Synergistic Interactions between Imatinib and the Novel Phosphoinositide-Dependent Kinase-1 Inhibitor OSU-03012 in Overcoming Imatinib Resistance

Running title: Overcome imatinib resistance by a PDK-1 inhibitor

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ABSTRACT

Resistance to the Abl kinase inhibitor imatinib 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 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, thereby overcoming the resistance. Here, we examined the effect of OSU-03012, a celecoxib-derived phosphoinositide-dependent kinase-1 (PDK-1) inhibitor, on imatinib-induced apoptosis in two clinically relevant Bcr-Abl mutant cell lines, Ba/F3p210E255K and Ba/F3p210T315I. The IC<sub>50</sub> values of imatinib to inhibit the proliferation of Ba/F3p210<sup>E255K</sup> and Ba/F3p210<sup>T315I</sup> were 14 ± 4 and 30 ± 2 µM, respectively. There was no cross-resistance to OSU-03012 in these mutant cells with IC<sub>50</sub> 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 resistance, especially with the Abl mutant T315I.
INTRODUCTION

It has been well established that Bcr-Abl kinase, the product of the Philadelphia chromosome, plays an obligatory role in the pathogenesis of chronic myelogenous leukemia (CML). 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. However, patients in more advanced phases of CML either fail to respond or quickly relapse following an initial response to imatinib. Acquisition of the imatinib-resistant phenotype is attributable to at least two major cellular mechanisms: amplification of the Bcr-Abl gene and mutations in the Abl catalytic domain. Mutations within the kinase domain represent the more commonly identified mechanism associated with relapse, among which Y253F/H, E255K/V, T315I, and M351T are characterized as the most clinically relevant mutants. Especially, E255K and T315I exhibit nearly two-orders-of-magnitude lower biochemical and cellular sensitivity to imatinib, with the resulting IC50 values greatly exceeding the therapeutically attainable concentration of the drug. Three-dimensional structural data indicate that the mechanisms by which E255K and T315I affect imatinib’s interactions with the catalytic domain vary. For example, conversion of T315 to an Ile residue in the catalytic domain results in the loss of a hydrogen bonding with imatinib, thereby restricting imatinib’s access to its binding site. In contrast, the structural effect of the E225K on imatinib binding is subtle since this residue is located in the nucleotide-binding loop for ATP. Conceivably, this mutation affects imatinib binding by altering the conformational flexibility of the neighboring nucleotide-binding and
activation loops\textsuperscript{8}. As a result of significantly reduced sensitivity to imatinib, both mutations exhibit high degrees of cellular resistance to imatinib\textsuperscript{20}. Thus, it is of urgency to develop an alternative strategy to overcome this imatinib resistance.

From a mechanistic perspective, expression of the Bcr-Abl oncogene upregulates multiple downstream signaling pathways, including those mediated by phosphatidylinositol 3-kinase (PI3K)/Akt, Ras/mitogen-activated protein kinase (MAPK), and signal transducer and activator of transcription (STAT)\textsuperscript{1,21}. Of these pathways, the PI3K/Akt signaling cascade plays an especially pivotal role in Abl oncogene-mediated proliferation, survival, and transformation\textsuperscript{22-25}. For example, recent evidence indicates that CML cells were susceptible to the growth-inhibitory effects of the PI3K inhibitor LY294002, but not the MAPK inhibitor PD98059\textsuperscript{26}. In addition, PI3K inhibitors have been shown to synergize with imatinib in inhibiting CML cell growth\textsuperscript{27}. Together, these findings suggest the clinical relevance of targeting Akt signaling in imatinib-resistant patients\textsuperscript{28}.

