In vitro Activity of Bcr-Abl Inhibitors AMN107 and BMS-354825 against Clinically Relevant Imatinib-Resistant Abl Kinase Domain Mutants

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Abstract

Imatinib, a Bcr-Abl tyrosine kinase inhibitor, is a highly effective therapy for patients with chronic myelogenous leukemia (CML). Despite durable responses in most chronic phase patients, relapses have been observed and are much more prevalent in patients with advanced disease. The most common mechanism of acquired imatinib resistance has been traced to Bcr-Abl kinase domain mutations with decreased imatinib sensitivity. Thus, alternate Bcr-Abl kinase inhibitors that have activity against imatinib-resistant mutants would be useful for patients who relapse on imatinib therapy. Two such Bcr-Abl inhibitors are currently being evaluated in clinical trials: the improved potency, selective Abl inhibitor AMN107 and the highly potent dual Src/Abl inhibitor BMS-354825. In this study, we compared imatinib, AMN107, and BMS-354825 in cellular and biochemical assays against a panel of 16 kinase domain mutants representing >90% of clinical isolates. We report that AMN107 and BMS-354825 are 20-fold and 325-fold more potent than imatinib against cells expressing wild-type Bcr-Abl and that similar improvements are maintained for all imatinib-resistant mutants tested, with the exception of T315I. Thus, both inhibitors hold promise for treating imatinib-refractory CML. (Cancer Res 2005; 65(11): 4500-5)

Introduction

Imatinib (STI571, Gleevec), an Abl kinase inhibitor, is now the first-line treatment for patients with chronic myelogenous leukemia (CML; ref. 1). Its success is predicated on the efficient inhibition of the deregulated, oncogenic tyrosine kinase Bcr-Abl, which plays a critical role in the pathogenesis of CML (2, 3). Most newly diagnosed CML patients with chronic phase disease treated with imatinib achieve durable responses (4); however, a small percentage of these patients and most advanced-phase patients relapse on imatinib therapy (4–6).

In patients who relapse on imatinib therapy, the Bcr-Abl kinase is reactivated, emphasizing the importance of the kinase activity of this protein to disease pathogenesis. The most common mechanism of resistance, occurring in 60% to 90% of patients who acquire imatinib resistance, involves specific mutations in the kinase domain of Bcr-Abl that interfere with imatinib binding without eliminating ATP binding or kinase activity (reviewed in refs. 7, 8). Clinically observed mutations identified within the Bcr-Abl kinase domain span a range of residual imatinib sensitivities (IC50: 900-4,400 nmol/L) and encompass several functionally distinct kinase domain regions, including the nucleotide binding P-loop, imatinib contact residues, and the activation loop (7, 9, 10).

An understanding of the mechanism of imatinib resistance has prompted the search for alternate Bcr-Abl inhibitors that are effective against clinically observed Bcr-Abl mutants. Two promising new Bcr-Abl inhibitors for treating imatinib-resistant CML are currently being evaluated in clinical trials: the selective Abl inhibitor AMN107 and the dual Src/Abl inhibitor BMS-354825 (Fig. 1A, top). AMN107 was developed by rational drug design based on the crystal structure of an Abl-imatinib complex, whereas BMS-354825 is a Src inhibitor that was found to exhibit Abl inhibitory properties.

The crystal structure of the Abl kinase domain in complex with imatinib indicates that few changes to the imatinib scaffold are likely to be tolerated (7, 11). Imatinib binds to the canonical ATP site lining the groove between the N and C lobes of the protein. The drug penetrates into the central cleft of the kinase and the high topological congruency between the aniline-pyrimidine substructure and the surface of the distorted ATP-binding pocket suggest that making changes within this region of imatinib might not be very productive. However, the methylpiperazinyl group of imatinib (Fig. 1A, top) lies along a surface-exposed pocket of the Abl kinase and is potentially more amenable to modification. Replacement of this heterocycle and further rational design to optimize drug-like properties led to the discovery of AMN107 (Novartis Pharmaceuticals, Basel, Switzerland; Fig. 1A), which, as predicted, possesses substantially increased binding affinity and selectivity for the Abl kinase compared with imatinib (12).

