Combined Abl Inhibitor Therapy for Minimizing Drug Resistance in Chronic Myeloid Leukemia: Src/Abl Inhibitors Are Compatible with Imatinib

Thomas O’Hare, Denise K. Walters, Eric P. Stoffregen, Daniel W. Sherbenou, Michael C. Heinrich, Michael W.N. Deininger, and Brian J. Druker

Abstract
Purpose: Chronic myeloid leukemia (CML) is effectively treated with imatinib. However, reactivation of Bcr-Abl via kinase domain mutations that reduce sensitivity to imatinib can cause relapse. As combination therapy is frequently used to prevent emergence of resistance, the combination of imatinib with an inhibitor of imatinib-resistant Bcr-Abl mutants (e.g., Src/Abl inhibitors AP23848 and BMS-354825) was investigated.

Experimental Design: To test this approach, cellular proliferation and Bcr-Abl tyrosine phosphorylation assays were done on Ba/F3 cells expressing wild-type (WT) Bcr-Abl and four common imatinib-resistant mutants (Y253F, E255K, T315I, and M351T). Colony-forming assays with primary CML cells were also done.

Results: Both Src/Abl inhibitors retained full inhibitory capacity when coadministered with imatinib at concentrations above typical clinical levels. For cells expressing WT Bcr-Abl or the marginally imatinib-resistant mutant M351T, inclusion of imatinib at therapeutic levels enhanced the effects of the Src/Abl inhibitors. By comparison, for the highly imatinib-resistant mutants Y253F and E255K, inclusion of imatinib at clinical levels resulted in only a slight enhancement beyond the effects of the Src/Abl inhibitors. None of the inhibitors affected Bcr-Abl T315I cells. Colony-forming assays with primary CML cells yielded analogous results.

Conclusions: Our results indicate that Src/Abl inhibitors are compatible with imatinib and suggest that combined Abl inhibitor therapy is a feasible treatment strategy for patients with CML.

Imatinib (Gleevec, STI571) is a well-tolerated inhibitor of Bcr-Abl, the oncogenic tyrosine kinase that causes chronic myeloid leukemia (CML; refs. 1, 2). Most newly diagnosed patients with chronic-phase disease, treated with imatinib, achieve durable complete cytogenetic responses (3). However, a small percentage of these patients and most advanced-phase patients relapse (3–5). Furthermore, only a minority of patients achieve undetectable levels of Bcr-Abl transcripts (6, 7).

The leading cause of acquired resistance to imatinib is reactivation of Bcr-Abl kinase activity via kinase domain mutations (8, 9) that decrease the sensitivity of the kinase to imatinib by 3- to >100-fold (10). Structural analysis has revealed that imatinib binds to a unique, inactive conformation of the Abl kinase domain in which the activation loop is in a closed position that precludes substrate binding (11). Mutations that confer resistance to imatinib either affect residues directly involved in drug binding, impair the ability of the Abl kinase to undergo the extensive conformational changes required for imatinib binding, or favor the active conformation of the kinase to which imatinib is unable to bind (11–14).

The Src and Abl kinases share a high degree of homology within their kinase domains (15). Although their inactive states have unique conformations, consistent with the inability of imatinib to inhibit Src kinases, their active conformations are predicted to be quite similar (11, 16). Thus, Src inhibitors that were subsequently shown to inhibit the Abl kinase (17) bind to Bcr-Abl irrespective of Abl conformation (16, 18). In addition, many Src/Abl inhibitors are smaller than imatinib, contact fewer residues, and require fewer Abl conformational changes for their binding (16, 18). On this basis, we reasoned that Src/Abl inhibitors should inhibit most imatinib-resistant mutants and showed previously that two Src/Abl inhibitors, PD180970 and AP23464, inhibited wild-type (WT) Bcr-Abl and all imatinib-resistant Bcr-Abl kinase domain mutants with the exception of T315I (19, 20). The Src/Abl inhibitors AP23848 (ref. 20; an AP23464 analogue) and BMS-354825 (dasatinib) (18, 21)
exhibit outstanding \textit{in vitro} inhibitory profiles against most imatinib-resistant mutants. In addition, BMS-354825 is orally bioavailable and is showing promise in clinical trials of patients with chronic and advanced disease, who have relapsed on imatinib therapy (22, 23).

