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

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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 preclinical evaluation of AP24534, a potent, orally available multitargeted kinase inhibitor active against T315I and other BCR-ABL mutants. AP24534 inhibited all tested BCR-ABL mutants in cellular and biochemical assays, 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.

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 5 years of therapy. Also, durable responses are uncommon in patients with advanced CML or Philadelphia chromosome (Ph)-positive acute lymphoblastic leukemia.

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

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 multitargeted kinase inhibitor that potently inhibits BCR-ABL T315I. It might 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 might offer important strategic advantages in a first-line capacity by minimizing BCR-ABL kinase domain mutation-based drug resistance.
imatinib family member nilotinib (Tasigna; AMN107) and the multitargeted 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 discussed 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-739558, 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 a 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 mutations in the same BCR-ABL allele mutants resistant to all ABL kinase inhibitors, including some that do not involve T315I mutations (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 preclinical 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, because 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 (Huang et al., 2009; M. Azam, personal communication). 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 (Figures 1B and 1C) and maintains a network of protein contacts similar to imatinib (Figures 1D and 1E). Specifically, the imidazo[1,2b]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: one with the backbone of M318 in the hinge region, one with the backbone of D381, one with the side chain of E286 (Figure 1C), and two from the methylpiperazine group (data 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^{L259P}, ABL^{Y253F}, and ABL^{L259K} (data 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 available online). 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
Figure 1. Chemical Structure of AP24534 and Cocrystal 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 gray. 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[1,2b]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 Cα positions of ABL residues 312–321 in the T315I mutant and in native ABL kinase complexed with imatinib (shown in brown; PDB code 1IEP).
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 (data 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-ABLT315I 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 BCR-ABL-negative cell lines derived from leukemic patients. Although 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-ABLT315I**

To confirm target inhibition in Ba/F3 cells expressing native BCR-ABL or BCR-ABLT315I, 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. Although 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-ABLT315I 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-ABLT315I 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<sup>T315I</sup>-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 nM, 58 nM, and 2 nM were achieved at 2 hr, 6 hr, and 24 hr postdose, respectively. At a dose of 30 mg/kg, mean plasma levels reached 782 nM, 561 nM, and 8 nM at the same time points. These results demonstrate that plasma levels exceeding the in vitro IC<sub>50</sub> values for all tested BCR-ABL mutants can be sustained in mice for >6 hr with oral dosing, 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<sup>T315I</sup> 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 days, 26 days, and 30 days, respectively compared with 16 days for vehicle-treated mice (p < 0.01 for all three dose levels).

The antitumor activity of AP24534 was further assessed in a xenograft model in which Ba/F3 BCR-ABL<sup>T315I</sup> cells were
injected subcutaneously into mice. Tumor growth was inhibited by AP24534 in a dose-dependent manner (Figure 5B, left) compared with vehicle-treated mice, with significant suppression of tumor growth upon daily oral dosing at 10 mg/kg 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 with 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 mg/kg, 30 mg/kg, and 50 mg/kg dose groups, respectively, with no signs of overt toxicity.

To confirm target inhibition, we assessed levels of phosphorylated BCR-ABL 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 IC50 for parental Ba/F3 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; data 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 IC50 for parental Ba/F3 cell line inhibition. This resistance profile was confirmed in a subsequent screen starting from a background of BCR-ABL255V, 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. Although 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 subregion 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 ABLT315I. Although 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 nonproximal 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 with ABLT315I. Several of these kinases are important clinical targets of imatinib, nilotinib, and/or dasatinib, although only dasatinib has been reported to inhibit all 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. The fact that SRC, VEGFR, FGFR, and PDGFR family kinases are potential targets in a variety of other malignancies 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...
AP24534 Is a Pan-BCR-ABL Inhibitor

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 pharmacology studies, where concentrations above the IC₅₀ are achieved in this assay only with combinations of nilotinib or 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 preclinical 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 methylpi perazin 1 y)methyl) 3 (trifluorometlylphényl)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.

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 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.

