# AP24534, a Pan-BCR-ABL Inhibitor for Chronic Myeloid Leukemia, Potently Inhibits the T315I Mutant and Overcomes Mutation-Based Resistance

**Thomas O'Hare**[*,†,ζ], **William C. Shakespeare**[‡,ζ], **Xiaotian Zhu**[‡], **Christopher A. Eide**[*,†], **Victor M. Rivera**[‡], **Frank Wang**[‡], **Lauren T. Adrian**[*,†], **Tianjun Zhou**[‡], **Wei-Sheng Huang**[‡], **Qihong Xu**[‡], **Chester A. Metcalf III**[‡], **Jeffrey W. Tyner**[*], **Marc M. Loriaux**[*], **Amie S. Corbin**[*,†], **Scott Wardwell**[‡], **Yaoyu Ning**[‡], **Jeffrey A. Keats**[‡], **Yihan Wang**[‡], **Raji Sundaramoorthi**[‡], **Mathew Thomas**[‡], **Dong Zhou**[‡], **Joseph Snodgrass**[‡], **Lois Commodore**[‡], **Tomi K. Sawyer**[‡], **David C. Dalgarno**[‡], **Michael W.N. Deininger**[*,ψ], **Brian J. Druker**[*,†,§,ψ], and **Tim Clackson**[‡,§]

[*] Division of Hematology and Medical Oncology, Oregon Health & Science University Knight Cancer Institute, Portland, OR 97239

[†] Howard Hughes Medical Institute, Portland, OR 97239

[‡] ARIAD Pharmaceuticals, Inc., Cambridge, MA 02139

## SUMMARY

Inhibition of BCR-ABL by imatinib induces durable responses in many patients with chronic myeloid leukemia (CML), but resistance attributable to kinase domain mutations can lead to relapse and a switch to second-line therapy with nilotinib or dasatinib. Despite three approved therapeutic options, the cross-resistant BCR-ABL[T315I] mutation and compound mutants selected on sequential inhibitor therapy remain major clinical challenges. We report design and pre-clinical evaluation of AP24534, a potent, orally available multi-targeted kinase inhibitor active against T315I and other BCR-ABL mutants. AP24534 inhibited all tested BCR-ABL mutants in cellular and biochemical assays,

§Correspondence: clackson@ariad.com ; Tel 617 621 2258; Fax 617 225 2589, drukerb@ohsu.edu ; Tel 503 494 5596; Fax 503 494 3688.
ζEqual contributions
ψEqual contributions

**SIGNIFICANCE**

The ABL kinase inhibitor imatinib is the current standard first-line therapy for patients with BCR-ABL-positive leukemia. The second-line ABL kinase inhibitors nilotinib and dasatinib are effective salvage therapies for patients who relapse on imatinib. BCR-ABL[T315I], a mutant resistant to all approved ABL kinase inhibitors, is emerging as a common pathway of failure on all three inhibitors. AP24534 is an orally active multi-targeted kinase inhibitor that potently inhibits BCR-ABL[T315I]. It may represent a treatment option for patients with this mutation and complement the current clinically employed inhibitors. Longer term, a pan-BCR-ABL inhibitor such as AP24534 may offer important strategic advantages in a first-line capacity by minimizing BCR-ABL kinase domain mutation-based drug resistance.

**SUPPLEMENTAL DATA**

The Supplemental Data include three supplemental figures (Figure S1: AP24534 effectively inhibits BCR-ABL-mediated signaling in CML cell lines expressing native or T315I-mutant BCR-ABL; Figure S2: AP25434 potently inhibits colony formation of primary BCR-ABL[T315I] cells with minimal toxicity to normal cells; Figure S3: Effect of dasatinib in mouse models using Ba/F3 BCR-ABL[T315I] cells) and five supplemental tables (Table S1: AP24534 kinase panel screening data; Table S2: AP24534 cell-based mutagenesis assay (starting from native BCR-ABL); Table S3: AP24534 cell-based mutagenesis assay (starting from BCR-ABL[T315I]); Table S4: AP24534 cell-based mutagenesis assay (starting from BCR-ABL[E255V]); Table S5: BCR-ABL compound mutations involving T315I or E255V conferring moderate to high level resistance to AP24534), and can be found online with this article at (*insert location*).

suppressed BCR-ABL$^{T315I}$-driven tumor growth in mice, and completely abrogated resistance in cell-based mutagenesis screens. Our work supports clinical evaluation of AP24534 as a pan-BCR-ABL inhibitor for treatment of CML.

## Keywords

dasatinib; nilotinib; imatinib resistance; compound mutation

## INTRODUCTION

The judicious use of tyrosine kinase inhibitors that target BCR-ABL constitutes an effective strategy for sustained disease control in chronic myeloid leukemia (CML). The exemplar of targeted therapy in CML is the BCR-ABL inhibitor imatinib (Gleevec; STI571), a safe and effective first-line therapy for most patients diagnosed with chronic phase disease (Druker et al., 2006). Although most patients attain a durable complete cytogenetic response, minimal residual disease persists in nearly all patients, and active disease recurs if treatment is discontinued. More importantly, discontinuation of imatinib due to intolerance or resistance is necessary in up to 30% of patients within the first five years on therapy. Also, durable responses are uncommon in patients with advanced CML or Philadelphia chromosome (Ph)-positive acute lymphoblastic leukemia (ALL).

Resistance to imatinib usually involves point mutations in the kinase domain of BCR-ABL that impair inhibitor binding. A broad spectrum of kinase domain mutations that confer resistance to the drug have been reported (Hughes et al., 2006). Clinically, identification of a BCR-ABL kinase domain mutation provides a potential explanation for imatinib resistance and suggests a clear treatment strategy: second-line therapy with an ABL kinase inhibitor active against the particular BCR-ABL mutant present in the patient (Jabbour et al., 2009). To date, two ABL kinase inhibitors have achieved regulatory approval for second-line use: the imatinib family member nilotinib (Tasigna; AMN107) and the multi-targeted kinase inhibitor dasatinib (Sprycel; BMS-354825) (Shah et al., 2004; Weisberg et al., 2005). With the availability of these three oral BCR-ABL inhibitors, most patients are successfully matched to an appropriate and effective drug, leading to retained or recaptured response. However, several kinase domain mutations confer high-level resistance to one or more of these therapies, in particular the BCR-ABL$^{T315I}$ mutation, which confers resistance to all three (reviewed by (O'Hare et al., 2007)).

Given the location of the T315 residue in the gatekeeper region of the ATP-binding site, the T315I mutant has proven difficult to inhibit with ATP mimetics. Modeling analysis indicates that the mutation eliminates a critical hydrogen bonding interaction required for high-affinity binding of imatinib, nilotinib, and dasatinib and alters the topology of the ATP-binding pocket (Tokarski et al., 2006; Weisberg et al., 2005). Although several reports have described approaches to overcome this, compound-to-clinic progress has been slow (reviewed by (Quintas-Cardama and Cortes, 2008)). Several ATP competitive inhibitors originally designed to target the Aurora kinase family have been found to be active against ABL$^{T315I}$, including MK-0457, PHA-739358, AT9283, and XL-228 (Quintas-Cardama and Cortes, 2008). These molecules have been formulated for intravenous administration in the clinic, and MK-0457 has shown some activity as salvage therapy for advanced phase CML patients harboring the T315I mutation (Giles et al., 2007), but clinical development has been halted due to toxicity concerns.

The importance of controlling mutation-mediated resistance is underscored by recent reports on the potential for sequential ABL kinase inhibitor therapy to select for compound (multiple mutations in same BCR-ABL allele) mutants resistant to all current ABL inhibitors, including

some that do not involve T315I (Shah et al., 2007). Therefore, an optimal next-generation ABL inhibitor capable of exerting a high level of disease control in CML would incorporate potent activity against BCR-ABL$^{T315I}$ and the full range of BCR-ABL kinase domain mutations as well as the native (unmutated) enzyme, while matching the pharmacologic advantages of the currently approved therapies. Here we report on the design and pre-clinical testing of AP24534, an orally active pan-inhibitor of BCR-ABL, including BCR-ABL$^{T315I}$.

## RESULTS

### Design of AP24534 and Crystallographic Analysis of AP24534:ABL$^{T315I}$

Recent X-ray crystallographic studies on the ABL kinase domain reveal that the threonine to isoleucine gatekeeper mutation, T315I, acts as a simple point mutant without significant perturbation of the overall protein structure (Zhou et al., 2007). Thus, as imatinib, nilotinib, and dasatinib each form a hydrogen bond with the side chain of T315 in native ABL, we designed ligands devoid of this interaction by introducing vinyl and ethyl linkages into a purine-based inhibitor scaffold (Wang et al., 2008) targeting both DFG-in (active) and DFG-out (inactive) binding modes. One DFG-out targeted compound also inhibited ABL$^{T315I}$ in biochemical and cellular assays (M. Azam, personal communication; (Huang et al., 2009)). Subsequent structure-guided design experiments led to AP24534 (Figure 1A), which accommodates the T315I side chain by virtue of a carbon-carbon triple bond (ethynyl) linkage.