Recently, based on our finding that the cyclooxygenase-2 (COX-2) inhibitor celecoxib mediates apoptosis by blocking 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\textsuperscript{29}. 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, in Bcr-Abl-expressing Ba/F3 cells (Ba/F3p210\textsuperscript{Bcr-Abl}) vis-à-vis two mutant cell lines (Ba/F3p210\textsuperscript{E255K} and Ba/F3p210\textsuperscript{T315I}), both of which are highly resistant to high doses of imatinib\textsuperscript{30,31}. Previous studies have shown that arsenic oxide
(As$_2$O$_3$), 5-aza-2-deoxycytidine (decitabine), or the farnesyl transferase inhibitor SCH66336 could not cooperate with imatinib in enhancing its in vitro efficacy in Ba/F3p210$^{T315I}$ cells$^{30,32}$. Here, we hypothesize that concurrent inhibition of PDK-1/Akt signaling would sensitize imatinib-resistant cells to the residual apoptotic effects of imatinib, thereby overcoming the resistance. This premise is corroborated by the ability of OSU-03012 to restore the sensitivity of Ba/F3p210$^{E255K}$ and Ba/F3p210$^{T315I}$ to imatinib by shifting the dose response curve to the left by more than one log unit.

**MATERIALS AND METHODS**

**Reagents and cell culture**

Imatinib mesylate, also known as STI-571, was obtained from commercial Gleevec® capsules (Novartis Pharmaceuticals, East Hanover, NJ) by solvent extraction followed by recrystallization. The PDK-1 inhibitor OSU-03012 was synthesized as described$^{29}$. Rabbit polyclonal anti-Akt and rabbit monoclonal anti-PARP were purchased from Cell Signaling Technology Inc. (Beverly, MA). Rabbit antibodies against phospo-Thr$^{308}$-Akt were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse monoclonal anti-cytochrome c, anti-Bcr, and anti-actin were from BD Pharamingen (San Diego, CA), Oncogene (Boston, MA), and ICN Biomedicals Inc. (Costa Mesa, CA), respectively. Goat anti-rabbit and goat anti-mouse IgG-horseradish peroxidase conjugates were from Jackson ImmunoResearch Laboratories (West Grove, PA). 32D, a murine myeloid hematopoietic cell line, and Ba/F3, a lymphoid cell line, were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA), 15% WEHI-conditioned media as a IL-3 source, and 50 units/ml penicillin G, 50 µg/ml
strepotmycin, and 10 µg/ml gentamicin (Sigma, St Louis, MO). Ba/F3p210^{Bcr-Abl} and two imatinib-resistant Ba/F3p210 mutant cell lines, Ba/F3p210^{E255K}, and Ba/F3p210^{T315I}, were generated as previously reported\textsuperscript{31}. These cells were cultured in RPMI 1640 medium containing 10% FBS, 50 units/ml penicillin G, 50 µg/ml streptomycin, and 10 µg/ml gentamycin (Sigma, St Louis, MO) at 37 °C in 5% CO\textsubscript{2}.

**Cell proliferation assay (MTS assay)**

Cell proliferation was analyzed by the MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, Madison, WI) in six replicates. Cells (5,000/well) were grown in 10% FBS-supplemented RPMI 1640 medium in 96-well, flat-bottomed plates, and exposed to various concentrations of individual agents or combination of drugs dissolved in DMSO (final concentration ≤ 0.1%) in the same medium. Control groups received DMSO vehicle at a concentration equal to that in drug-treated cells. After 48-h treatment, MTS and the phenazine methosulfate (PMS) detection reagent were mixed at a ratio of 20:1 (MTS:PMS), and immediately added to the culture medium at a ratio of 1:5. Cells were incubated in the CO\textsubscript{2} incubator at 37 °C for 3 h, and the production of formazan was analyzed by measuring the absorbance at 492 nm in a plate reader.