Another approach to counteract imatinib resistance is to use inhibitors that bind Bcr-Abl with less stringent conformational requirements than imatinib. Imatinib selectively targets an inactive conformation of the Abl kinase domain in which the activation loop is in a nonphosphorylated, closed position that is incompatible with substrate binding (13). Specific differences between the inactive conformations of Abl and Src provide a structural basis for the initially surprising finding that Src family kinases are not imatinib targets, despite a high degree of sequence homology (11). The Abl and Src active conformations are more similar and many inhibitors that bind to the active conformation of Src are also capable of inhibiting Abl (11). BMS-354825 is a dual Src-Abl kinase
Materials and Methods

Reagents. BMS-354825 (N-(2-chloro-6-methylphenyl)-2-[[6-[[4-[(2-hydroxyethyl)-1-piperazinyl]-2-methyl-4-pyrimidinyl]amino]-5-thiazolylcarboxamide) was synthesized by Bristol-Myers Squibb (New York, NY; ref. 14). AMN107 (NVP-AMN107-AA: 4-methyl-N-[[4-(4-(2-hydroxyethyl)-1-piperazinyl)-2-methyl-4-pyrimidinyl]amino]-5-thiazolylcarboxamide, benzamide, hydrochloride) was synthesized by Novartis Pharmaceuticals (12). Imatinib was synthesized by Bristol-Myers Squibb (New York, NY; ref. 14).

Cell lines. Ba/F3 transfectants (expressing full-length wild-type Bcr-Abl or Bcr-Abl with kinase domain point mutations) were generated, selected, and maintained as previously described (10). Parental Ba/F3 cells were supplemented with interleukin-3 (IL-3).

Kinase autophosphorylation assays with glutathione S-transferase–Abl kinase domains. Kinase assays using wild-type and mutant glutathione S-transferase (GST)–Abl fusion proteins (c-Abl amino acids 220-498) were performed as described, with minor alterations (15). GST-Abl fusion proteins were released from glutathione-Sepharose beads before use; the concentration of ATP was 5 μmol/L. Immediately before use in kinase autophosphorylation and in vitro peptide substrate phosphorylation assays, GST-Abl kinase domain fusion proteins were treated with LAR tyrosine phosphatase according to the manufacturer's instructions (New England Biolabs, Beverly, MA). After 1-hour incubation at 30°C, LAR phosphatase was inactivated by addition of sodium vanadate (1 mmol/L). Immunoblot analysis comparing untreated GST-Abl kinase to dephosphorylated GST-Abl kinase was routinely done using phosphotyrosine-specific antibody 4G10 to confirm complete (>95%) dephosphorylation of tyrosine residues and c-Abl antibody CST 2862 to confirm equal loading of GST-Abl kinase. The inhibitor concentration ranges for IC50 determinations were 0 to 5,000 nmol/L (imatinib and AMN107) or 0 to 32 nmol/L (BMS-354825). The BMS-354825 concentration range was extended to 1,000 nmol/L for mutant T315I. These same inhibitor concentrations were used for the in vitro peptide substrate phosphorylation assays. The three inhibitors were tested at these same concentration ranges against GST-Src kinase (Cell Signaling Technology, Boulder, CO) and GST-Lyn kinase (Stressgen, Victoria, BC, Canada).

In vitro peptide substrate phosphorylation assays with glutathione S-transferase–Abl kinase domains. The effects of imatinib (0-5,000 nmol/L), AMN107 (0-5,000 nmol/L), and BMS-354825 (0-32 nmol/L) on the catalytic activity of unphosphorylated GST-Abl kinase were assessed using a synthetic, NH2-terminal biotin-linked peptide substrate (biotin-FAIYAAPFAKK-amide; ref. 16). Assays were carried out at 30°C for 5 minutes in 25 μL of reaction mixture consisting of kinase buffer [25 mmol/L Tris-HCl (pH 7.5), 5 mmol/L L-β-glycerophosphate, 2 mmol/L DTT, 0.1 mmol/L Na2VO4, 10 mmol/L MgCl2; Cell Signaling Technology], 50 μmol/L peptide substrate, 10 nmol/L wild-type or mutant GST-Abl kinase, and 50 μmol/L ATP/[γ-32P]ATP (5,000 cpm/pmol). Reactions were terminated by addition of guanidine hydrochloride to a final concentration of 2.5 mol/L.
A portion of each terminated reaction mixture was transferred to a streptavidin-coated membrane (SAM® biotin capture membrane; Promega, Madison, WI), washed, and dried according to the manufacturer's instructions; phosphate incorporation was determined by scintillation counting. Results were corrected for background binding to the membranes as determined by omitting peptide substrate from the kinase reaction. Time course experiments to establish the linear range of enzymatic activity as determined by omitting peptide substrate from the kinase reaction. Similar in vitro peptide substrate phosphorylation assays were conducted with two Src family kinases: GST-Src kinase (Cell Signaling Technology) and GST-Lyn kinase (Stressgen). For Src family kinases, SignaTECT PTK biotinylated peptide substrate 2 (Promega) was the peptide substrate; all other conditions were as described for the GST-Abl kinase assays.