X-ray crystallographic analysis of AP24534 in complex with the murine ABL$^{T315I}$ kinase domain confirmed that AP24534 binds in the DFG-out mode (Figure 1B and 1C) and maintains a network of protein contacts similar to imatinib (Figure 1D and 1E). Specifically, the imidazo [1,2*b*]pyridazine core of AP24534 occupies the adenine pocket of the enzyme, the methylphenyl group occupies the hydrophobic pocket behind the gatekeeper residue, the trifluoromethylphenyl group binds tightly to the pocket induced by the DFG-out conformation of the protein, and the ethynyl linkage of AP24534 makes favorable van der Waals interactions with the I315 mutated residue. A total of five hydrogen bonds are made between the inhibitor and the protein, including: with the backbone of M318 in the hinge region, with the backbone of D381, and with the side chain of E286 (Figure 1C), as well as two hydrogen bonds from the methylpiperazine group (not shown). The P-loop of the kinase is collapsed in this conformation, bringing Y253 into van der Waals contact with AP24534. Additional favorable contacts are made between the inhibitor and F382 of the DFG motif, displaced outwards into the ligand-binding site in the DFG-out mode. Although the methylphenyl groups occupying the hydrophobic pocket and hinge hydrogen bonding moieties of AP24534 and imatinib are placed similarly (Figure 1D), superposition of the two inhibitors shows AP24534 engaging in productive van der Waals interactions with I315, while steric clash between imatinib and the I315 side chain is evident (Figure 1E).

### AP24534 Inhibits the Catalytic Activity of ABL$^{T315I}$

We tested the activity of AP24534, imatinib, nilotinib, and dasatinib in biochemical assays with purified, dephosphorylated, native ABL and ABL$^{T315I}$. All inhibitors diminished the enzymatic activity of native ABL, but only AP24534 was effective against the ABL$^{T315I}$ mutant (Figure 2). Similar potent inhibition by AP24534 was observed for additional imatinib-resistant ABL mutants tested, including ABL$^{G250E}$, ABL$^{Y253F}$, and ABL$^{E255K}$ (not shown), establishing that AP24534 directly targets native and mutant ABL kinase, including ABL$^{T315I}$.

### Kinase Selectivity Profile of AP24534

The in vitro potency and selectivity of AP24534 was assessed in kinase assays with multiple recombinant kinase domains and peptide substrates (Table 1 and Table S1). AP24534 potently

inhibited native ABL ($IC_{50}$: 0.37 nM), $ABL^{T315I}$ ($IC_{50}$: 2.0 nM), and other clinically important ABL kinase domain mutants ($IC_{50}$: 0.30–0.44 nM) (Table 1). AP24534 also inhibited SRC ($IC_{50}$: 5.4 nM) and members of the VEGFR, FGFR, and PDGFR families of receptor tyrosine kinases (Table 1 and Table S1). AP24534 did not inhibit Aurora kinase family members, nor did it inhibit insulin receptor or cyclin-dependent kinase 2 (CDK2)/Cyclin E ($IC_{50} > 1000$-fold relative to native ABL).

## AP24534 Inhibits the Growth of Cells Expressing Native or Mutant BCR-ABL

Cellular proliferation assays were performed with parental Ba/F3 cells and Ba/F3 cells expressing native BCR-ABL or BCR-ABL with a range of single mutations in the kinase domain. AP24534 potently inhibited proliferation of Ba/F3 cells expressing native BCR-ABL ($IC_{50}$: 0.5 nM). All BCR-ABL mutants tested remained sensitive to AP24534 ($IC_{50}$: 0.5–36 nM; Table 2) including $BCR-ABL^{T315I}$ ($IC_{50}$: 11 nM). Annexin V staining confirmed that inhibition of proliferation by AP24534 correlated with induction of apoptosis (not shown). Growth of parental Ba/F3 cells was inhibited only at significantly higher $IC_{50}$ (1713 nM), indicating a substantial differential selectivity for inhibition of BCR-ABL-positive cells. Ba/F3 $BCR-ABL^{T315I}$ cells grown in the presence of IL-3 exhibited an $IC_{50}$ (1804 nM) similar to that of parental Ba/F3 cells.

We also tested AP24534 against BCR-ABL-positive and -negative cell lines derived from leukemic patients. While we observed potent growth inhibition of K562, KY01, and LAMA cells (derived from CML patients in blast crisis), there was no significant activity against three BCR-ABL-negative leukemia cell lines (Table 2).

## AP24534 Inhibits BCR-ABL-Mediated Signaling in Cells Expressing BCR-ABL^T315I

To confirm target inhibition in Ba/F3 cells expressing native BCR-ABL or $BCR-ABL^{T315I}$, we examined the effect of AP24534 on the tyrosine phosphorylation status of BCR-ABL (Figure S1) and the direct BCR-ABL substrate CrkL (Figure 3), with the three approved ABL inhibitors included for comparison. Monitoring CrkL tyrosine phosphorylation status as a surrogate for BCR-ABL kinase activity has been the preferred pharmacodynamic assay in clinical trials of BCR-ABL inhibitors (Druker et al., 2001; Talpaz et al., 2006). In the CrkL gel shift assay, the percentage of tyrosine-phosphorylated CrkL (upper band) decreases in response to inhibition of BCR-ABL. While all tested inhibitors were effective against Ba/F3 cells expressing native BCR-ABL (Figure 3A), only AP24534 demonstrated activity against the T315I mutant (Figure 3B). Inhibition of BCR-ABL phosphorylation was observed in parallel experiments (Figure S1).

## Treatment of CML Primary Cells with AP24534 Inhibits Cellular Proliferation

To assess the efficacy of AP24534 on primary cells from patients with BCR-ABL-driven leukemia, we exposed mononuclear cells derived from blood or bone marrow from CML myeloid blast crisis patients harboring native BCR-ABL or $BCR-ABL^{T315I}$ and from healthy individuals to graded concentrations of AP24534 and assayed viable cells after 72 hr. Consistent with biochemical and cell line viability data, AP24534 induced a selective reduction of viable cell numbers in primary CML cells, with $IC_{50}$ values approximately 500-fold lower than those observed with normal cells (Figure 4A). Neither imatinib nor dasatinib reached an $IC_{50}$ (highest concentration: 1000 nM) in primary CML $BCR-ABL^{T315I}$ cells (data not shown).

## AP24534 Inhibits BCR-ABL^T315I Kinase Activity and Colony Formation in Primary CML Cells

To monitor target inhibition following ex vivo exposure to AP24534 of mononuclear cells obtained from a CML T315I lymphoid blast crisis patient, we carried out an assay similar to that described for Ba/F3 cell lines, wherein cells were incubated with inhibitors and then

analyzed for CrkL phosphorylation by immunoblot. Exposure to AP24534 resulted in a reduction in phosphorylated CrkL signal while none of the other ABL inhibitors had an effect (Figure 4B); similar results were obtained upon analysis for global tyrosine phosphorylation by flow cytometry (Figure 4C).

We also evaluated the efficacy of AP24534 in myeloid colony formation assays using mononuclear cells from a CML T315I accelerated phase patient and from a healthy individual. Whereas neither nilotinib nor dasatinib showed an effect against patient-derived T315I cells, AP24534 inhibited the formation of colonies in a concentration-dependent manner (Figure S2A) and exhibited no toxicity to normal hematopoietic cells at concentrations below 500 nM (Figure S2B), consistent with cellular proliferation assay data obtained using normal cells (Figure 4A).

## Oral AP24534 Prolongs Survival and Reduces Tumor Burden in Mice with BCR-ABL$^{T315I}$-Dependent Disease

To examine the pharmacologic properties of AP24534, mice were administered a single oral dose and plasma concentrations were then measured at multiple time points. In mice administered a dose of 2.5 mg/kg, mean plasma levels of 90, 58, and 2 nM were achieved at 2, 6, and 24 hr post-dose, respectively. At a dose of 30 mg/kg, mean plasma levels reached 782, 561, and 8 nM at the same time points. These results demonstrate that plasma levels exceeding the in vitro $IC_{50}$ values for all tested BCR-ABL mutants can be sustained in mice for >6 hr at oral doses that are well tolerated, indicating that adequate target inhibition for a therapeutic effect should be reached (Shah et al., 2008).

We next evaluated the efficacy of AP24534 in a survival model in which Ba/F3 cells expressing native BCR-ABL were injected intravenously. As shown in Figure 5A (left), the median survival time for vehicle-treated mice was 19 days. Daily oral treatment with 2.5 or 5 mg/kg AP24534 for 19 days prolonged median survival to 27.5 and 30 days, respectively (p<0.01 for both dose levels). These results were comparable to those achieved following daily oral administration of 5 mg/kg dasatinib (a regimen previously reported to be efficacious (Lombardo et al., 2004)), in which median survival was 27 days (p<0.01).

