Synergistic interactions between imatinib mesylate and the novel phosphoinositide-dependent kinase-1 inhibitor OSU-03012 in overcoming imatinib mesylate resistance

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Resistance to the Abelson protein tyrosine (Abl) kinase inhibitor imatinib mesylate has become a critical issue for patients in advanced phases of chronic myelogenous leukemia. Imatinib-resistant tumor cells develop, in part, as a result of point mutations within the Abl kinase domain. As protein kinase B (Akt) plays a pivotal role in Abl oncogene–mediated cell survival, we hypothesize that concurrent inhibition of Akt will sensitize resistant cells to the residual apoptotic activity of imatinib mesylate, thereby overcoming the resistance. Here, we examined the effect of OSU-03012, a celecoxib-derived phosphoinositide-dependent kinase-1 (PDK-1) inhibitor, on imatinib mesylate–induced apoptosis in 2 clinically relevant breakpoint cluster region (Bcr)–Abl mutant cell lines, Ba/F3p210E255K and Ba/F3p210T315I. The 50% inhibitory concentration (IC\textsubscript{50}) values of imatinib mesylate to inhibit the proliferation of Ba/F3p210E255K and Ba/F3p210T315I were 14 ± 4 and 30 ± 2 μM, respectively. There was no cross-resistance to OSU-03012 in these mutant cells with an IC\textsubscript{50} of 5 μM irrespective of mutations. Nevertheless, in the presence of OSU-03012 the susceptibility of these mutant cells to imatinib-induced apoptosis was significantly enhanced. This synergistic action was, at least in part, mediated through the concerted effect on phospho-Akt. Together these data provide a novel therapeutic strategy to overcome imatinib mesylate resistance, especially with the Abl mutant T315I. (Blood. 2005;105:4021-4027)

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Introduction

It has been well established that breakpoint cluster region/Abelson protein tyrosine (Bcr-Abl) kinase, the product of the Philadelphia chromosome, plays an obligatory role in the pathogenesis of chronic myelogenous leukemia (CML).\textsuperscript{1} This causal relationship underlies the clinical success of using the Bcr-Abl tyrosine kinase inhibitor imatinib mesylate (ST1571; Gleevec) to target this molecular defect in CML, as evidenced by the complete remission and remarkably few associated side effects in patients with first chronic-phase CML.\textsuperscript{2-5} However, patients in more advanced phases of CML either fail to respond or quickly relapse following an initial response to imatinib mesylate.\textsuperscript{6,7} Acquisition of the imatinib mesylate-resistant phenotype is attributable to at least 2 major cellular mechanisms: amplification of the Bcr-Abl gene and mutations in the Abl catalytic domain.\textsuperscript{8} Mutations within the kinase domain represent the more commonly identified mechanism associated with relapse,\textsuperscript{8,10} among which Y253F/H, E255K/V, T315I, and M351T are characterized as the most clinically relevant mutations.\textsuperscript{11-19} Especially, E255K and T315I exhibit nearly 2-orders-of-magnitude lower biochemical and cellular sensitivity to imatinib mesylate than CML either fail to respond or quickly relapse following an initial response to imatinib mesylate.\textsuperscript{6,7} Acquisition of the imatinib mesylate-resistant phenotype is attributable to at least 2 major cellular mechanisms: amplification of the Bcr-Abl gene and mutations in the Abl catalytic domain.\textsuperscript{8} Mutations within the kinase domain represent the more commonly identified mechanism associated with relapse,\textsuperscript{8,10} among which Y253F/H, E255K/V, T315I, and M351T are characterized as the most clinically relevant mutations.\textsuperscript{11-19} Especially, E255K and T315I exhibit nearly 2-orders-of-magnitude lower biochemical and cellular sensitivity to imatinib mesylate than typically occur in CML without a molecular defect.\textsuperscript{19,20} However, in the presence of OSU-03012, these mutant cells develop, in part, as a result of point mutations within the Abl kinase domain. As protein kinase B (Akt) plays a pivotal role in Abl oncogene–mediated cell survival, we hypothesize that concurrent inhibition of Akt will sensitize resistant cells to the residual apoptotic activity of imatinib mesylate, thereby overcoming the resistance. Here, we examined the effect of OSU-03012, a celecoxib-derived phosphoinositide-dependent kinase-1 (PDK-1) inhibitor, on imatinib mesylate–induced apoptosis in 2 clinically relevant breakpoint cluster region (Bcr)–Abl mutant cell lines, Ba/F3p210E255K and Ba/F3p210T315I. The 50% inhibitory concentration (IC\textsubscript{50}) values of imatinib mesylate to inhibit the proliferation of Ba/F3p210E255K and Ba/F3p210T315I were 14 ± 4 and 30 ± 2 μM, respectively. There was no cross-resistance to OSU-03012 in these mutant cells with an IC\textsubscript{50} of 5 μM irrespective of mutations. Nevertheless, in the presence of OSU-03012 the susceptibility of these mutant cells to imatinib-induced apoptosis was significantly enhanced. This synergistic action was, at least in part, mediated through the concerted effect on phospho-Akt. Together these data provide a novel therapeutic strategy to overcome imatinib mesylate resistance, especially with the Abl mutant T315I. (Blood. 2005;105:4021-4027)