Cellular proliferation assays. Ba/F3 cell lines were plated in triplicate and incubated with escalating concentrations of imatinib, AMN107, or BMS-354825 for 72 hours. Proliferation was measured using a methane-thiosulfonate-based viability assay (CellTiter96 Aqueous One Solution Reagent; Promega). IC50 and IC90 values are reported as the mean of three independent experiments done in quadruplicate. The inhibitor concentration ranges for IC50 and IC90 determinations were 0 to 2,000 nmol/L (imatinib and AMN107) or 0 to 32 nmol/L (BMS-354825). The imatinib concentration range was extended to 6,400 nmol/L for mutants with IC50 >2,000 nmol/L. The BMS-354825 concentration range was extended to 200 nmol/L for mutant T315I.

Immunoblotting. Ba/F3 cell lines (1 × 10⁶ cells) were incubated for 3 hours in media containing escalating doses of either imatinib or AMN107. Cells were collected by centrifugation and lysed in SDS sample buffer. Following SDS-PAGE, proteins were transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) for immunoblotting. Tyrosine-phosphorylated Bcr-Abl was detected with mouse monoclonal phosphotyrosine antibody 4G10 (Supplementary Fig. S1). Bcr-Abl expression was detected using rabbit c-Abl antibody CST 2862 (Cell Signaling Technology).

Results and Discussion

In this study, we compared the activity of imatinib and two promising Bcr-Abl inhibitors, AMN107 and BMS-354825, against wild-type Bcr-Abl and imatinib-resistant Bcr-Abl mutants. Biochemical assays with purified, dephosphorylated wild-type GST-Abl kinase showed that AMN107 inhibited Abl-catalyzed peptide substrate phosphorylation with ~20-fold higher potency than imatinib (IC50: 15 versus 280 nmol/L), whereas BMS-354825 had a two-log (~325-fold) increased potency relative to imatinib (IC50: 0.6 versus 280 nmol/L). Corresponding experiments with mutant Abl kinase domains revealed that the ~20-fold improved potency of AMN107 compared with imatinib is also seen with the imatinib-resistant mutants (Table 1). The one exception was the Abl mutant.
T315I, which was completely insensitive to AMN107 (highest concentration tested: 5,000 nmol/L). To facilitate comparisons within Table 1, the IC50 data are also expressed as fold change relative to a baseline of one for wild-type Abl kinase. By viewing the data in this way, it is apparent that the overall pattern of mutant sensitivity to AMN107 closely parallels that of imatinib. Because imatinib and AMN107 are predicted to share an absolute requirement for a specific inactive Bcr-Abl conformation and to bind in very similar ways, it is logical to expect largely the same pattern of effectiveness for AMN107 as for imatinib, but with the range of effectiveness shifted to a value more than an order of magnitude lower than that of imatinib. In contrast, BMS-354825 potently inhibited wild-type Abl kinase and all mutants except T315I over a narrow range (IC50 < 1.7 nmol/L). We also did a complete set of Abl kinase autophosphorylation assays with each of the inhibitors and obtained similar results to those from peptide substrate assays (Table 1). Thus, biochemical assays establish that AMN107 and BMS-354825 directly target wild-type and mutant Abl kinase domains and inhibit autophosphorylation and substrate phosphorylation in a concentration-dependent manner. Similar assays with the Src family kinases Src and Lyn (17, 18), which is expressed at high levels in primary CML blast crisis cells, showed that BMS-354825 is a potent inhibitor of these Src family kinases, whereas imatinib and AMN107 are inactive against these kinases (Table 1).