In a survival model in which mice were instead injected with Ba/F3 BCR-ABL$^{T315I}$ cells, administration of dasatinib at doses as high as 300 mg/kg had no effect on survival time, as expected (Figure S3). By contrast, treatment with AP24534 prolonged survival in a dose-dependent manner (Figure 5A, right). AP24534 dosed orally for 19 days at 5, 15, and 25 mg/kg prolonged median survival to 19.5, 26, and 30 days, respectively compared to 16 days for vehicle-treated mice (p<0.01 for all three dose levels).

The anti-tumor activity of AP24534 was further assessed in a xenograft model in which Ba/F3 BCR-ABL$^{T315I}$ cells were injected subcutaneously into mice. Tumor growth was inhibited by AP24534 in a dose-dependent manner (Figure 5B, left) compared to vehicle-treated mice, with significant suppression of tumor growth upon daily oral dosing at 10 and 30 mg/kg (%T/C = 68% and 20%, respectively; p<0.01 for both dose levels). Daily oral dosing of 50 mg/kg AP24534 caused significant tumor regression (%T/C = 0.9%, p<0.01), with a 96% reduction in mean tumor volume at the final measurement compared to the start of treatment. AP24534 was well tolerated at all efficacious dose levels for the duration of the study; maximal decreases in body weight were <5%, <5%, and <12% for the 10, 30, and 50 mg/kg dose groups, respectively, with no signs of overt toxicity.

To confirm target inhibition, levels of phosphorylated BCR-ABL$^{T315I}$ and phosphorylated CrkL were assessed in tumors from mice harvested 6 hr after one-time dosing with vehicle or

AP24534. As shown in Figure 5B (right), a single oral dose of 30 mg/kg markedly decreased levels of phosphorylated BCR-ABL and phosphorylated CrkL.

## Single-Agent AP24534 Completely Suppresses Outgrowth of Resistant Clones

To survey for potential sites of vulnerability to resistance, we tested AP24534 in our established accelerated mutagenesis assay. This assay has previously been used to characterize the resistance profile of imatinib, nilotinib, and dasatinib, and has proved to be predictive of clinical experience with these inhibitors (Bradeen et al., 2006). In this screen, a BCR-ABL-driven cell line is exposed to mutagen, and then plated into tissue culture wells with graded concentrations of inhibitor. Outgrowth of cells reflects the emergence of resistant subclones, which are sequenced to identify BCR-ABL mutations.

Initially, we performed mutagenesis experiments using Ba/F3 cells expressing native BCR-ABL at several concentrations of AP24534 (5–40 nM) and found a concentration-dependent reduction in both the percentage of wells with outgrowth and in the scope of mutations observed (Figure 6A). At 5 nM AP24534, all wells (576/576) exhibited outgrowth and 90% of the sequenced representative subclones expressed native BCR-ABL (Table S2). Raising the concentration of AP24534 to 10 nM resulted in both a marked reduction in outgrowth (168/1440 wells; 11.7%) and an increased frequency of mutated subclones (33.1%; Table S2). Mutations recovered included occurrences at several P-loop residues (G250, Q252, Y253, and E255), a cluster at the C-helix (K285, E292, and L298), and T315 (T315I), as well as F317, V339, F359, L387, and S438. Among the recovered mutations, nearly all have been previously encountered in resistance to imatinib, nilotinib, and/or dasatinib (reviewed by (O'Hare et al., 2007)). No mutations were encountered that were specific for AP24534 only.

We next investigated 20 nM AP24534 and found that outgrowth was sharply curtailed (3/1440 wells; 0.2%), with only two mutations, E255V and T315I, persisting (Figure 6A and Table S2). Thus, within our extensive survey, no previously undiscovered mutations capable of conferring high-level resistance to AP24534 were identified. At 40 nM AP24534, which is 43-fold lower than the $IC_{50}$ for parental BaF/3 cells, complete suppression of in vitro resistance was achieved. This absence of resistant outgrowth was further confirmed at higher concentrations of AP24534 (80–320 nM; not shown).

## Effects of AP24534 on Compound Mutants

Having identified a limited resistance susceptibility profile for AP24534 at the level of single mutations, we wanted to investigate the vulnerability to compound mutations, defined as two kinase domain mutations in the same allele, which have been detected in some treatment failures (Khorashad et al., 2008; Shah et al., 2007; Stagno et al., 2008). To simulate the situation in which AP24534 is used to treat a patient with a predominant T315I subclone, we repeated the accelerated mutagenesis assay, this time starting with an existing T315I mutation (Figure 6B and Table S3). We found that there was still a concentration-dependent hierarchy and that AP24534 at a concentration of 160 nM or lower overcame all compound mutants involving T315I except Y253H/T315I and E255V/T315I. At 320 nM, the only remaining compound mutant was E255V/T315I, which couples the two most resistant single mutants, and outgrowth was completely suppressed at the highest concentration (640 nM) tested, still ~3-fold below the $IC_{50}$ for parental Ba/F3 cell line inhibition. This resistance profile was confirmed in a subsequent screen starting from a background of BCR-ABL[E255V], the most resistant single BCR-ABL kinase domain mutation to AP24534, with the E255V/T315I compound mutant again persisting to 320 nM and being eliminated at 640 nM (Table S4).

## DISCUSSION

AP24534 is a next-generation ABL kinase inhibitor optimized using structure-based drug design to bind to the inactive, DFG-out conformation of ABL and ABL[T315I]. The key structural feature of the molecule is a carbon-carbon triple bond linkage that makes productive hydrophobic contact with the side chain of I315, allowing inhibition of the T315I mutant. The triple bond also acts as an inflexible connector that enforces correct positioning of the two binding segments of AP24534 into their established binding pockets. AP24534 maintains an extensive hydrogen-bonding network and occupies a region of the kinase that overlaps significantly with the imatinib binding site.

A key design feature of AP24534 underlying its pan-BCR-ABL inhibitor profile is incorporation of multiple contact points to confer very high potency and to balance and distribute the overall binding affinity. While each of the hydrogen-bonding and contact residue interactions contribute substantially to the inhibitor's affinity for its target, mutation-based disruption of one element of the binding network or distortion of a sub-region within the binding pocket results in only a slight reduction in affinity. As a consequence, AP24534 also retains potency against other imatinib-resistant ABL mutants in addition to ABL[T315I]. While mutations that destabilize the inactive conformation of ABL to which AP24534 binds, including T315I and E255V, result in modest reductions in binding affinity, substantial reductions would be expected to require at least two changes at non-proximal residues – a prediction consistent with findings from our mutagenesis screen.

Kinase selectivity studies showed that AP24534 does not inhibit Aurora kinases, clearly distinguishing it from other T315I inhibitors in development. These studies also revealed inhibition of SRC, LYN, PDGFRα, and c-KIT with <10-fold selectivity compared to ABL[T315I]. Several of these kinases are important clinical targets of imatinib, nilotinib and/or dasatinib, although only dasatinib has been reported to inhibit SRC family kinases. Although assay differences preclude direct comparison of the kinase profiles of AP24534 and dasatinib, a comprehensive kinase interaction map for dasatinib was recently reported (Karaman et al., 2008). In general, the linearity of the triple bond in AP24534 is predicted to minimize steric clash between the inhibitor and hydrophobic gatekeeper residues. This feature probably contributes to the relatively broad kinase specificity profile of AP24534, which includes VEGFR and FGFR family kinases, receptors not inhibited by the three currently approved BCR-ABL drugs. As SRC, VEGFR, FGFR, and PDGFR family kinases are potential targets in a variety of other malignancies, this supports the potential testing of AP24534 in a wider range of cancers.

Evaluation of AP24534 in cellular proliferation assays confirmed its potent pan-BCR-ABL inhibition against cells expressing native or mutant BCR-ABL, including BCR-ABL[T315I], while retaining a high degree of selectivity (>1000-fold) for Ph-positive cells. Among the BCR-ABL mutants tested, the E255V mutant, which confers high-level resistance to imatinib and intermediate-level resistance to nilotinib and dasatinib (O'Hare et al., 2007), was most resistant to AP24534. Notably, AP24534 potently inhibited mutants at residues Y253 and F359 (which have been reported in patients failing nilotinib (Cortes et al., 2007; Kantarjian et al., 2007)), as well as F317 (implicated in clinical resistance to dasatinib (Burgess et al., 2005; Cortes et al., 2007; Khorashad et al., 2008; Shah et al., 2007; Talpaz et al., 2006)). While clinically achievable and effective doses will need to be determined, the sizeable selectivity for BCR-ABL-expressing cells (regardless of mutational status) over normal cells suggests the potential for efficacy with minimal toxicity.

