toxicity and inability to achieve appropriate dose intensity.

Patient 2 is a 63-year-old woman diagnosed with CP-CML in 2010 initially treated with imatinib (400 mg daily) until 2013, when she was switched to nilotinib because of peripheral edema and suboptimal molecular response. She developed pancreatitis on nilotinib and was switched to bosutinib in 2015, on which she achieved MMR in November 2016. However, in May 2017, her BCR-ABL1 transcript level increased to 7% IS. Mutation analysis revealed E255V mutation, and she was switched to dasatinib (140 mg daily) in August 2017, on which her BCR-ABL1 transcript level remained between 5% and 6% IS. Mutation analysis in December 2017 revealed E255V at 100% allele frequency. She continued on dasatinib (140 mg daily) until December 2018 and started K0706 (174 mg daily) in January 2019. BCR-ABL1 transcript percentage in January prior to initiation of K0706 was 6% IS with bone marrow cytogenetics demonstrating Ph+ in 29% (6/21) metaphases. Following 3 months of K0706 (174 mg daily), her BCR-ABL1 polymerase chain reaction (PCR) increased to 32% IS with 3-month bone marrow cytogenetics revealing an increase to 80% (16/20) Ph+ metaphases. Drug tolerance is excellent, with grade 1 fatigue.

Our preclinical evaluation of K0706 and associated molecular modeling provide a mechanistic basis for the divergent responses in the two patients. In patient 1, BCR-ABL1 kinase domain sequencing of trial entry and longitudinal on-treatment samples demonstrated exclusively native BCR-ABL1. In contrast, patient 2 carried BCR-ABL1^{E255V} from the outset, explaining the limited effectiveness of K0706.

K0706 has potential to be an important, well-tolerated new addition to the group of effective BCR-ABL1 TKIs. The spectrum of BCR-ABL1 mutants sensitive to K0706 compares favorably with all approved BCR-ABL1 TKIs except ponatinib and the allosteric inhibitor asciminib, both of which inhibit the BCR-ABL1^{T315I} gatekeeper mutant. Despite structural similarity to dasatinib, K0706 exhibits limited activity against dasatinib-sensitive mutants Y253H and E255V, corresponding to the lack of clinical response observed in patient 2. Ongoing dose escalation studies should further clarify the role of K0706 against these mutants. Despite early predictions, K0706 is not active against BCR-ABL1^{T315I} [9]. Another investigational BCR-ABL1 TKI with close structural similarity to ponatinib, PF-114, is currently in phase 1 clinical evaluation in patients with CML with failure of prior TKI therapy (NCT02885766), including failures due to BCR-ABL1^{T315I} [10]. Early results suggest PF-114 has clinical activity, but that skin toxicity may be dose limiting. Another BCR-ABL1 inhibitor, HQP1351, has demonstrated significant clinical activity in a phase 1 study of patients with TKI-resistant CML that included patients with the T315I mutation. There was a 64% rate of grade 3 or 4 toxicity, including 16% nonhematologic toxicity [11]. A phase 2 study of HQP1351 in CML patients with the T315I mutation is recruiting (NCT03883087). Given that active second- or third-generation TKIs are approved, new additions to this armamentarium will have to match available TKIs with respect to activity, tolerability, and cost.

Acknowledgments

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Conflict of Interest Disclosure

MWD is on the advisory board and is a consultant for Incyte, Novartis, and Pfizer, and serves on the advisory boards for Takeda, Blueprint, and Galena BioPharma. His laboratory receives research funding from Novartis and Pfizer.

Author Contributions

MWD and TO designed the project. OA, NAV, ADP, ABP, AVS, PMC, and TO analyzed the data. OA, ADP, MWD, and TO wrote the article. OA performed experiments. ADP, ABP, and MWD provided clinical

insights and perspective. AO, NAV, ADP, AVS, MWD, and TO critically reviewed the article.

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SUPPLEMENTAL INFORMATION

Materials and Methods

Inhibitors. K0706 was provided by Sun Pharma Advanced Research Company Ltd. All other inhibitors were purchased from Selleckchem.