To extend the results with isolated kinase domains into a cellular context, we carried out cellular proliferation assays using Ba/F3 cells expressing wild-type or kinase domain mutants of Bcr-Abl. Similar to the data using isolated kinase domains, AMN107 inhibited the growth of cells expressing wild-type Bcr-Abl with 20-fold higher potency than imatinib (IC50 values: AMN107 13 versus 260 nmol/L; BMS-354825 0.8 nmol/L) displayed 325-fold greater potency compared with imatinib against cells expressing wild-type Bcr-Abl (Fig. 1; Table 2). For both inhibitors, similar improvements were maintained for all imatinib-resistant mutants tested, with the notable exception of T315I. We have previously reported that inhibition of Bcr-Abl by imatinib or the Ssrc/Abl inhibitor AP23464 results in the accumulation of cells expressing WT Bcr-Abl in the G0-G1 cell cycle phase (19). We confirmed that this is also the case for AMN107 in the current study (data not shown).

The data for AMN107 is summarized in Fig. 2, which shows a model of AMN107 in complex with Abl kinase mutant M351T, and shows that the sensitivity of Bcr-Abl mutants to AMN107 segregates into four categories: high (IC50 < 70 nmol/L: M244V, G250E, Q252H, F311L, F317L, M351T, V379I, L387M, H396P, H396R), medium (IC50 < 200 nmol/L: Y253F, E255K, F359V), low (IC50 < 450 nmol/L: Y253H, E255V), and insensitive (IC50 > 2,000 nmol/L: T315I).

This pattern is highly reminiscent of the corresponding ranking of imatinib sensitivities (Table 2) and expected given the highly related structures of these two compounds and the binding

| Table 2. Imatinib, AMN107, and BMS-354825 IC50 values (nmol/L) for cellular proliferation and cellular Bcr-Abl tyrosine phosphorylation assays |
|-------------------------------------------|----------------|----------------|----------------|----------------|----------------|
|                                           | Cellular proliferation |                |                | Bcr-Abl tyrosine phosphorylation |
|                                           | Imatinib | AMN107 | BMS-354825 | Imatinib | AMN107 | BMS-354825 |
| WT Bcr-Abl | 260 (9) | 13 (28) | 0.8 (2.6) | 280 (10) | 10 (10) | <10 (10) |
| M244V    | 2,000 (8) | 38 (56) | 1.3 (1.8) | 2,000 (8) | 48 (56) | 10 (10) |
| G250E    | 1,350 (5) | 48 (152) | 1.8 (3) | 1,350 (5) | 48 (152) | 10 (10) |
| Q252H    | 1,325 (5) | 70 (156) | 3.4 (11.2) | 1,325 (5) | 70 (156) | 10 (10) |
| Y253F    | 3,475 (13) | 125 (215) | 1.4 (3) | 3,475 (13) | 125 (215) | 10 (10) |
| Y253H    | >6,400 (25) | <100 (25) | 1.3 (3) | >6,400 (25) | <100 (25) | 10 (10) |
| E255K    | 5,200 (20) | 200 (415) | 5.6 (12.8) | 5,200 (20) | 200 (415) | 10 (10) |
| E255V    | >6,400 (25) | <100 (25) | 11 (27) | >6,400 (25) | <100 (25) | 10 (10) |
| F311L    | 480 (2) | 23 (48) | 1.3 (2.8) | 480 (2) | 23 (48) | nd (nd) |
| T315I    | >6,400 (25) | <100 (25) | >500 (25) | >6,400 (25) | <100 (25) | >1,000 (1,000) |
| F317L    | 1,050 (4) | 50 (116) | 1.2 (1.4) | 1,050 (4) | 50 (116) | 0.8 (1.2) |
| M351T    | 880 (3) | 15 (31) | 1.1 (3) | 880 (3) | 15 (31) | 0.8 (1.2) |
| F359V    | 1,825 (7) | 175 (385) | 2.2 (4.8) | 1,825 (7) | 175 (385) | 10 (10) |
| V379I    | 1,630 (6) | 51 (115) | 0.8 (1.6) | 1,630 (6) | 51 (115) | 0.8 (1.6) |
| L387M    | 1,000 (4) | 49 (115) | 2 (4) | 1,000 (4) | 49 (115) | <1 (1) |
| H396P    | 850 (3) | 41 (69) | 0.6 (1.2) | 850 (3) | 41 (69) | <1 (1) |
| H396R    | 1,750 (7) | 41 (73) | 1.3 (2.7) | 1,750 (7) | 41 (73) | 0.8 (1.2) |
| Parental Ba/F3 | >6,400 (25) | >100 (25) | >1,000 (1,000) | ns | ns | nd |