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In addition, PI3K inhibitors have been shown to synergize with imatinib mesylate in inhibiting CML cell growth. Together these findings suggest the clinical relevance of targeting Akt signaling in imatinib-resistant patients.

Recently, based on our finding that the cyclooxygenase-2 (COX-2) inhibitor celecoxib mediates apoptosis by blocking phosphoinositol-dependent kinase-1 (PDK-1)/Akt signaling independently of COX-2 inhibition, we have used celecoxib as a scaffold to develop a novel class of PDK-1 inhibitors with high potency in deactivating Akt and inducing apoptosis in cancer cells. These celecoxib-derived PDK-1 inhibitors, however, are devoid of COX-2 inhibitory activity. Here, we examined the effect of an optimal inhibitor (OSU-03012), alone or in combination with imatinib mesylate, in Bcr-Abl–expressing Ba/F3 cells (Ba/F3p210T315I) and Ba/F3p210E255K, both of which are highly resistant to high doses of imatinib mesylate. 

Reagents and cell culture

Materials and methods

Reagents and cell culture

Imatinib mesylate, also known as STI571, was obtained from commercial imatinib mesylate capsules (Novartis Pharmaceuticals, East Hanover, NJ) by solvent extraction followed by recrystalization. The PDK-1 inhibitor OSU-03012 was synthesized as described. Rabbit polyclonal anti-Akt and rabbit monoclonal anti-PARP (anti–poly–adenosine diphosphate [ADP]–ribose polymerase) were purchased from Cell Signaling Technology (Beverly, MA). Rabbit antibodies against phospho-Thr308-Akt were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti–cytochrome c, anti-Bcr, and antiactin were from BD Pharmingen (San Diego, CA), Oncogene (Boston, MA), and ICN Biomedicals (Costa Mesa, CA), respectively. Goat anti–rabbit and goat anti–mouse immunoglobulin G (IgG) horseradish peroxidase conjugates were from Jackson ImmunoResearch Laboratories (West Grove, PA). A murine myeloid hematopoietic cell line (32D) and a lymphoid cell line (Ba/F3) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA); 15% Walter and Eliza Hall Institute (WEHI)–conditioned media medium supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA); 2.5 mM CaCl2 at a concentration of 5 M sodium vanadate, and 10 M b-estradiol phosphate. The mixture was sonicated for 5 seconds and protein contents were analyzed by using the Bradford assay kit (Bio-Rad, Hercules, CA). Twenty-five micrograms total protein was resolved in SDS–polyacrylamide gels on a Mini gel apparatus and transferred to a nitrocellulose membrane using a semidyey transfer cell. The transfblotted membrane was washed 3 times with Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST). After blocking with TBST containing 5% nonfat milk for 60 minutes, the membrane was incubated with the appropriate primary antibody at 1:1000 dilution in TBST–5% nonfat milk at 4°C overnight and then washed 3 times with TBST. The membrane was probed with horseradish peroxidase–conjugated secondary antibody at 1:3000 for 1 hour at room temperature and then washed with TBST 3 times. The immunoblots were visualized by enhanced chemiluminescence. The density of the blot was further analyzed by densitometry using the Gel-Pro Analyzer (Media Cybernetics, San Diego, CA).

Assessment of apoptosis

Flow cytometric analysis.

Cytocrome c release analysis.

Cells that were drug treated were collected by centrifugation at 1000g for 5 minutes. The pellet fraction was recovered, placed on ice, and resuspended in 100 μL of a chilled hypotonic lysis solution (220 mM mannitol; 68 mM sucrose; 50 mM PIPES [piperazine diethanesulfonic acid]–KOH, pH 7.4; 140 mM NaCl; 2.5 mM CaCl2) at a concentration of 5 M cytochrome c. A 200-μL solution (1 × 106) was transferred to a culture tube, to which were added annexin V–FITC and PI. The cells were gently vortexed and incubated for 15 minutes at room temperature in the dark. An additional 800 μL of binding buffer was added to each tube and the samples were analyzed by flow cytometry.