In clinical studies of BCR-ABL inhibitors, pharmacodynamic evaluation of target inhibition is an important component of dose optimization. In the preclinical studies reported here we

monitored phosphorylation of CrkL, a direct substrate of native and mutant BCR-ABL, by immunoblot analysis. In both Ba/F3 cells and primary CML BCR-ABL$^{T315I}$ cells, treatment with AP24534 resulted in a marked reduction in phosphorylated CrkL, while imatinib, dasatinib, and nilotinib had no effect. This assay was recently used to monitor BCR-ABL activity in patients treated with nilotinib; values of percent phosphorylated CrkL from serially collected peripheral blood samples were consistent with BCR-ABL kinase domain mutation status and matched closely with other measures of response, including BCR-ABL transcript levels and white cell counts (La Rosee et al., 2008). Given its extensive validation in the clinic, this assay is being employed to monitor the pharmacodynamic effects of AP24534 in its phase 1 evaluation.

The oral bioavailability of AP24534 was confirmed in mouse pharmacology studies, where concentrations above the IC$_{50}$s for all tested mutants could be safely sustained following daily oral dosing. AP24534 demonstrated potent activity after daily oral administration in a series of mouse models of CML driven by native BCR-ABL or BCR-ABL$^{T315I}$. In a survival model using Ba/F3 cells expressing native BCR-ABL, AP24534 significantly prolonged survival at low doses of 2.5 and 5 mg/kg and demonstrated similar efficacy to dasatinib. In an analogous model using BCR-ABL$^{T315I}$ cells, AP24534 significantly extended survival whereas dasatinib, as expected, was inactive. AP24534 was also active in a subcutaneous BCR-ABL$^{T315I}$ tumor model, where tumor stasis or regression occurred at doses of 30 and 50 mg/kg, and suppression of BCR-ABL signaling was demonstrated using the shift CrkL phosphorylation assay. AP24534 was well tolerated at all dose levels used in these studies. Thus, AP24534 is orally bioavailable, inhibits its molecular target, and has a wide therapeutic range (5–50 mg/kg) in BCR-ABL$^{T315I}$ dependent CML animal models.

Mutation-mediated resistance to clinical ABL inhibitors is the main route of BCR-ABL signaling reactivation, particularly in chronic phase disease. As AP24534 advances into clinical evaluation, anticipating potential resistance liabilities, especially compared to those of nilotinib and dasatinib, will be important for prospective treatment decisions. Several mutations have been reported in association with clinical resistance to nilotinib (L248V, Y253F/H, E255K/V, T315I, F359C/V; (Cortes et al., 2007; Kantarjian et al., 2007)) or dasatinib (V299L, T315A/I, F317I/L; (Cortes et al., 2007; Khorashad et al., 2008; Shah et al., 2007)) which are largely consistent with our in vitro profiling (Bradeen et al., 2006). In our accelerated mutagenesis screens for AP24534, we found a concentration-dependent reduction in both the percentage of wells with outgrowth and in the range of mutations observed. Although at 10 nM AP24534 we observed 16 different substitutions across 13 different residues, the only resistant subclones recovered at 20 nM harbored either a T315I or E255V mutation, and at 40 nM AP24534 and above complete suppression of outgrowth was observed. Depending on achievable plasma levels, our data suggest that AP24534 may have the potential to overcome single-mutation-based resistance in the clinical setting. This result has been previously achieved in this assay only with combinations of nilotinib or dasatinib and a pre-clinical T315I inhibitor (O'Hare et al., 2008). To our knowledge no other ABL kinase inhibitor has been shown to have this potential as a single agent.

As compound (multiple) mutations of BCR-ABL represent a rare but challenging scenario clinically, we carried out additional accelerated mutagenesis screens starting with cells expressing either of the two individually most resistant mutants, BCR-ABL$^{T315I}$ or BCR-ABL$^{E255V}$. This predictive assay implicated certain compound mutations, especially those involving any two of Y253H, E255V, and T315I in moderate to high-level resistance to AP24534. Among these, Y253H/T315I and E255V/T315I are predicted to be the most resistant pairings, although high concentrations of AP24534 still prevented these mutations emerging. Thus, AP24534 has the capability to eliminate compound mutations involving T315I and E255V predicted to be highly resistant to all other inhibitors. Currently, the number of clinically

documented compound mutations within the kinase domain of BCR-ABL associated with treatment failure is low (Table S5). Nonetheless, they represent a formidable problem for those patients harboring them, and incidence may increase with the prolonged survival of CML patients and with more patients undergoing sequential ABL kinase inhibitor treatment (Shah et al., 2007). Overall, although no mutagenesis screen can be completely exhaustive, our data indicate AP24534 has the potential to address this currently unmet clinical issue.

Our pre-clinical profiling indicates that AP24534 has potential as an important option for controlling resistance in CML. The combined results of our biochemical, cell-based, and in vivo studies suggest that AP24534 exhibits sufficient activity against native BCR-ABL and all tested BCR-ABL mutants to warrant consideration for single-agent use as a pan-BCR-ABL inhibitor. Moreover, our results indicate that AP24534 holds promise for controlling compound mutants involving T315I, while raising awareness that it is advantageous to eliminate resistant subclones at the single-mutation stage. In the longer term, this may advocate for the potential future use of a pan-BCR-ABL inhibitor such as AP24534 in a first-line therapeutic capacity.

Clinical use of a pan-BCR-ABL inhibitor active against T315I could make long-term remissions an achievable goal at least for some patients with advanced CML. A phase 1 clinical trial evaluating oral AP24534 in patients with refractory CML and other hematologic malignancies is ongoing (NCT00660920, www.clinicaltrials.gov).

# EXPERIMENTAL PROCEDURES

## Inhibitors

AP24534, 3-(imidazo[1,2$b$]pyridazin-3-ylethynyl)-4-methyl-$N$-(4-((4-methylpiperazin-1-yl) methyl)-3-(trifluoromethyl)phenyl)benzamide was synthesized at ARIAD Pharmaceuticals. Imatinib, dasatinib, and nilotinib were purchased from the Oregon Health & Science University (OHSU) pharmacy or made at ARIAD. All inhibitors were prepared as 10.0 mM stock solutions and stored at −20°C. Serial dilutions of 10.0 mM stock solutions were carried out just prior to use in each experiment.

## Crystallization and Structural Determination of ABL[T315I]:AP24534 Complex

The kinase domain of murine ABL[T315I] (residues 229–515) was co-expressed with YopH protein tyrosine phosphatase in E. coli as described (Zhou et al., 2007) and purified in the presence of AP24534 to near homogeneity (> 95%) using metal affinity, Mono Q, and size exclusion chromatography. The typical yield of purified ABL[T315I] bound with AP24534 was about 1 mg/L.

Co-crystals of ABL[T315I] and AP24534 were grown by the hanging drop vapor diffusion method at 4°C by mixing equal volumes of the AP24534:ABL[T315I] complex (25 mg/mL) and well solution (30% w/v polyethylene 4000, 0.2 M sodium acetate, 0.1 M Tris, pH 8.5). After 1–2 days, crystals reached a typical size of $50 \times 50 \times 300$ μm$^3$ and were harvested in mother liquor supplemented with 30% v/v glycerol as cyroprotectant. X-ray diffraction data were collected at 100K at beamline 19 BM (Advanced Photon Service, Argonne, IL). The data were indexed and scaled in space group P21 by using the HKL2000 package (Otwinowski and Minor, 1997).

The structure of AP24534 in complex with ABL[T315I] was determined by molecular replacement by AMoRe (Navaza, 1994) using the structure of native ABL bound with imatinib (PDB code: 1IEP). There were two ABL[T315I] molecules in the asymmetric unit. The structure was refined with CNX combined with manual rebuilding in Quanta (Accelrys Inc., San Diego, CA), and AP24534 was built into the density after several cycles of refinement and model building, which then continued until convergence was reached. The final model, refined to

1.95 Å, consists of residues 228 through 511, with 386–397 in the activation loop disordered. The electron density for bound AP24534 as well as the side chain of I315 was well resolved in both complexes, leaving no ambiguities for the binding mode of the inhibitor.

### Autophosphorylation Assays For ABL$^{T315I}$

Kinase autophosphorylation assays with full length, tyrosine-dephosphorylated ABL, ABL$^{G250E}$, ABL$^{Y253F}$, ABL$^{E255K}$ and ABL$^{T315I}$ (Invitrogen; San Diego, CA) were performed in the presence of imatinib, nilotinib, dasatinib, or AP24534 (0–1000 nM) as per (O'Hare et al., 2004).

### Kinase Selectivity Profile of AP24534

AP24534 was profiled against >100 kinases by Reaction Biology Corporation (Malvern, PA) using the Kinase Hotspot assay, which utilizes 10 μM [$^{33}$P]-ATP, recombinant kinase domain, peptide substrate, and a range of 10 concentrations of inhibitor to establish an IC$_{50}$ value (http://www.reactionbiology.com/pages/kinase.htm).

### Collection of Patient Samples

Clinical samples were obtained with informed consent and under the approval of the OHSU Institutional Review Board. Blood or bone marrow from patients or healthy individuals was separated on a Ficoll gradient (GE Healthcare) for isolation of mononuclear cells.