One attractive strategy for minimizing the onset of acquired drug resistance is to use a combined Abl inhibitor approach. Thus, frontline imatinib therapy would be supplemented with a Src/Abl inhibitor capable of preventing the expansion of clones dependent on mutated, imatinib-resistant Bcr-Abl. In addition, mutations associated with resistance to the alternate Abl inhibitors may be vulnerable to imatinib thus decreasing the overall chances of resistance (24, 25). Lastly, if additive antiproliferative effects are observed with the combination, this could lead to eradication of a higher proportion of residual leukemic cells (26). Additivity would be predicted only in the case of WT Bcr-Abl and mutants with marginal imatinib resistance (e.g., M351T).

For a combined Abl inhibitor approach to work, it is crucial that imatinib not interfere with the ability of the Src/Abl inhibitor to access its binding site within the Bcr-Abl kinase domain. Although they display different conformational and binding requirements, imatinib and the Src/Abl inhibitors bind to overlapping sites within the Bcr-Abl kinase domain. This raises the important concern that clinically achievable concentrations of imatinib may exert an interfering effect by restricting the second inhibitor’s access to its binding site. This would be of particular concern for WT Bcr-Abl. However,
as all of the imatinib-resistant mutants except T315I retain residual affinity for Bcr-Abl, interference would also be a concern for these mutants. In this study, we show that the Src/Abl inhibitors AP23848 and BMS-354825 retain their full inhibitory capacity even when coadministered with imatinib at concentrations above typical clinical levels and in some cases additive antiproliferative effects are observed. Our results suggest that combined Abl inhibitor therapy is a feasible approach for minimizing acquired drug resistance and potentially for decreasing Bcr-Abl-dependent molecular disease persistence in CML.

### Materials and Methods

**Reagents.** Experiments were done with serial dilutions of stock solutions (10 mmol/L) of imatinib (purchased from the Oregon Health and Science University pharmacy), AP23848 (ARIAD Pharmaceuticals, Cambridge, MA), and BMS-354825 (Bristol-Myers Squibb, New York, NY).

**Cell lines.** Parental Ba/F3 cells and stable Ba/F3 cell lines expressing full-length WT Bcr-Abl or Bcr-Abl with kinase domain point mutations were generated and maintained as described (19, 20). The term WT Bcr-Abl is used to distinguish Bcr-Abl with unmutated Abl kinase sequence from Bcr-Abl with mutations in the Abl kinase domain.

**Cell proliferation assays.** Proliferation assays with Ba/F3 cells expressing WT or mutant Bcr-Abl (Y253F, E255K, T315I, or M351T) were done as previously described (20). Cell viability was measured after a 3-day incubation in escalating concentrations of AP23848 or BMS-354825 alone or in combination with imatinib; concentration ranges are shown in Fig. 1 legend. The combination index (CI) for each of the combinations was evaluated using CalcuSyn dose effect analysis software (Biosoft, Ferguson, MO). This method, based on the multiple drug effect equation of Chou-Talalay (27), is suitable for calculating combined drug activity over a wide range of growth inhibition. Synergy level classifications were adapted from the CalcuSyn manual.

**Bcr-Abl phosphotyrosine immunoblot analysis.** Ba/F3 cells expressing WT or mutant Bcr-Abl (1 × 10^6 cells per well) were plated in medium containing escalating concentrations of imatinib and Src/Abl inhibitor AP23848 or BMS-354825 spanning the same concentration ranges as in corresponding cell proliferation assays. Following a 3-hour incubation at 37°C, cells were pelleted and lysed directly in SDS-PAGE loading buffer. For immunoblot analysis, tyrosine-phosphorylated Bcr-Abl was detected with mouse monoclonal phosphotyrosine antibody 4G10 (28); Bcr-Abl expression was detected with rabbit Abl antibody K12 (Santa Cruz Biotechnology, Santa Cruz, CA).

**Hematopoietic colony-forming assays.** Colony-forming assays were done on cryopreserved bone marrow mononuclear cells. Samples were obtained with informed consent from four CML patients or from a tissue repository (AllCells, Berkeley, CA). The CML samples were screened for kinase domain mutations by conventional sequencing (29). Thawed cells were plated in triplicate at 5 × 10^4 viable cells/mL in methylcellulose media containing recombinant human interleukin-3 and recombinant human granulocyte macrophage colony-stimulating factor (Methocult GF H4534; Stem Cell Technologies, Vancouver, British Columbia, Canada) and supplemented with vehicle (DMSO), imatinib (1 μmol/L), BMS-354825 (5 nmol/L), or imatinib (1 μmol/L) plus BMS-354825 (5 nmol/L). Following a 2-week incubation at 37°C, granulocyte-macrophage colony-forming units were counted. The results of triplicate experiments are reported as the percentage of granulocyte-macrophage colony-forming units compared with vehicle-treated control for each group (Fig. 4).