Western blot analysis of PARP cleavage.

Cells that were drug treated for 48 hours were collected, washed with ice-cold phosphate-buffered saline (PBS), and resuspended in the aforementioned lysis buffer. Soluble cell lysates were collected after centrifugation at 10 000g for 5 minutes. Equivalent amounts of proteins (50 μg) from each lysate were resolved in
externalization indicate that the thresholds for imatinib mesylate to trigger apoptosis in Ba/F3p210E255K and Ba/F3p210T315I cells were approximately 10 and 20 μM, respectively, vis-à-vis 0.1 μM for Ba/F3p210Bcr-Abl (Figure 1B-C). Flow analysis indicates that at 10 μM the extents of imatinib mesylate-induced apoptotic death (annexin V-positive cells) were 97%, 35%, and 6% in Ba/F3p210Bcr-Abl, Ba/F3p210E255K, and Ba/F3p210T315I, respectively (Figure 1C both quadrants B2 and B4).

As Akt signaling represents a major pathway through which Bcr-Abl mediates oncogenic effects in CML cells, we further assessed the effect of imatinib mesylate on Akt activation in wild-type versus mutant Bcr-Abl-overexpressing Ba/F3 cells. Because overexpression of Bcr-Abl up-regulates PK3/Akt signaling,36,37 Ba/F3p210Bcr-Abl, Ba/F3p210E255K, and Ba/F3p210T315I exhibited substantially higher levels of Akt phosphorylation, irrespective of mutations, compared with that of 32D and untransfected Ba/F3 cells (Figure 2A). However, the respective susceptibility of these 3 cell lines to the inhibitory effect of imatinib mesylate on Akt varied to a great extent.

Densitometry analysis of the immunoblots shows that exposure of Ba/F3p210Bcr-Abl to imatinib mesylate, even at concentrations as low as 0.5 μM, led to complete Akt deactivation (Figure 2B). A decreasing trend of phospho-Akt was noted with increasing imatinib mesylate concentrations in Ba/F3p210E255K and Ba/F3p210T315I (trend P values were .004 and .022, respectively, according to the Jonckheere-Terpstra test). The estimated IC50 values for imatinib-mediated Akt dephosphorylation were approximately 10 and 20 μM for Ba/F3p210E255K and Ba/F3p210T315I,

10% SDS–polyacrylamide gels. Bands were transferred to nitrocellulose membranes and analyzed by immunoblotting with monoclonal anti-PARP antibody.

**Statistical analysis and determination of synergism**

The medium-effect method was used to analyze dose-response data for single drug or multiple drugs. The synergistic effect of multiple drugs was determined by the definition of Chou and Talalay.34 A combination index (CI) less than 1 was defined as synergism. By using the software package CalcuSyn (Biosoft, Cambridge, United Kingdom), the values of IC50, the combination index, were calculated. These drugs were assumed as totally independent modes of action and are therefore mutually nonexclusive. The sign test, a nonparametric test,35 was used to test the hypothesis that combination indices for drugs in fixed ratio experiments in ordered groups,35 was used to evaluate the decreasing trend in the relationship between drug concentrations and the densitometry values of phospho-Akt.

**Results**

**Differential inhibitory effects of imatinib mesylate on Akt activation**

In line with the earlier reports,30,31 Ba/F3p210Bcr-Abl, Ba/F3p210E255K, and Ba/F3p210T315I exhibited differential sensitivity to imatinib mesylate’s antiproliferative effects. MTS assays indicate that the IC50 values for Ba/F3p210E255K and Ba/F3p210T315I were 14 ± 2 and 30 ± 2 μM, respectively, 2 orders of magnitude greater than the wild-type counterpart (0.13 ± 0.01 μM; Figure 1A). Imatinib mesylate, however, was ineffective against the cell lines 32D and untransfected Ba/F3 (with IC50 > 50 μM; Figure 1A), indicating the pivotal role of Bcr-Abl in the drug action. Furthermore, characterizations of cytochrome c release and phosphatidylinerse
respectively. Together these data suggested a putative link between imatinib mesylate resistance and Akt signaling. Accordingly, we hypothesized that concurrent inhibition of Akt could lower the threshold of imatinib-mediated apoptosis, thereby overcoming imatinib mesylate resistance.