**Immunoblotting**

The general procedure for the Western blot analysis of Bcr-Abl, Akt, phospho-Akt, and actin was performed as follows. Cells were collected by centrifugation at 2000 x g, and resuspended in RIPA lysis buffer consisting of 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and a mixture of protease inhibitor cocktail [100 µM 4-(2-aminoethyl)benzenesulfonyl fluoride, 80 nM
aprotinin, 5 µM bestatin, 1.5 µM E-64 protease inhibitor, 2 µM leupeptin, 1 µM pepstatin A (Calbiochem, La Jolla, CA) and phosphatase inhibitors (10 µM sodium fluoride, 5 µM sodium vanadate, and 10 µM β-glycerol phosphate). The mixture was sonicated for 5 s, and protein contents were analyzed by using the Bradford assay kit (Bio-Rad). Twenty-five µg total proteins were resolved in SDS-polyacrylamide gels on a Minigel apparatus, and transferred to a nitrocellulose membrane using a semi-dry transfer cell. The transblotted membrane was washed three times with TBS containing 0.05% Tween 20 (TBST). After blocking with TBST containing 5% nonfat milk for 60 min, 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 three times with TBST. The membrane was probed with horseradish peroxidase-conjugated secondary antibody at (1:3000) for 1 h at room temperature, and was then washed with TBST three 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

Fluorescein-conjugated Annexin V (Annexin V-FITC) and propidium iodide (PI) (BD Pharmingen) were used to quantify the percentage of cells undergoing apoptosis by following the protocol provided by the vender. In short, after drug treatment, the cells were collected and resuspended in 1 ml binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) at a concentration of 5 x 10⁶ cells/ml. Two-hundred µl solution (1 x 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 min at room temperature in the dark. Additional 800 µl of binding buffer was added to each tube and the samples were analyzed by flow cytometry.

**Cytochrome c release analysis**

Cytosol-specific mitochondria-free lysates were prepared as previously described. Drug-treated cells were collected by centrifugation at 1000 x g for 5 min. 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-KOH, pH 7.4, 50 mM KCl, 5 mM EDTA, 2 mM MgCl₂, 1 mM dithiothreitol, and the aforementioned protease inhibitors cocktails). After a 20-min incubation on ice, the mixture was centrifuged at 600 x g for 10 min. The supernatant was collected in a microcentrifuge tube and centrifuged at 14000 x g for 30 min. An equivalent amount of protein (25 µg) from each supernatant was resolved by 15% SDS-polyacrylamide gel electrophoresis and blotted with anti-cytochrome c antibody by following the procedure described above.

**Western blot analysis of PARP cleavage**

Drug-treated cells for 48 h were collected, washed with ice-cold PBS, and resuspended in the aforementioned lysis buffer. Soluble cell lysates were collected after centrifugation at 10000 x g for 5 min. Equivalent amounts of proteins (50 µg) from each lysate were resolved in 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. A combination index (CI) significantly less than 1 was defined as synergism. By using the software package Calcusyn (Biosoft, Cambridge, UK), the values of IC₅₀, the drug concentration required for 50% growth inhibition, and CI were calculated. These drugs were assumed as totally independent modes of action and are therefore mutually non-exclusive. The sign test, a nonparametric test, was used to test the hypothesis that combination indices for drugs were less than 1. The Jonckheere-Terpstra test, a nonparametric test for trend in ordered groups, 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 on Akt activation. In line with the earlier reports, Ba/F3p210²¹⁰Bcr-Abl, Ba/F3p210²¹⁰E₂⁵⁵K, and Ba/F3p210³¹⁵I exhibited differential sensitivity to imatinib’s antiproliferative effects. MTS assays indicate that the IC₅₀ values for Ba/F3p210²¹⁰E₂⁵⁵K and Ba/F3p210³¹⁵I were 14 ± 4 and 30 ± 2 µM, respectively, two orders of magnitude greater than the wild-type counterpart (0.13 ± 0.01 µM) (Fig. 1A). Imatinib, however, was ineffective against the cell lines 32D and untransfected Ba/F3 (with IC₅₀ greater than 50 µM; Fig. 1A), indicating the pivotal role of Bcr-Abl in the drug action. Furthermore, characterizations of cytochrome c release and phosphatidylserine externalization indicate that the thresholds for imatinib to trigger apoptosis in Ba/F3p210²¹⁰E₂⁵⁵K and Ba/F3p210³¹⁵I cells were approximately 10 and 20 µM, respectively, vis-à-vis 0.1 µM for Ba/F3p210²¹⁰Bcr-Abl (Fig. 1B and C). Flow analysis indicates that at 10 µM, the extents of imatinib-induced apoptotic death (Annexin V-
positive cells) were 97%, 35%, and 6% in Ba/F3p210<sup>Bcr-Abl</sup>, Ba/F3p210<sup>E255K</sup>, and Ba/F3p210<sup>T315I</sup>, respectively (panel C; both quadrants B2 and B4).