### Results and Discussions

Both AP23848 and BMS-354825 showed potent single-agent cellular activity against WT Bcr-Abl and imatinib-resistant mutants (except T315I), with BMS-354825 being ~11- to 14-fold more potent than AP23848 (Table 1A). To investigate whether escalating concentrations of imatinib adversely affect the effectiveness of these Src/Abl inhibitors, we did cellular proliferation and immunoblot assays comparing each Src/Abl

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**Table 1.** Cell proliferation IC\textsubscript{50} values (nmol/L) for (A) imatinib, AP23848, and BMS-354825 as single agents and (B) AP23848 and BMS-354825 in combination with escalating concentrations of imatinib against Ba/F3 cells transfected with WT or various Bcr-Abl mutants

<table>
<thead>
<tr>
<th></th>
<th>AP23848</th>
<th>BMS-354825</th>
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<tbody>
<tr>
<td><strong>A. Single agent</strong></td>
<td></td>
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<tr>
<td>Ba/F3 cell line</td>
<td>Imatinib*</td>
<td>AP23848</td>
</tr>
<tr>
<td>WT</td>
<td>300</td>
<td>18</td>
</tr>
<tr>
<td>M351T</td>
<td>930</td>
<td>21</td>
</tr>
<tr>
<td>Y253F</td>
<td>3040</td>
<td>33</td>
</tr>
<tr>
<td>E255K</td>
<td>4400</td>
<td>72</td>
</tr>
<tr>
<td>T315I</td>
<td>&gt;10,000</td>
<td>&gt;8,000</td>
</tr>
<tr>
<td>Vector only</td>
<td>&gt;10,000</td>
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| **B. Combinations with imatinib** | | |
| [Imatinib] | AP23848 | BMS-354825 |
| WT | | |
| 0 | 18 | 1.5 |
| 75 | 12 | 1.25 |
| 150 | 8 | 0.75 |
| 300 | | |
| 600 | | |
| 1,250 | | |
| 2,500 | | |
| Y253F | | |
| 0 | 33 | 2.3 |
| 750 | 23 | 1.8 |
| 1,500 | 14 | 1.5 |
| 3,000 | 4 | 0.85 |
| 6,000 | | |
| E255K | | |
| 0 | 72 | 6.5 |
| 1,000 | 44 | 6.1 |
| 2,000 | 25 | 4.6 |
| 4,000 | 6 | 2.2 |
| 8,000 | | |

*Values for imatinib as previously reported by La Rosée et al. (19).

Cells transfected with Bcr-Abl mutant T315I or vector only showed no inhibition of proliferation at any concentration of single agent.

Cells transfected with Bcr-Abl mutant T315I or vector only showed no inhibition of proliferation at any concentration of single agent or combination (data not shown).
inhibitor alone to imatinib-AP23848 and imatinib-BMS-354825 combinations. For these studies, cells expressing either WT Bcr-Abl or Bcr-Abl mutants spanning a range of imatinib sensitivities (M351T, marginally resistant; Y253F and E255K, highly resistant; T315I, insensitive) were used (8, 10, 12, 19). These are among the most common mutants observed in imatinib-resistant patients and reside in several regions of the kinase domain: the phosphate-binding P-loop (Y253F and E255K), an imatinib contact residue within the drug-binding site (T315I), and the base supporting the COOH-terminal activation loop (M351T; ref. 30). For each case, the imatinib concentration range was matched to the degree of imatinib resistance and was designed to provide the most rigorous test of whether imatinib interferes with the action of the Src/Abl inhibitor. Complete IC$_{50}$ results for all imatinib-AP23848 and imatinib-BMS-354825 inhibitor combinations are summarized in Table 1.

Results from studies with cells expressing WT Bcr-Abl clearly show that, despite the ability of imatinib to bind to WT Bcr-Abl, the effectiveness of the Src/Abl inhibitors AP23848 and BMS-354825 is not diminished in the presence of clinically achievable levels of imatinib (Fig. 1; Table 1). In fact, coadministering imatinib with either Src/Abl inhibitor resulted in lower IC$_{50}$ values than corresponding single-agent Src/Abl inhibitor treatments. For example, AP23848 treatment of cells expressing WT Bcr-Abl yielded an IC$_{50}$ of 18 nmol/L, whereas the IC$_{50}$ using AP23848 in the presence of 150 nmol/L imatinib declined to 8 nmol/L (Fig. 1A; Table 1). Similarly, treatment of cells expressing WT Bcr-Abl with BMS-354825 yielded an IC$_{50}$ of 1.8 nmol/L, whereas the IC$_{50}$ using BMS-354825 in the presence of 150 nmol/L imatinib decreased to 0.8 nmol/L (Fig. 1B; Table 1). This trend was maintained with successively higher concentrations of imatinib (Fig. 1; Table 1).