### Cell Lines

Ba/F3 transfectants (expressing native BCR-ABL or BCR-ABL with a single kinase domain mutation) were maintained in RPMI 1640 supplemented with 10% FCS, 1 unit/mL penicillin G, and 1 mg/mL streptomycin (complete media) at 37°C and 5% CO$_2$. The Ba/F3 BCR-ABL$^{T315A}$ cell line was a kind gift of Dr. Neil Shah, UCSF. Parental Ba/F3 cells (and as a control, Ba/F3 BCR-ABL$^{T315I}$ cells) were supplemented with IL-3 provided by WEHI-conditioned media. Prior to cell proliferation assays, RNA was isolated from each Ba/F3 cell line, and kinase domain mutations were confirmed by RT-PCR followed by DNA sequence analysis using Mutation Surveyor software (SoftGenetics, State College, PA).

### Cell Proliferation Assays

Ba/F3 cell lines were distributed in 96-well plates ($4 \times 10^3$ cells/well) and incubated with escalating concentrations of AP24534 for 72 hr. The inhibitor ranges used were: 0–625 nM for cells expressing BCR-ABL and 0–10,000 nM for BCR-ABL negative cells. Proliferation was measured using a methanethiosulfonate (MTS)-based viability assay (CellTiter96 Aqueous One Solution; Promega). IC$_{50}$ values are reported as the mean of three independent experiments performed in quadruplicate. For cell proliferation experiments with CML or normal primary cells, mononuclear cells were plated in 96-well plates ($5 \times 10^4$ cells/well) over graded concentrations of AP24534 (0–1000 nM) in RPMI supplemented with 10% FBS, L-glutamine, penicillin/streptomycin, and 100 μM β-mercaptoethanol. Following a 72 hr incubation, cell viability was assessed by subjecting cells to an MTS assay. All values were normalized to the control wells with no drug.

### CrkL Phosphorylation in Ba/F3 Cell Lines

Ba/F3 cells expressing native BCR-ABL or BCR-ABL$^{T315I}$ ($5 \times 10^6$ per well) were cultured 4 hr in complete media alone or with imatinib (2000 nM), dasatinib (50 nM), nilotinib (500 nM), or AP24534 (0.1–1000 nM). Lysates produced by boiling cells in SDS-PAGE loading buffer supplemented with protease and phosphatase inhibitors. Lysates were subjected to SDS-PAGE and immunoblotted with anti-CrkL antibody C-20 (Santa Cruz). Phosphorylated and

non-phosphorylated CrkL signals were distinguished based on differential band migration, quantified by densitometry on a Lumi Imager (Roche) and expressed as a % phosphorylated CrkL.

## Ex Vivo Exposure of BCR-ABL$^{T315I}$ Patient Samples to AP24534

Peripheral blood mononuclear cells from a patient with CML in lymphoid blast crisis (CML L-BC) with a BCR-ABL$^{T315I}$ mutation were isolated by Ficoll centrifugation. RT-PCR and sequencing analysis confirmed that the sample predominantly contained the BCR-ABL$^{T315I}$ mutant. Mononuclear cells ($5 \times 10^6$ cells/well) were cultured overnight in serum-free IMDM media (Invitrogen) supplemented with 20% BIT (StemCell), 40 μg/mL human low-density lipoprotein, and 100 μM β-mercaptoethanol alone or with imatinib (1000 nM), dasatinib (50 nM), nilotinib (200 nM), or AP24534 (50 nM, 500 nM). Cells were lysed directly into boiling SDS-PAGE loading buffer supplemented with protease and phosphatase inhibitors. Lysates were subjected to SDS-PAGE and immunoblotted with anti-CrkL antibody C-20 (Santa Cruz). Phosphorylated and non-phosphorylated CrkL were distinguished based on differential band migration. Band signal intensities were quantified by densitometry on a Lumi Imager (Roche).

## Global Tyrosine Phosphorylation by FACS

Mononuclear cells ($2 \times 10^5$) were cultured overnight in serum-free media alone or with imatinib (1000 nM), dasatinib (50 nM), nilotinib (200 nM), or graded concentrations of AP24534 (50, 500 nM). Cells were fixed and permeabilized according to the manufacturer's instructions (Caltag), incubated with 2 μg of anti-phosphotyrosine 4G10-FITC antibody (BD Biosciences) for 1 hr, washed twice with PBS supplemented with 1% BSA and 0.1% sodium azide, and fixed in 1% formaldehyde. FITC signal intensity was analyzed on a FACSAria instrument (BD) and mean fluorescence intensity (MFI) was calculated. Values are reported as fold increase in MFI relative to unstained controls.

## Hematopoietic Colony Forming Assays of Primary CML Cells and Normal Bone Marrow

To assess the effect of AP24534 against primary CML cells harboring BCR-ABL$^{T315I}$ and normal hematopoietic progenitors, bone marrow mononuclear cells isolated by Ficoll density centrifugation were cultured with graded concentrations of AP24534 (CML patient: 0–50 nM; healthy individual: 0–1000 nM). Cells were plated in triplicate ($5\times10^4$ cells/plate) in 1 mL of IMDM:methylcellulose media (1:9 v/v) containing 50 ng/mL SCF, 10 ng/mL GM-CSF, and 10 ng/mL IL-3 (Methocult GF H4534; Stem Cell Technologies) to assess granulocyte/ macrophage colony formation (CFU-GM). After culturing at 37°C for 14–18 days, colonies (>50 cells) were counted and results reported as the percentage of colonies relative to untreated control ± SEM.

## Pharmacokinetics

All animal experiments were approved by ARIAD's IACUC and conformed to relevant regulatory standards. The pharmacokinetic profile of AP24534 (in citrate buffer, pH 2.74) was assessed in CD-1 female mice after a single dose by oral gavage. Blood samples were collected at various time points and AP24534 concentrations in plasma determined by an internal standard LC/MS/MS method using protein precipitation and calibration standards prepared in blank mouse plasma. Reported concentrations are average values from 3-mice/time point/dose group.

## Ba/F3 survival model

Ba/F3 cells expressing native BCR-ABL or BCR-ABL$^{T315I}$ were injected into the tail vein of female SCID mice (100 μL of a $1\times10^7$ cells/mL suspension in serum-free medium). Beginning 72 hr later mice were treated once daily by oral gavage with vehicle (25 mM citrate buffer, pH

2.75), AP24534, or dasatinib for up to 19 consecutive days. Moribund animals were sacrificed as per IACUC guidelines. On necropsy, mice had marked splenomegaly due to tumor cell infiltration. Survival data were analyzed using Kaplan-Meier method, and statistical significance was evaluated with a Log-rank test (GraphPad PRISM) comparing the survival time of each treatment group with the vehicle group.

## Ba/F3 Tumor Model

Ba/F3 BCR-ABL$^{T315I}$ cells were implanted subcutaneously into the right flank of female nude mice (100 μL of a $1\times10^7$ cells/mL cell suspension in serum-free medium). Mice were randomized to treatment groups when the average tumor volume reached approximately 500 mm$^3$. Mice were treated once daily by oral gavage with vehicle (25 mM citrate buffer, pH 2.75) or AP24534 for up to 19 consecutive days. Tumor volume (mm$^3$) was calculated using the following formula: tumor volume = $L\times W^2\times0.5$. To determine tumor growth inhibition when the treatment period was finished, mean tumor volume for treatment group/mean tumor volume for control group (%T/C) was calculated at the final measurement. The mean tumor volume from the last measurement of all groups was compared using a one-way ANOVA test (GraphPad PRISM) and each treatment group was further compared to that of vehicle-treated mice for statistical significance using Dunnett's test.

For analysis of tyrosine-phosphorylated BCR-ABL and CrkL levels, tumor-bearing animals were treated with a single dose of vehicle or 30 mg/kg AP24534 by oral gavage. Six hr after dosing, animals (N=3/group) were sacrificed and tumor samples collected for immunoblot analysis with antibodies against pBCR-ABL and eIF4E (Cell Signaling Technology) and total CrkL (C-20; Santa Cruz).

## Accelerated cell-based mutagenesis screen: Single-agent AP24534

Ba/F3 cells expressing native BCR-ABL were treated overnight with *N*-ethyl-*N*-nitrosourea (ENU; 50 μg/mL), pelleted, resuspended in fresh media, and distributed into 96-well plates at a density of $1\times10^5$ cells/well in 200 μL complete media supplemented with graded concentrations of AP24534. The wells were observed for cell growth under an inverted microscope and media color change every two days throughout the 28-day experiment. The contents of wells exhibiting cell outgrowth were transferred to a 24-well plate containing 2 mL complete media supplemented with AP24534 at the same concentration as in the initial 96-well plate. If growth was simultaneously observed in all wells of a given condition, 24 representative wells were expanded for further analysis. At confluency, cells in 24-well plates were collected by centrifugation. DNA was extracted from the cell pellets using a DNEasy Tissue kit (QIAGEN). The BCR-ABL kinase domain was amplified using primers B2A (5′ TTCAGAAGCTTCTCCCTGACAT 3′) and ABL4317R (5′ AGCTCTCCTGGAGGTCCTC 3′), PCR products were bi-directionally sequenced by a commercial contractor (Agencourt Bioscience) using primers ABL3335F (5′-ACCACGCTCCATTATCCAGCC-3′) and ABL4275R (5′-CCTGCAGCAAGGTAGTCA-3′), and the chromatograms were analyzed for mutations using Mutation Surveyor software (SoftGenetics). Results from this screen are reported as the cumulative data from three independent experiments (see Table S2). The mutagenesis screen was also conducted as described above for single-agent AP24534 starting with Ba/F3 cells expressing BCR-ABL$^{T315I}$ (see Table S3) or BCR-ABL$^{E255V}$ (see Table S4) in single independent experiments.