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Cryotography. The typical yield of purified ABLT315I bound with AP24534 was about 1 mg/L. Crystals of ABLT315I 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 glycol 4000, 0.2 M sodium acetate, 0.1 M Tris [pH 8.5]). After 1-2 days, crystals reached a typical size of 50 × 50 × 300 μm³ and were harvested in mother liquor supplemented with 30% v/v glycerol as cryoprotectant. X-ray diffraction data were collected at 100 K 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) with the structure of native ABL bound with imatinib (Protein Data Bank 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 (Otwinowski and Minor, 1997).

Autophosphorylation Assays For ABL T315I
Kinase autophosphorylation assays with full length, tyrosine dephosphorylated ABL T315I, ABL T315I-V256E, ABL T315I-V256F, 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 [32P] ATP, recombinant kinase domain, peptide substrate, and a range of 10 concentrations of inhibitor to establish an IC50 value (http://www.reactionbiology.com/pages/kinase.html).

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% CO2. The Ba/F3 BCR ABL T315I cell line was a kind gift of Dr. Neil Shah (University of California, San Francisco). 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 reverse transcriptase polymerase chain reaction (RT PCR) followed by DNA sequence analysis with Mutation Surveyor software (SoftGenetics, State College, PA).

Cell Proliferation Assays
Ba/F3 cell lines were distributed in 96 well plates (4 × 10³ 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). IC₅₀ 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 × 10⁴ cells/well) over graded concentrations of AP24534 (0–1000 nM) in RPMI supplemented with 10% fetal bovine serum, 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 × 10⁴ 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 C20 (Santa Cruz). Phosphorylated and nonphosphorylated CrkL signals were distinguished based on differential band migration, quantified by densitometry on a Lumino 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 × 10⁶ cells/well) were cultured overnight in serum free IMDM media (Invitro) 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 C20 (Santa Cruz). Phosphorylated and nonphosphorylated CrkL were distinguished based on differential band migration. Band signal intensities were quantified by densitometry on a Lumino Imager (Roche).

Global Tyrosine Phosphorylation by Fluorescence-Activated Cell Sorting
Mononuclear cells (2 × 10⁵) 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 nM, 500 nM). Cells were fixed and permeabilized according to the manufacturer’s instructions (CalTag), incubated with 2 μg anti-phosphotyrosine 4G10 FITC antibody (BD Biosciences) for 1 hr, washed twice with phosphate buffered saline supplemented with 1% bovine serum albumin and 0.1% sodium azide, and fixed in 1% formaldehyde. Fluorescein isothiocyanate (FITC) signal intensity was analyzed on a FACScan 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, we cultured bone marrow mononuclear cells isolated by Ficoll density centrifugation with graded concentrations of AP24534 (CML patient: 0; normal healthy individual: 0; 1000 nM). Cells were plated in triplicate (5 × 10³ cells/plate) in 1 ml IMDM:methylcellulose media ([1:1 v/v]) containing 50 ng/ml SCF, 10 ng/ml GM CSF, and 10 ng/ml IL 3 (Methocult GF H4534; Stem Cell Technologies) for assessment of 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 and standard error of the mean (SEM).

Pharmacokinetics
All animal experiments were approved by ARIA’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
lipid chromatography tandem mass spectrometry 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 x 10^7 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 microscopy, 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 x 10^7 cells/ml suspension in serum free medium). Mice were randomized to treatment groups when the average tumor volume reached ~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 x W^2 x 0.5. For determining 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 analysis of variance test (GraphPad PRISM) and each treatment group was further compared to that of vehicle treated mice for statistical significance using Dunnnett’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 hours 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 x 10^5 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 2 days throughout the 28 day experiment. The contents of wells exhibiting cell outgrowth were transferred to a 24 well plate containing 2 ml serum free medium. If growth was simultaneously observed in all wells the mutagenesis screen was halted. 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 L259V (see Table S4) in single independent experiments.

**ACCESSION NUMBERS**
Crystallographic coordinates for the AP24534:ABL T315I complex have been deposited at the RCSB Protein Data Bank under accession number 3IK3.

**SUPPLEMENTAL DATA**
Supplemental Data include three figures and five tables and can be found with this article online at http://www.cell.com/cancer-cell/supplemental/S1535-6108(09)00339-0.

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and recommendations for harmonizing current methodology for detecting BCR ABL transcripts and kinase domain mutations and for expressing results. Blood 108, 28 37.