The PDK-1/Akt signaling inhibitor OSU-03012 induces apoptosis irrespective of Bcr-Abl mutation

To test this hypothesis we examined the antiproliferative effects of the PDK-1 inhibitor OSU-03012, a structurally optimized derivative of celecoxib, which exhibits IC\textsubscript{50} in PDK-1 inhibition of 5 μM.\textsuperscript{29} However, the consequent effect on intracellular Akt is more pronounced presumably due to the concurrent action of protein phosphatase 2A in Akt dephosphorylation. As a result, this agent sensitized, to a great extent, Ba/F3p210E255K and Ba/F3p210T315I cells to OSU-03012–mediated apoptosis (Figure 3B-C).

In addition, Western blot analysis indicates that OSU-03012 was able to diminish the phospho-Akt level at a concentration as low as 1 μM in these 3 cell lines irrespective of Bcr-Abl mutation (data not shown). Exposure to OSU-03012 over the range of 5 to 7.5 μM resulted in complete Akt dephosphorylation, which corresponds to the precipitous drop in cell viability between 5 and 7.5 μM (from 60% to 10%) in the dose-response curves (Figure 3A). Together these data clearly demonstrate the lack of cross-resistance to OSU-03012 in these imatinib mesylate-resistant cells.

OSU-03012 sensitizes imatinib mesylate-resistant cells to imatinib-induced apoptosis

To explore the effect of OSU-03012 on imatinib mesylate resistance, Ba/F3p210E255K and Ba/F3p210T315I cells, both of which exhibited IC\textsubscript{50} greater than 10 μM against imatinib mesylate, were treated with varying concentrations of imatinib mesylate in the presence of 5 μM OSU-03012 or vice versa. As shown by the dose-response curves in Figure 4A, OSU-03012 sensitized, to a great extent, Ba/F3p210E255K and Ba/F3p210T315I cells to imatinib-induced cell death. For example, imatinib mesylate alone was ineffective in preventing cell proliferation within therapeutically attainable concentrations (≤ 5 μM; Figure 4A left panel dotted lines). However, in the presence of 5 μM OSU-03012, the susceptibility of these mutant cells to imatinib-induced apoptosis increased by more than one order of magnitude (Figure 4A left panel solid lines) with an apparent IC\textsubscript{50} of 1 μM. Annexin V analysis indicates that in combination with 5 μM OSU-03012, imatinib mesylate at 2.5 and 5 μM caused 70% and greater than 95% cell death, respectively (Figure 4B). Similarly, imatinib mesylate at 5 μM also sensitized Ba/F3p210E255K and Ba/F3p210T315I cells to OSU-03012–mediated apoptosis (Figure 4A right panel) with a reduction of IC\textsubscript{50} from 5 μM to less than 3 μM. Medium dose analysis of apoptosis induction in Ba/F3p210E255K and Ba/F3p210T315I cells was carried out over a range of OSU-03012 and imatinib mesylate concentrations at a fixed ratio (1:1) for 48 hours, after which CI values for apoptosis were determined in relation to the fraction affected. As shown in Figure 4C, the resulting CI values were significantly less than 1, which are considered as a synergistic interaction. A combination of imatinib mesylate and OSU-03012 at 5 μM each represented an effective treatment to completely eliminate these imatinib-resistant cells.

Such a synergy, however, was not noted with 32D and Ba/F3 cells that lacked Bcr-Abl expression (Figure 5A), even though OSU-03012 was able to facilitate Akt dephosphorylation in both cell systems (Figure 5B). This synergistic action was, at least in part, mediated through the concerted effect of OSU-03012 and imatinib mesylate on phospho-Akt. Exposure of Ba/F3p210E255K and Ba/F3p210T315I cells to the drug combination did not affect the Bcr-Abl expression level (Figure 6A). However, imatinib mesylate at varying concentrations could augment the effect of 5 μM OSU-03012 on phospho-Akt in both cell lines (Figure 6B). Complete Akt dephosphorylation was achieved with a combination of 5 μM imatinib mesylate and 5 μM OSU-03012, in line with the optimal combination to elicit complete apoptotic death in these mutant cells. Similar augmenting effects were also noted with the combination of varying concentrations of OSU-03012 with 5 μM imatinib mesylate (Figure 6B).
Discussion