## Crystallographic Coordinates

Crystallographic coordinates for the AP24534:ABL$^{T315I}$ complex have been deposited at the RCSB Protein Data Bank. The PDB accession number is 3IK3.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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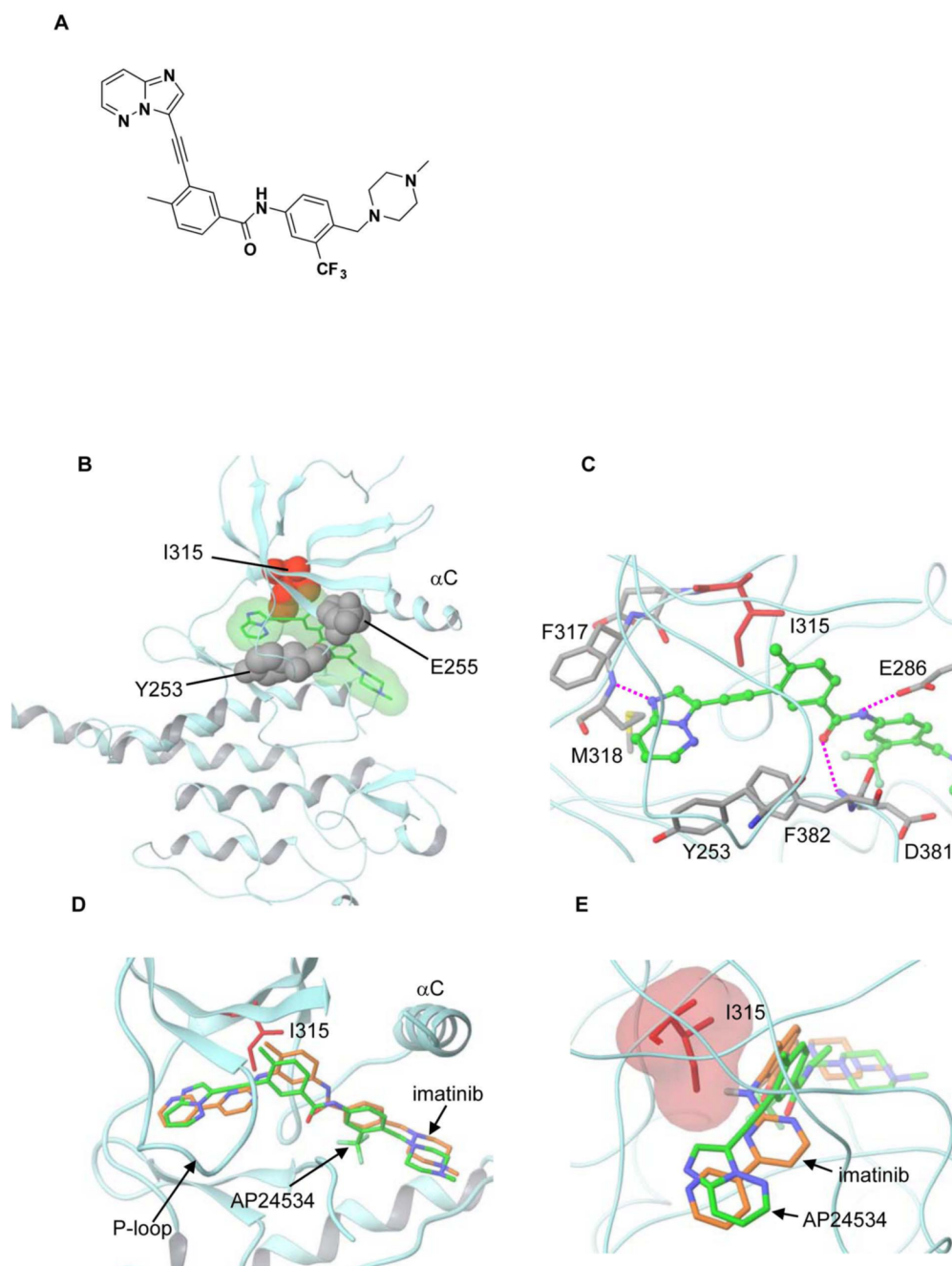
**Figure 1. Chemical Structure of AP24534 and Co-Crystal Structure with ABL[T315I]**

(A) Chemical structure of AP24534.

(B) Crystal structure of AP24534 in complex with the ABL[T315I] mutant kinase. AP24534 is shown in green with translucent molecular surface. The side chain of the mutated gatekeeper residue Ile315 is shown in red. The side chains of Y253 and E255, locations of point mutations appearing in the resistant outgrowth screen of AP24534 are shown in grey. The C-helix is labeled (αC).

(C) Key interactions of AP24534 with ABL[T315I] at the ATP binding site. Hydrogen bonds are highlighted with pink dashed lines. Residues making critical contact with the imidazo[1,2*b*] pyridazine core and the ethynyl linker group of AP24534 are also labeled.

(D) and (E) Superposition of imatinib and AP24534 highlighting the effect of the Thr to Ile mutation. The superposition was based on the C positions of ABL residues 312–321 in the T315I mutant and in native ABL kinase complexed with imatinib (shown in brown; PDB code 1IEP).
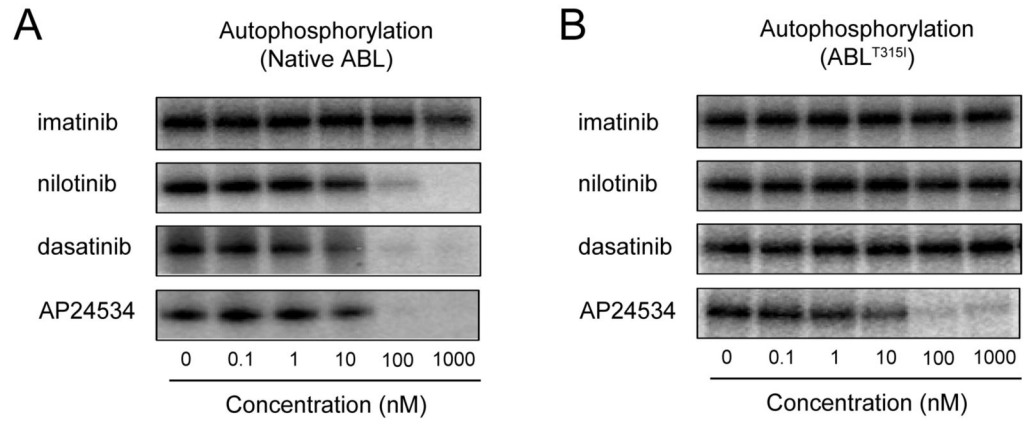
**Figure 2. AP24534 Inhibits the Autophosphorylation of ABL and ABL^T315I**
In vitro [γ-$^{32}$P]-ATP autophosphorylation of full-length (A) ABL or (B) ABL$^{T315I}$ kinase treated with AP24534, imatinib, nilotinib, or dasatinib. After incubation of tyrosine-dephosphorylated enzyme with the indicated inhibitor in the presence of [γ-$^{32}$P]-ATP and separation by SDS-PAGE, signal intensity was measured by autoradiography.