**Fig. 1. Differential susceptibility of Ba/F3p210<sup>Bcr-Abl</sup>, Ba/F3p210<sup>E255K</sup>, and Ba/F3p210<sup>T315I</sup> cells to imatinib.** A, dose-response curves obtained by MTS assays after 48-h exposure of Ba/F3p210<sup>Bcr-Abl</sup> (WT), Ba/F3p210<sup>E255K</sup> (E225K), Ba/F3p210<sup>T315I</sup> (T315I) cells versus the control 32D and untransfected Ba/F3 cells to imatinib. Each data point represents means ± S.D. (n = 6). B, dose-dependent effect of imatinib on cytochrome c release. The immunoblots are representative of three independent experiments. C, flow cytometric analysis of apoptotic death in the three cell lines overexpressing wild-type or mutant Bcr-Abl after treatment with DMSO vehicle, or 5 or 10 µM imatinib for 48 h. Results are representative of at least three independent experiments. B1, B2, B3, and B4 represent Annexin V<sup>−</sup>/PI<sup>−</sup>, Annexin V<sup>+</sup>/PI<sup>−</sup> (early apoptosis), Annexin V<sup>−</sup>/PI<sup>+</sup>, and Annexin V<sup>+</sup>/PI<sup>+</sup> (late apoptosis), respectively.
As Akt signaling represents a major pathway through which Bcr-Abl mediates oncogenic effects in CML cells, we further assessed the effect of imatinib on Akt activation in wild type versus mutant Bcr-Abl-overexpressing Ba/F3 cells. Because overexpression of Bcr-Abl up-regulates PI3K/Akt signaling\textsuperscript{36,37}, Ba/F3p210\textsuperscript{Bcr-Abl}, Ba/F3p210\textsuperscript{E255K}, and Ba/F3p210\textsuperscript{T315I} exhibited substantially higher levels of Akt phosphorylation, irrespective of mutations, as compared to that of 32D and untransfected Ba/F3 cells (Fig. 2A). However, the respective susceptibility of these three cell lines to the inhibitory effect of imatinib on Akt varied to a great extent.

Densitometry analysis of the immunoblots shows that exposure of Ba/F3p210\textsuperscript{Bcr-Abl} to imatinib, even at concentrations as low as 0.5 µM, led to complete Akt deactivation (Fig. 2B). A decreasing trend of phospho-Akt was noted with increasing imatinib concentrations Ba/F3p210\textsuperscript{E255K} and Ba/F3p210\textsuperscript{T315I} (trend \( P \) values were 0.004 and 0.022, respectively, according to the Jonckheere-Terpstra test). The estimated IC\textsubscript{50} values for imatinib-mediated Akt dephosphorylation were approximately 10 and 20 µM for Ba/F3p210\textsuperscript{E255K} and Ba/F3p210\textsuperscript{T315I}, respectively. Together, these data suggested a putative link between imatinib resistance and Akt signaling. Accordingly, we hypothesized that concurrent inhibition of Akt could lower the threshold of imatinib-mediated apoptosis, thereby overcoming imatinib resistance.
Fig. 2. Dose-dependent effect of imatinib on Akt dephosphorylation in Ba/F3p210Bcr-Abl, Ba/F3p210E255K, and Ba/F3p210T315I cells. A, Bcl-Abl expression versus phospho-Thr\(^{308}\) Akt levels in 32D, Ba/F3, Ba/F3p210Bcr-Abl (WT), Ba/F3p210E255K (E225K), and Ba/F3p210T315I (T315I) cells. As shown, Akt phosphorylation is upregulated by Bcr-Abl irrespective of mutations. B, dose-dependent effect of imatinib on Akt Thr\(^{308}\) phosphorylation in the three cell lines after 36-h exposure. All immunoblots are representative of three independent experiments (left panel). Bars represent the means of relative p-Akt level as compared to the DMSO control of three independent determinations ± S.D. (right panel). According to the Jonckheere-Terpstra test, the trend \(P\) values were 0.004 and 0.022 for E225K and T315I, respectively.
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 $IC_{50}$ in PDK-1 inhibition of 5 µM. 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 could affect Akt phosphorylation levels as low as 1 µM.