Cell proliferation IC_{50} determination. IL-3 dependent murine Ba/F3 cells cultured in RPMI-1640 complete medium (ThermoFisher, 11875093) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin (ThermoFisher, 15070063), 1% L-glutamine (ThermoFisher, 25030081) and IL-3 from WEHI-conditioned media were infected with retrovirus expressing p210 *BCR-ABL1* (MSCV-IRES-GFP). Selection of infected cells was performed by IL-3 withdrawal. Clinically relevant *BCR-ABL1* point mutations were introduced using site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit; Agilent Technologies). Retrovirus generated using calcium phosphate transfection (ProFection Mammalian Transfection System; Promega) of 293FT cells and harvesting the supernatant. Ba/F3 *BCR-ABL1* and *BCR-ABL1*-mutants were plated in 96-well plates ($2x10^3$ cells/well) and incubated with indicated inhibitor for 72 hours. Inhibitor concentrations: K0706, nilotinib, dasatinib, bosutinib and ponatinib (0, 4.9, 9.8, 19.5, 39.1, 78.1, 156.3, 315.5, 625, 1250, 2500, and 5000 nmol/L); Retested sensitive cell lines ($IC_{50} \leq 3$) from original screen at (0, 0.2, 0.4, 0.8, 1.6, 3.1, 6.3, 12.5, 25, 50, 10, and 200 nmol/L). Proliferation was measured using methanethiosulfonate (MTS)-based viability assay (CellTiter 96 AQueous One Solution, Promega). IC_{50} values are reported as the mean of three independent experiments performed in quadruplicate.

BCR-ABL1 tyrosine phosphorylation immunoblot analysis. Ba/F3 cells expressing native and mutated *BCR-ABL1* were plated in 24-well plates ($2x10^6$ cells/well) and cultured in 1 mL RPMI complete medium with titrated concentrations of TKI (0, 15.6, 32.3, 62.5, 125, 250, 500, 1000, and 2000 nmol/L) for 4 hours. Cells were washed with cold PBS and lysed in M-PER (Mammalian protein extraction reagent; ThermoFisher) containing phosphatase (PhosStop, Roche) and protease inhibitor cocktails (Complete Mini, Roche) on ice for 10 min. Samples were diluted 1:1 in SDS-Page loading buffer (2x Laemmli sample buffer, Bio Rad) supplemented with 2-mercaptoethanol and denatured by boiling for 10 min at 95°C. Lysates were separated on 4-20% Tris-Glycine (Mini-PROTEAN TGX, Bio Rad) gradient gels, transferred and immunoblotted using anti-c-Abl (Ab-3) mouse mAb (24-21) (OP20, Millipore), anti-phospho-c-Abl