A

Ba/F3 cells:
Native BCR-ABL



B

Ba/F3 cells:
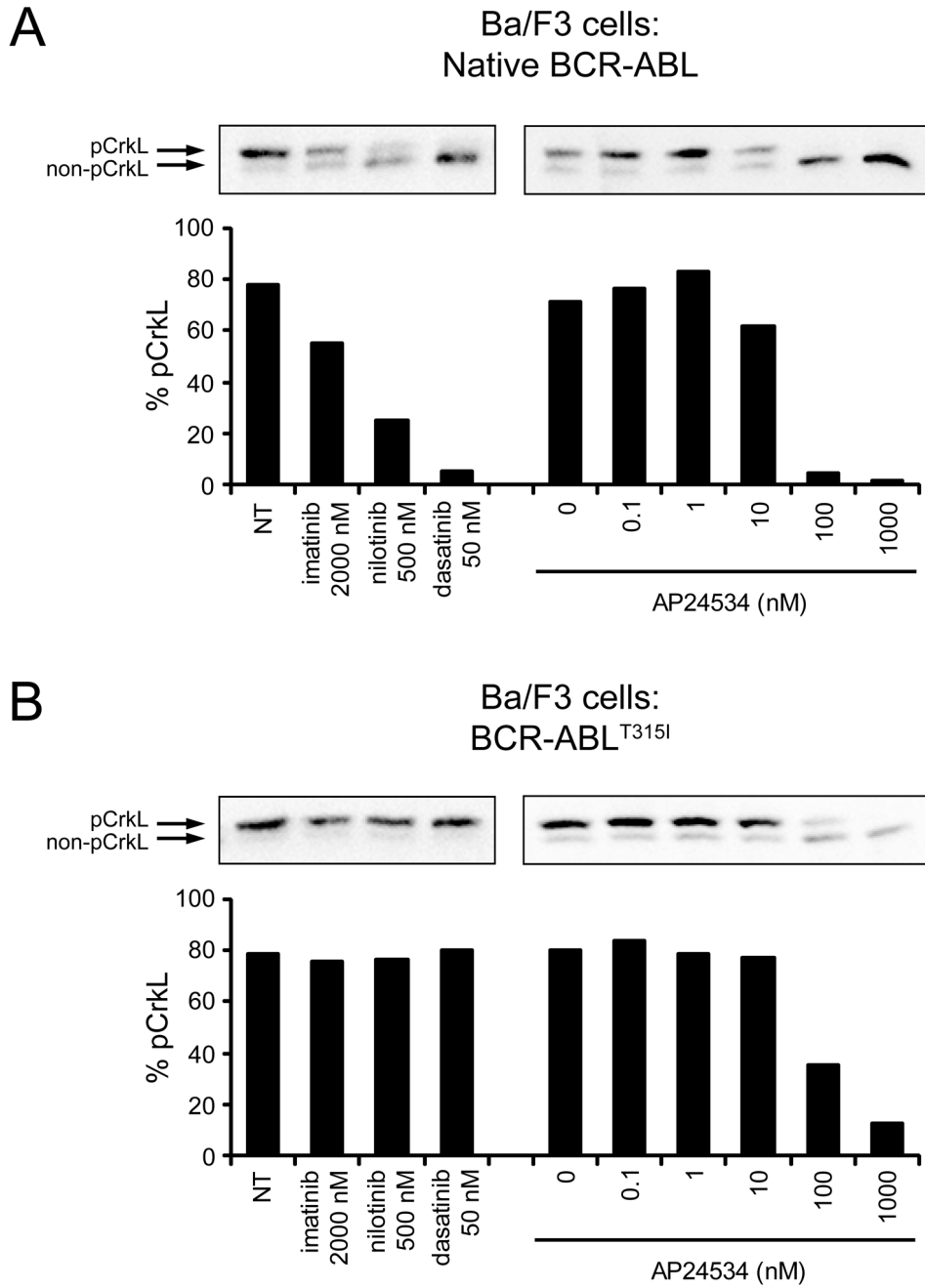BCR-ABL$^{T315I}$



**Figure 3. AP24534 Inhibits BCR-ABL Signaling in CML Cell Lines Expressing Native BCR-ABL or BCR-ABL$^{T315I}$**

(A) Immunoblot analysis of CrkL phosphorylation in Ba/F3 cells expressing native BCR-ABL treated with imatinib, nilotinib, dasatinib, or AP24534. Cells were cultured for 4 hr with inhibitors, harvested, lysed, and analyzed by immunoblot using an antibody for CrkL, a substrate of BCR-ABL whose phosphorylation is an established clinical marker of BCR-ABL kinase activity. Both the phosphorylated and non-phosphorylated forms are resolved by electrophoretic mobility, and bands are quantitated by densitometry and expressed as a % phosphorylated CrkL. (B) Immunoblot analysis of CrkL phosphorylation in Ba/F3 BCR-

ABL$^{T315I}$ cells treated with imatinib, nilotinib, dasatinib, or AP24534. Assays and analysis were carried out as described above in panel (A). Abbreviations: NT, no treatment.
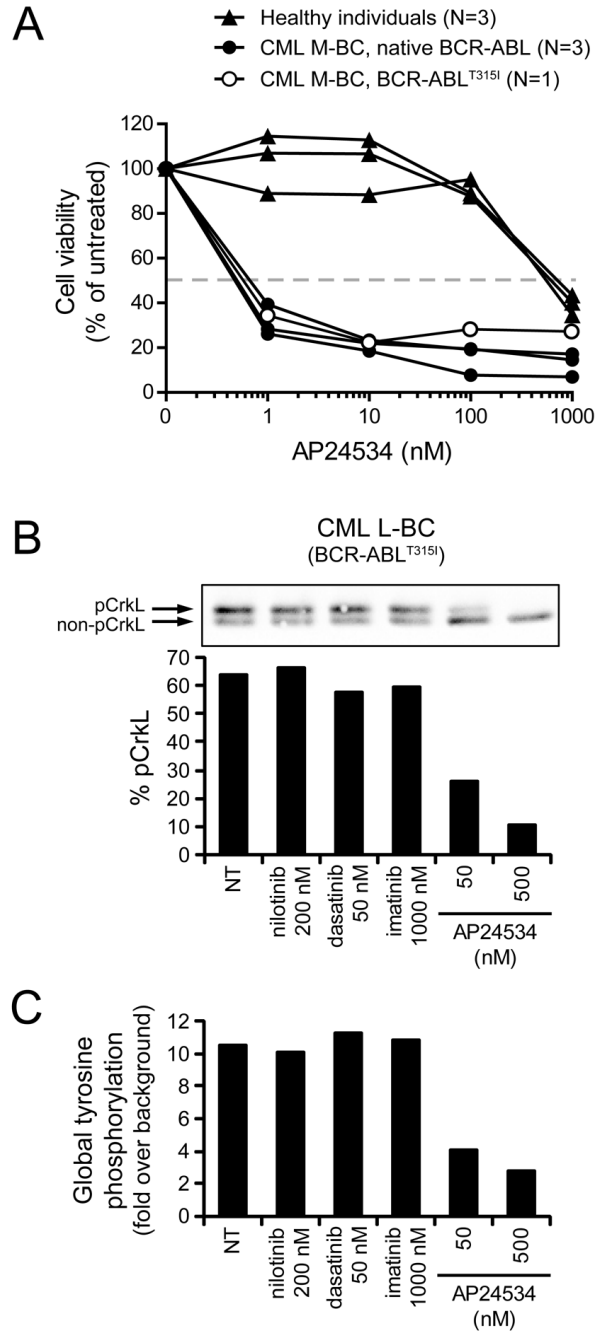
**Figure 4. Ex Vivo Treatment of CML Primary Cells with AP24534 Inhibits Cellular Proliferation and BCR-ABL-Mediated Signaling**

(A) Cellular proliferation assays for ex vivo AP24534-treated mononuclear cells from CML myeloid blast crisis (M-BC) patients harboring native BCR-ABL (N=3) or BCR-ABL[T315I] (N=1) and from healthy individuals (N=3). For reference, the dashed line indicates 50% cell viability relative to untreated cells.

(B) Immunoblot analysis of CrkL phosphorylation in mononuclear cells from a CML lymphoid blast crisis (L-BC) patient harboring BCR-ABL[T315I] following ex vivo exposure to AP24534, imatinib, nilotinib, or dasatinib. Cells were cultured overnight with inhibitors, harvested, lysed, and analyzed by CrkL immunoblot. Both the phosphorylated and non-phosphorylated forms

are resolved by electrophoretic mobility, and bands are quantitated by densitometry and expressed as a % phosphorylated CrkL.

(C) FACS analysis of global tyrosine phosphorylation in mononuclear cells from the CML L-BC BCR-ABL$^{T315I}$ patient in panel (B). After overnight culture with inhibitors, cells were fixed and permeabilized, incubated with a FITC-labeled antibody for phosphorylated tyrosine, and analyzed by FACS. Values reported are as fold increase in mean fluorescence intensity relative to unstained controls. Abbreviations: NT, no treatment.
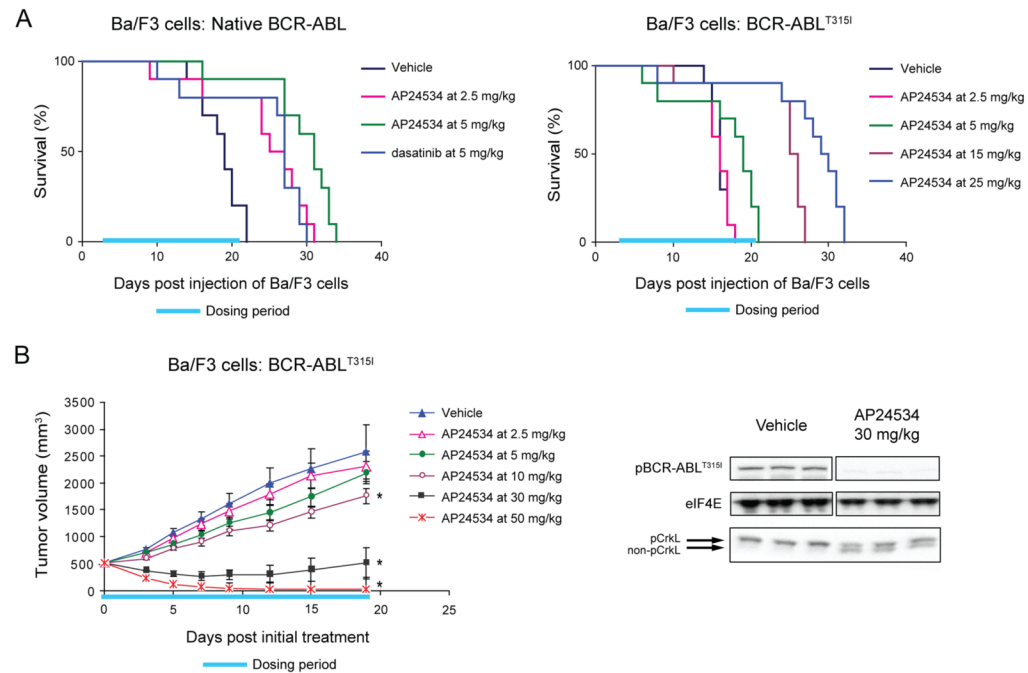
**Figure 5. AP24534 is Effective in Mouse Xenograft Models of BCR-ABL-Driven and BCR-ABL$^{T315I}$-Driven Tumor Growth**