Development of new therapeutic strategies to overcome imatinib mesylate resistance in accelerated CML has been the focus of many recent investigations. In the literature, at least 3 distinct approaches have been reported. First, recent efforts have led to the identification of several novel Abl inhibitors capable of inhibiting some or all of mutant Abl kinases, which include PD180970,31 BMS-354825,38 and AP23464.39 In addition, the Bcr-Abl chaperone heat shock protein 90 inhibitors geldanamycin and 17-allylamino-geldanamycin have also been shown to inhibit the growth of imatinib-resistant hematopoietic cells found in patients with T315I and E255K mutation.40 Second, cotreatment of imatinib-resistant cells with antileukemic agents such as As2O3, decitabine, the farnesyl transferase inhibitor SCH66336, and the histone deacetylase inhibitors suberoylanilide hydroxamic acid (SAHA) and butyrerate could enhance the antiproliferative activity of imatinib mesylate.30,32,41 Third, the combination of different target-directed therapeutic agents such as the proteasome inhibitor bortezomib in conjunction with the cyclin-dependent kinase inhibitor flavopiridol or with SAHA has also been shown to effectively induce apoptosis in imatinib-resistant cells.42,43 As many of these strategies remain ineffective against the T315I mutant cells, this study is aimed at developing an alternative strategy to overcome imatinib mesylate resistance.
Despite reduced binding affinity, imatinib mesylate still displays differential residual activity against these mutations. However, its ability to activate apoptotic signaling in mutant cell lines diminishes. In light of the pivotal role in Akt in regulating apoptosis threshold, we hypothesize that concurrent inhibition of Akt would achieve a mechanistic synergy by sensitizing imatinib-resistant cells to the residual apoptotic efficacy of imatinib mesylate. To examine this premise, we evaluate the effect of OSU-03012, a celecoxib-derived PDK-1 inhibitor lacking COX-2 inhibitory activity, on imatinib mesylate resistance in 2 clinically relevant cell lines, Ba/F3p210BCR/ABL and Ba/F3p210ABL, compared with untransfected Ba/F3 and 32D cells. Despite low levels of phospho-Akt, both Ba/F3 and 32D cells were sensitive to the apoptosis-inducing effect of OSU-03012, which has also been observed in many types of cancer cells with normal phosphatase with tensin homology (PTEN) function.25 The effect of OSU-03012 on apoptosis in Ba/F3 and 32D might involve both Akt-dependent and -independent mechanisms. First, Akt, even not constitutively activated, still plays a role in the survival of these proliferating cells. Second, PDK-1 has non-Akt targets such as p70S6K that are also involved in cell survival and proliferation.

Both mutant cell lines exhibited the same susceptibility to OSU-03012 as their wild-type counterpart Ba/F3p210BCR/ABL irrespective of the Bcr-Abl mutations. The lack of cross-resistance of OSU-03012 and imatinib mesylate underscores the functional relevance of targeting PDK-1/Akt signaling in imatinib mesylate-resistant mutant cells. It is especially noteworthy that OSU-03012 showed augmenting effects with imatinib mesylate on apoptosis in mutant imatinib mesylate-resistant cells at therapeutically attainable concentrations (≤ 5 μM), shifting the dose-response curves by more than 1 log unit. For example, the IC50 and IC80 values for imatinib mesylate in the presence of 5 μM OSU-03012 in Ba/F3p210BCR/ABL cells were 1 and 2.5 μM, respectively, vis-à-vis 30 and 65 μM for imatinib mesylate alone. This synergy is in sharp contrast to earlier reports that many antileukemic agents such as As2O3, decitabine, and SCH66336 could not synergize with imatinib mesylate in inhibiting the growth of Ba/F3p210BCR/ABL cells.3,32

Our data suggest that the ability of OSU-03012 to facilitate imatinib mesylate-mediated antiproliferative effects in these imatinib-resistant cells could be rationalized by the mechanistic synergy at the phospho-Akt level (ie, OSU-03012 was able to suppress Akt phosphorylation at low μM levels independent of Bcr-Abl mutations). As Akt contributes to enhanced survival of Bcr-Abl–expressing cells, the reduced apoptosis threshold renders imatinib mesylate-resistant cells sensitive to the residual antiproliferative activity of imatinib mesylate. Consequently, the molecular basis underlying this augmenting effect of OSU-03012 differs from that of other antileukemic agents such as As2O3 (Bcr-Abl expression),30 decitabine (DNA hypomethylation),30 SCH66336 (protein farnesylation),32 and flavopiridol (cyclin-dependent kinase).44 It is noteworthy that OSU-03012 is currently undergoing preclinical testing under the Rapid Access to Intervention Development (RAID) program at the National Cancer Institute. Our data indicate that oral administration of this agent in tumor-bearing nude mice even at 200 mg/kg for 1 month gave rise to no weight loss or apparent toxicity at necropsy (C.S.C., unpublished data, August 2003). Thus, this combination represents a viable strategy for clinical testing in imatinib-resistant CML.

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