The inhibition profile of the marginally imatinib-resistant mutant M351T was similar to that of WT Bcr-Abl. No interference by imatinib with either structurally unrelated Src/Abl inhibitor was observed, even at the highest imatinib concentration (2,500 nmol/L). The case of M351T illustrates the potential use of a combined Abl inhibitor approach for eradicating weakly imatinib-resistant mutants. Treatment with imatinib (1,250 nmol/L) alone or AP23848 (30 nmol/L) alone reduced proliferation to ~40% of control levels, whereas coadministering both drugs at these same doses reduced proliferation by ~90% (Fig. 1A; Table 1). Similarly, treatment with imatinib (1,250 nmol/L) alone or BMS-354825 (2.5 nmol/L) alone reduced proliferation to ~40% of control levels, whereas combining the two drugs at these same doses reduced proliferation by ~90% (Fig. 1B; Table 1). Whereas higher
concentrations of imatinib were required to inhibit proliferation compared with those used for cells expressing WT Bcr-Abl, an imatinib concentration of 1,250 nmol/L is within the clinically achievable range for imatinib plasma levels (∼1,500 nmol/L; refs. 1, 31).

The P-loop mutants Y253F (IC₅₀ = 3,040 nmol/L) and E255K (IC₅₀ = 4,400 nmol/L) are highly but not completely imatinib resistant. Although imatinib at clinically achievable concentrations (∼1,500 nmol/L in plasma; refs. 2, 31) is not an effective therapy for these mutants, a critical concern is that low-affinity interactions of imatinib with Bcr-Abl mutants could obstruct the access of a second inhibitor to its binding site within the Bcr-Abl kinase domain. To test this possibility, we compared the effect of treating cells expressing these mutants with AP23848 or BMS-354825 alone and in combination with extremely high concentrations of imatinib. For example, a range of 0 to 6,000 nmol/L imatinib was used for mutant Y253F compared with 0 to 600 nmol/L for the experiments with WT Bcr-Abl described above. In summary (Fig. 1; Table 1), imatinib did not protect cells expressing Y253F or E255K from either Src/Abl inhibitor, even at imatinib concentrations several-fold above mean trough levels (2, 31). In all cases, inclusion of imatinib resulted in dose-dependent, extremely minor lowering of the cellular IC₅₀. The results were reminiscent of those observed for cells expressing WT Bcr-Abl, except that much higher levels of imatinib were required to observe a contribution to inhibition of cell proliferation.

With respect to clinical effectiveness, the contribution from imatinib would be expected to be extremely small for the cases of highly imatinib-resistant mutants. For example, AP23848 treatment of cells expressing Y253F yielded an IC₅₀ of 33 nmol/L, whereas this value declined to 23 nmol/L when 750 nmol/L imatinib was included and 13 nmol/L when 1,500 nmol/L imatinib was included. Similarly, treatment of cells expressing Y253F with BMS-354825 alone yielded an IC₅₀ of 2.3 nmol/L, whereas this value declined to 1.8 nmol/L when 750 nmol/L imatinib was included and 1.5 nmol/L when 1,500 nmol/L imatinib was included. As noted, Y253F and E255K are not completely imatinib resistant; thus, the slight, dose-dependent inhibitory activity of imatinib against these mutants could account for the extremely minor additive effects observed in our studies.

No single-agent or combined drug treatment inhibited proliferation of cells expressing Bcr-Abl mutant T315I (Fig. 1), confirming that blockade of Bcr-Abl is required to inhibit proliferation. The results with mutant T315I also indicate that inhibition of Src kinases, several of which are involved in the Bcr-Abl signaling cascade, is not sufficient to block Bcr-Abl-dependent cell proliferation. There was no inhibition of parental Ba/F3 cells with any of the combinations, indicating that the combined Abl inhibitors do not exhibit increased toxicity compared with single agents.

Immunoblot analyses spanning the same inhibitor concentration ranges used in cell proliferation assays showed that decreased cellular Bcr-Abl tyrosine phosphorylation directly correlated with inhibition of proliferation (Fig. 2). Specifically, progressively reduced Bcr-Abl tyrosine phosphorylation levels are evident in all cases except T315I when comparing AP23848 alone to corresponding imatinib-AP23848 treatments. Similar results were obtained when comparing BMS-354825 alone to imatinib-BMS-354825 treatments (data not shown).