As shown in Fig. 3, 32D, Ba/F3, Ba/F3p210$^{Bcr-Abl}$, Ba/F3p210$^{E255K}$, and Ba/F3p210$^{T315I}$ were equally susceptible to the antiproliferative effects of OSU-03012, with the respective $IC_{50}$ values of 4.4 ± 0.1 µM, 4.8 ± 0.1 µM, 4.9 ± 1.0 µM, 4.8 ± 0.1 µM, and 4.5 ± 0.3 µM, respectively (panel A). Analyses of cytochrome c release, PARP cleavage, and Annexin V/PI staining in Ba/F3p210$^{Bcr-Abl}$, Ba/F3p210$^{E255K}$, and Ba/F3p210$^{T315I}$ cells demonstrate that the OSU-03012-mediated cell death was mainly attributable to apoptosis (panels B and 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 three cell lines irrespective of Bcr-Abl mutation (data not shown). Exposure to OSU-03012 over the range of 5 – 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 (Fig. 3A). Together, these data clearly demonstrate the lack of cross-resistance to OSU-03012 in these imatinib-resistant cells.
Fig. 3. Ba/F3p210<sup>Bcr-Abl</sup>, Ba/F3p210<sup>E255K</sup>, and Ba/F3p210<sup>T315I</sup> cells are equally susceptible to OSU-03012 irrespective of Bcr-Abl mutations.  

A, dose-response curves obtained by MTS assays after 48-h exposure of the control 32D and untransfected Ba/F3 cells versus Ba/F3p210<sup>Bcr-Abl</sup> (WT), Ba/F3p210<sup>E255K</sup> (E225K), and Ba/F3p210<sup>T315I</sup> (T315I) cells to OSU-03012. Each data point represents means ± S.D. (n = 6).  

B, effect of 5 µM OSU-03012 on cytochrome c release. The immunoblots are representative of three independent experiments.  