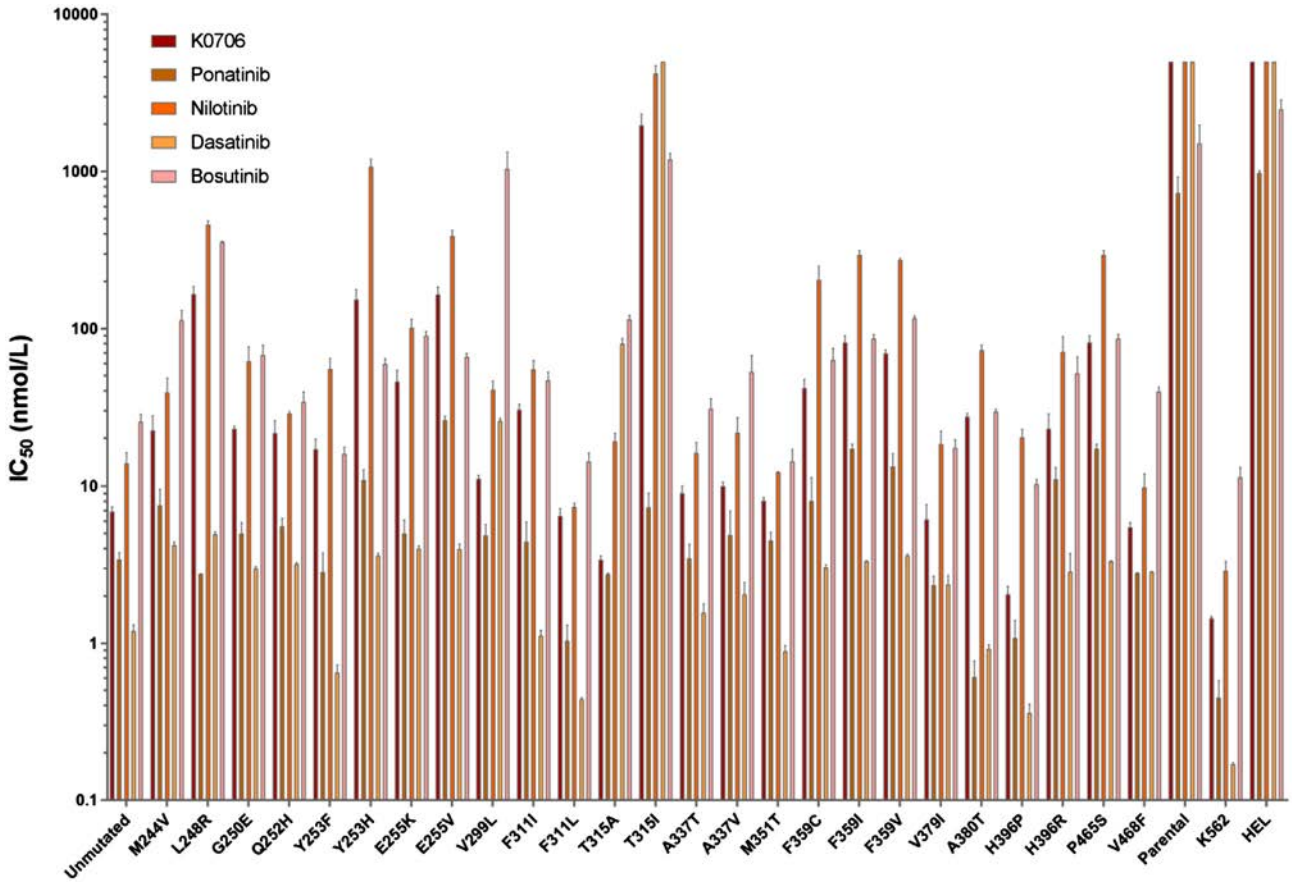

Figure S1. Bar graph comparing cellular IC_{50} values of K0706 to the FDA approved BCR-ABL1 TKIs bosutinib, dasatinib, nilotinib, and ponatinib.

Table S1. BCR-ABL1 mutants detected in the presence of K0706 for resistance screens starting from native BCR-ABL1.

Concentration	Clones sequenced (N)	Mutant	Occurrences (n)	Frequency among clones (%)	Frequency among mutants (%)
400 nM	54	L248R	2	3.7	3.7
		Y253H	6	11.1	11.1
		E255V	3	5.6	5.6
		T315I	43	79.6	79.6
800 nM	57	Unmutated	3	5.3	—
		Y253H	3	5.3	5.6
		E255V	1	1.8	1.9
		T315I	50	87.7	92.6
1600 nM	54	T315I	54	100.0	100.0
3200 nM	49	T315I	49	100.0	100.0

(Tyr204) (C42B5) rabbit mAb (3009, Cell Signaling Technology), and anti-β-actin (D6A8) rabbit mAb (8457, Cell Signaling Technology).

Cell-based resistance assay. Native *BCR-ABL1* expressing Ba/F3 cells were treated with *N*-ethyl-*N*-nitrosourea (ENU, 50 μg/mL) overnight, pelleted, resuspended in fresh RPMI complete medium, and plated in 96-well plates ($1.25x10^5$ cells/well) supplemented with graded concentrations of K0706 (400, 800, 1600, and 3200 nmol/L) in quintuplicates. ENU was inactivated overnight with a sodium thiosulfate (20% w/v) and sodium hydroxide (100 mmol/L) and safely disposed of according to protocol. Wells were monitored for outgrowth, visually, using a microscope every two days for 30 days. Fifty outgrowth colonies from each treatment group were randomly selected and expanded in 24-well plates containing an equivalent concentration of K0706 as the original 96-well plate. Expanded cells in 24-well plates were harvested by centrifugation and stored at -80˚C.

Sanger sequencing of *BCR-ABL1* kinase domain. DNA isolated from Ba/F3 *BCR-ABL1* expressing lysates (DNeasy Blood & Tissue Kit, Qiagen) was used as a template for amplification of *BCR-ABL1* kinase domain. Amplification (Phusion High-Fidelity DNA Polymerase, New England BioLabs) was performed using a two-step PCR to excluded endogenous *ABL1*. PCR products were electrophoresed on a 1% agarose gel to confirm amplification, purified (QIAquick PCR Purification Kit, Qiagen), and Sanger sequenced (DNA Sequencing Core Facility, University of Utah).

Molecular modeling. Computational modeling of K0706 ligand binding were generated using the inactive conformation of ABL1 Kinase (PDB entry 3OXZ) [1]. After removing the complexed ligand (ponatinib), three mutations were introduced in the receptor (ABL^{Y253H}, ABL^{T315I} and ABL^{R248L}) and the side-chain placement was optimized using the rotamer library. The resulting mutant conformation was energy minimized by Steepest Descent and Newton Raphson algorithm respectively, to remove strain and relax local interactions. Using flexible ligand alignment module of the Schrodinger program, the ligand K0706 was overlaid with ponatinib and placed in the binding site.

Supplemental References

[1] Zhou T, Commodore L, Huang WS, et al. Structural mechanism of the Pan-BCR-ABL inhibitor ponatinib (AP24534): lessons for overcoming kinase inhibitor resistance. Chem Biol Drug Des. 2011; 77:1-11.