The imatinib-Src/Abl inhibitor combinations were subjected to CI dose effect analysis using CalcuSyn software to determine whether various inhibitor combinations had antagonistic (CI > 1), additive (CI = 1), or synergistic (CI < 1) activity (Fig. 3). One limitation of this statistical algorithm for combination drug analysis is a tendency toward artificially high CI values in the low-dosage range (32). As a control for the CI range indicative of additivity, we used cells expressing WT Bcr-Abl to do an experiment in which imatinib was combined with imatinib; the resulting CI values ranged from 1.2 to 0.7 (data not shown). For each of the combinations shown in Fig. 4, the CI values were 1.0 to 0.7 at all but the lowest dose range, consistent with the combinations having additive activity. This data is also in accord with findings in the dose-response plots (Fig. 1).

As BMS-354825 has advanced to clinical trials while AP23848 is in preclinical development, we next assessed whether clinically achievable imatinib concentrations interfere with the ability of BMS-354825 to inhibit colony formation in mononuclear cells from CML patients (WT, Y253F, T315I, and M351T) or normal controls (Fig. 4). BMS-354825 was used at a...
relative low concentration (5 nmol/L); a concentration of 500 nmol/L has been reached safely in ongoing phase I clinical trials (23). Because the 500 nmol/L value is a steady-state trough level and does not reveal the free drug concentration, these values cannot be directly compared. However, it is notable that steady-state trough levels measured in patients are well above the free drug concentrations required for inhibitory activity in our study and that patients are achieving responses under these conditions. For cells expressing WT Bcr-Abl, mutant M351T, or mutant Y253F, single agents produced a marked reduction in colony number, whereas administering imatinib and BMS-345825 simultaneously produced a further reduction. The primary cells expressing Y253F were more sensitive to treatment to combined dimatinib-BMS-345825 treatment than predicted from our in vitro results, possibly reflecting additional, uncharacterized susceptibility factors within the CML cells from this patient. Although this sample was verified by sequence analysis to be predominantly Y253F, we cannot rule out that cells expressing WT Bcr-Abl were also present. None of the treatments inhibited colony formation by normal cells or by primary CML cells expressing mutant T315I.

At present, imatinib is the treatment of choice for chronic-phase CML. We investigated two Src/Abl inhibitors from distinct structural classes to test whether Src/Abl inhibitor usage is compatible with imatinib therapy. Overall, inclusion of imatinib with Src/Abl inhibitor treatments did not adversely affect the inhibition profile. Instead, inclusion of imatinib at clinically achievable concentrations yielded extremely minor (Y253F and E255K) to moderate (WT and M351) additive enhancement of inhibition. For all cell lines tested, results obtained with imatinib-AP23848 and imatinib-BMS-345825 combinations were similar except that the latter combination was effective at ∼11- to 14-fold lower Src/Abl inhibitor concentrations (Table 1).

The critical finding of these studies, especially with respect to highly imatinib-resistant mutants, is that imatinib does not interfere with the action of either Src/Abl inhibitor under any circumstances tested. The lack of interference by imatinib may be attributable to a confluence of factors: relatively low binding affinity of imatinib for WT Bcr-Abl (33) and for mutated Bcr-Abl compared with the Src/Abl inhibitors, the different conformational requirements placed on Bcr-Abl by the two types of inhibitors, and the fact that the respective binding sites are only partially overlapping.

The most immediate potential benefit of combined Abl inhibitor therapy is that an inhibitor cocktail may target a wider range of resistant clones than either single agent and thereby prohibit or delay the onset of acquired drug resistance. For example, mutations that confer resistance to BMS-345825 but not to imatinib have been identified in vitro (25). The goals of this type of approach are different from combination strategies directed against distinct cellular targets (34–37) but similar to strategies using combinations of protease or reverse transcriptase inhibitors for HIV (38). Due to the enhanced inhibitory activity of combined Abl inhibitor therapy, this strategy may also improve molecular response rates in newly diagnosed patients and patients with imatinib-resistant CML. In the clinical setting, it is possible that use of imatinib-Src/Abl inhibitor combinations will lead to increased toxicity in normal tissues, and this will require careful monitoring. Consideration must be given to the tolerability of such drug regimens and to the potential emergence of new resistance mechanisms that circumvent this strategy. In addition, this approach does not solve the problem of mutants that are resistant to both drugs, such as T315I. However, these studies suggest that clinical trials of these drug combinations should be pursued.

References
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