NOTE: Fold change refers to the fold difference in the IC50, relative to wild type, which is set to 1.
Abbreviation: nd, no signal detected, Bcr-Abl not expressed.
*Estimated from ref. (14).
†Values in parentheses are IC50 values, given for AMN107 and BMS-354825 only.
The least sensitive mutants were the ones that showed higher IC50 values for AMN107 and BMS-354825. For example, Y253H and E255V had IC50 values of 155 and 250 nmol/L, respectively, compared to the IC50 of 15 nmol/L for wild-type Bcr-Abl. However, T315I was completely insensitive to both AMN107 and BMS-354825, as indicated by the absence of phosphorylated Bcr-Abl tyrosine kinase signal in cell lysates.

In agreement with cellular proliferation and tyrosine phosphorylation assay results, AMN107 induced apoptosis at significantly lower concentrations than imatinib in cells expressing wild-type Bcr-Abl or any of the kinase domain mutants except T315I. As a basis of comparison, wild-type Bcr-Abl and all imatinib-resistant mutants except T315I were tested except T315I. If a trough level of only 500 nmol/L is achievable, three mutants (Y253H, E255V, and T315I) are predicted to be substantially resistant. BMS-354825 is ~325-fold more potent than imatinib and 16-fold more potent than AMN107 against wild-type Bcr-Abl. Again, invoking inhibition at or above the IC90 value (Table 2) as an indicator of clinical benefit, BMS-354825 would be predicted to be an effective single agent therapeutic for cells expressing wild-type Bcr-Abl and all mutants tested except T315I. If a trough level of only 500 nmol/L is achievable, three mutants (Y253H, E255V, and T315I) are predicted to be substantially resistant.

In summary, the cellular and biochemical experiments directly comparing AMN107 and BMS-354825 to imatinib show that both inhibitors are more potent than imatinib against all cell lines and purified Abl kinase domains tested except T315I. The mutants, other than T315I, that were least responsive to AMN107 in all three cellular assays were Y253H and E255V (Fig. 2). Analogous to imatinib, the extent of sensitivity to AMN107 depended on the specific substitution at a given position (e.g., E255V less sensitive than E255K to AMN107).

Both AMN107 and BMS-354825 hold promise for treating patients with imatinib-resistant CML except when the disease is driven by Bcr-Abl mutant T315I. Although both inhibitors efficiently block Bcr-Abl tyrosine kinase catalytic activity, they do so by binding to distinct, partially overlapping sites in the kinase domain and by placing different conformational requirements on the Abl kinase domain. If results of clinical trials and pharmacokinetic studies indicate that AMN107 and BMS-354825 are safe and effective, the feasibility of using these drugs in combination should be evaluated. In support of this approach, we recently investigated the use of imatinib in combination with BMS-354825 as a strategy for confronting drug resistance in patients with imatinib-resistant CML.
CML. Specifically, treating Ba/F3 cells expressing wild-type or imatinib-resistant Bcr-Abl kinase domain mutants with various combinations of imatinib and BMS-354825 produced additive inhibitory effects. Even at imatinib concentrations above clinically achievable levels, no antagonism of the Src/Abl inhibitor was observed. A particularly appealing therapeutic option is to use a Bcr-Abl inhibitor cocktail containing these inhibitors as well as an as yet undiscovered T315I inhibitor. Advantages of combinatorial therapy are that clones resistant to one of the Bcr-Abl inhibitors may be vulnerable to another component of the cocktail (22), the potential to eliminate a wider spectrum of mutants, including those that predate therapeutic intervention (23), and eradication of a higher proportion of residual leukemic cells (8). Therefore, using Bcr-Abl inhibitor combinations to treat newly diagnosed, chronic-phase CML patients may represent the best strategy to prevent or significantly delay the onset of acquired drug resistance. In addition, these Bcr-Abl kinase inhibitors could also increase response rates and duration of response because of their increased potency.

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