(A) Effect of AP24534 on survival of SCID mice after intravenous injection of Ba/F3 cells expressing native BCR-ABL (left) or BCR-ABL$^{T315I}$ (right). Ba/F3 cells expressing native BCR-ABL or BCR-ABL$^{T315I}$ were injected into the tail vein of SCID mice, and animals were treated once daily by oral gavage with vehicle, AP24534, or dasatinib for the indicated dosing period (days 3–21).

(B) In vivo efficacy and BCR-ABL signaling suppression by AP24534 in a subcutaneous xenograft model using Ba/F3 BCR-ABL$^{T315I}$ cells. Tumor-bearing animals were treated once daily by oral gavage with vehicle or the indicated doses of AP24534 for 19 consecutive days (dosing period indicated) with mean tumor volume plotted (error bars represent S.E.M.). Each AP24354 treatment group was compared to the vehicle group using Dunnett's test, with statistical significance (p<0.05) indicated by an asterisk. BCR-ABL and CrkL phosphorylation were evaluated by immunoblot in animals treated with a single oral dose of vehicle or 30 mg/kg AP24534 (N=3 per group).
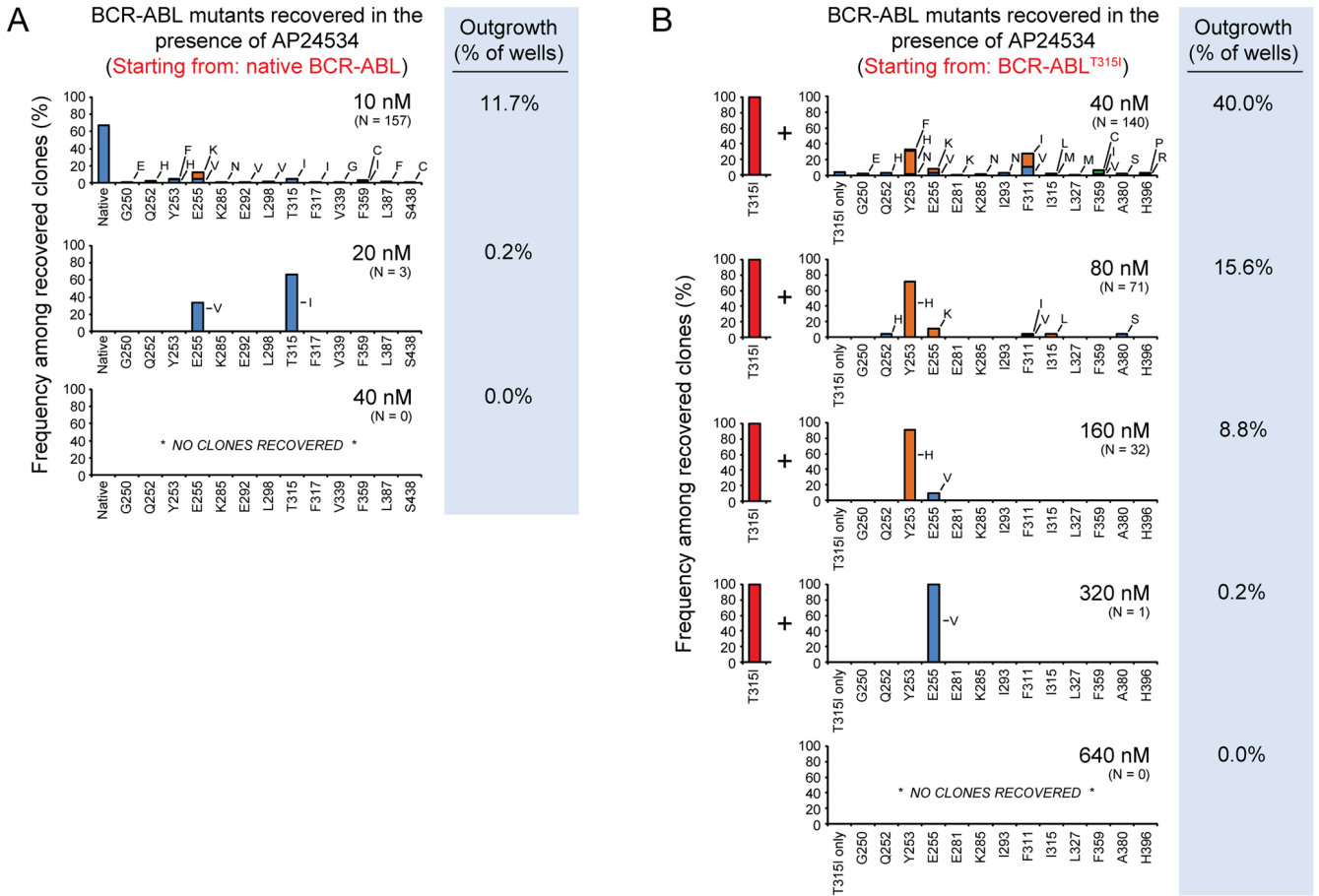
**Figure 6. Single-Agent AP24534 Completely Suppresses Resistant Outgrowth in Cell-Based Mutagenesis Screens**

(A) Resistant clones recovered from ENU-treated Ba/F3 cells starting from native BCR-ABL cultured with graded concentrations of AP24534 (10, 20, 40 nM). Each bar represents the relative percentage of the indicated BCR-ABL kinase domain mutant among recovered subclones. Since the percentage of surviving resistant subclones and the concentration of AP24534 are inversely related, a different number of sequenced subclones are represented in the graph for each concentration of AP24534 (Table S2). The percent of wells surveyed that contained outgrowth is indicated to the right of each graph.

(B) ENU-treated Ba/F3 BCR-ABL^T315I cells were cultured with graded concentrations of AP24534 (40, 80, 160, 320, 640 nM). Experiments and data analysis were performed as described in panel (A). All recovered subclones contain T315I in addition to the secondary mutation indicated on each graph.

**Table 1**

Kinase Inibition Profile of AP24534 for Native ABL, ABL$^{T315I}$, and Selected Kinases

| Kinase | IC$_{50}$ (nM) |
| --- | --- |
| ABL | 0.37 |
| ABL$^{T315I}$ | 2.0 |
| ABL$^{Q252H}$ | 0.44 |
| ABL$^{Y253\,F}$ | 0.30 |
| ABL$^{M351T}$ | 0.30 |
| ABL$^{H396P}$ | 0.34 |
| c-SRC | 5.4 |
| LYN | 0.24 |
| c-KIT | 12.5 |
| VEGFR2 | 1.5 |
| FGFR1 | 2.2 |
| PDGFRα | 1.1 |
| IR | >1000 |
| IGF-1R | >1000 |
| Aurora A | >1000 |
| CDK2/Cyclin E | >1000 |

**Table 2**

AP24534 $IC_{50}$ Values for Ba/F3 Cellular Proliferation Assays and CML and Non-CML Cellular Proliferation Assays

| Cell lines | AP24534 $IC_{50}$ (nM) |
| --- | --- |
| Ba/F3 cells | |
| Native BCR-ABL | 0.5 |
| M244V | 2.2 |
| G250E | 4.1 |
| Q252H | 2.2 |
| Y253F | 2.8 |
| Y253H | 6.2 |
| E255K | 14 |
| E255V | 36 |
| T315A | 1.6 |
| T315I | 11 |
| F317L | 1.1 |
| F317V | 10 |
| M351T | 1.5 |
| F359V | 10 |
| H396P | 1.1 |
| Parental | 1713 |
| T315I + IL-3 | 1804 |
| CML leukemia cells | |
| K562 | 3.9 |
| KY01 | 0.4 |
| LAMA | 0.3 |
| Non-CML leukemia cells | |
| Marimo | 2215 |
| HEL | 2522 |
| 1CMK | 1652 |