C, flow cytometric analysis of apoptotic death in the three cell lines treated with DMSO vehicle or OSU-03012 at 2.5, 5, 7.5, or 10 µM for 48 h. Results are representative of at least three independent experiments.
**OSU-03012 sensitizes imatinib-resistant cells to imatinib-induced apoptosis.** To explore the effect of OSU-03012 on imatinib resistance, Ba/F3p210<sup>E255K</sup> and Ba/F3p210<sup>T315I</sup> cells, both of which exhibited IC<sub>50</sub> greater than 10 µM against imatinib, were treated with varying concentrations of imatinib in the presence of 5 µM OSU-03012 or vice versa. As shown by the dose-response curves in Fig. 4A, OSU-03012 sensitized, to a great extent, Ba/F3p210<sup>E255K</sup> and Ba/F3p210<sup>T315I</sup> cells to imatinib-induced cell death. For example, imatinib alone was ineffective in preventing cell proliferation within therapeutically attainable concentrations (≤ 5 µM) (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 (solid lines) with apparent IC<sub>50</sub> of 1 µM. Annexin V analysis indicates that in combination with 5 µM OSU-03012, imatinib at 2.5 and 5 µM caused 70% and >95% cell death, respectively (Fig. 4B). Similarly, imatinib at 5 µM also sensitized Ba/F3p210<sup>E255K</sup> and Ba/F3p210<sup>T315I</sup> cells to OSU-03012-mediated apoptosis (Fig. 4A, right panel), with a reduction of IC<sub>50</sub> from 5 µM to less than 3 µM. Medium dose analysis of apoptosis induction in Ba/F3p210<sup>E255K</sup> and Ba/F3p210<sup>T315I</sup> cells was carried out over a range of OSU-03012 and imatinib concentrations at a fixed ratio (1:1) for 48 h, after which CI values for apoptosis were determined in relation to the fraction affected. As shown in Fig. 4C, the resulting CI values were significantly less than 1, which are considered as a synergistic interaction. A combination of imatinib and OSU-03012 at 5 µM each represented an effective treatment to completely eliminate these imatinib-resistant cells.
Fig. 4. OSU-03012 sensitizes Ba/F3p210\textsuperscript{E255K} and Ba/F3p210\textsuperscript{T315I} cells to imatinib-induced apoptosis. A, left panel: dose-response curves obtained by MTS assays after 48-h exposure of Ba/F3p210\textsuperscript{E255K} (E225K) and Ba/F3p210\textsuperscript{T315I} (T315I) cells to a combination of 5 µM OSU-03012 and varying concentration of imatinib (solid curves), or varying concentrations of imatinib alone (dotted curves). Right panel: dose-response curves obtained by MTS assays after 48-h exposure to a combination of 5 µM imatinib and varying concentration of OSU-03012 (solid curves), or varying concentrations of OSU-03012 alone (dotted curves). Each data point represents means ± S.D. (n = 6). B, flow cytometric analysis of apoptotic death in Ba/F3p210\textsuperscript{E255K} (upper panels) and Ba/F3p210\textsuperscript{T315I} (lower panels) cells treated with 1, 2.5, and 5 µM imatinib in combination with 5 µM OSU-03012 for 48 h. Results are representative of at least three independent experiments. C, Ba/F3p210\textsuperscript{E255K} and Ba/F3p210\textsuperscript{T315I} cells were exposed to varying
concentrations of OSU-03012 and imatinib at a fixed ratio (1 : 1) for 48 h, after which CI values for apoptosis were determined in relation to the fraction affected using the medium dose effect analysis. CI values less than 1 are considered as a synergistic interaction. Mutually non-exclusive CI for combination at the IC₅₀ was 0.602 for Ba/F3p210E₂₅₅K and 0.649 for Ba/F3p210T₃₁₅I. The P values for sign test comparing CI equal to 1 versus CI < 1 are 0.016 for Ba/F3p210E₂₅₅K and 0.008 for Ba/F3p210T₃₁₅I.

Such a synergy, however, was not noted with 32D and Ba/F3 cells that lacked Bcr-Abl expression (Fig. 5A) even though OSU-03012 was able to facilitate Akt dephosphorylation in both cell systems (panel B).

**Fig 5.** OSU-03012-mediated Akt dephosphorylation does not sensitize 32D and untransfected Ba/F3 cells to imatinib-induced apoptotic death. **A,** effect of 5 µM OSU-03012 on Thr₃₀₈ phosphorylation of Akt in 32D and Ba/F3 cells after 36 h-exposure. The immunoblots are representative of three independent experiments. **B,** dose-response curves obtained by MTS assays after 48 h-exposure to a combination of 5 µM OSU-03012 and varying concentrations of imatinib. Each data point represents mean ± S.D.
This synergistic action was, at least in part, mediated through the concerted effect of OSU-03012 and imatinib on phospho-Akt. Exposure of Ba/F3p210E255K and Ba/F3p210T315I cells to the drug combination did not affect the Bcr-Abl expression level (Fig. 6A). However, imatinib at varying concentrations could augment the effect of 5 µM OSU-03012 on phospho-Akt in both cell lines (panel B). Complete Akt dephosphorylation was achieved with a combination of 5 µM imatinib 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.
Fig. 6. Combination effect of OSU-03012 and imatinib at different concentrations on the state of Bcr-Abl expression (A) and Akt phosphorylation (B) in Ba/F3p210\textsuperscript{E255K} and Ba/F3p210\textsuperscript{T315I} cells. The immunoblots are representative of three independent experiments. Bars represent means ± S.D. (n = 3). No appreciable changes in Bcr-Abl expression levels were noted. However, a decreasing trend of p-Akt with increasing doses of imatinib and OSU-03012 in combination with 5 µM OSU-03012 and 5 µM imatinib, respectively.
DISCUSSION

Development of new therapeutic strategies to overcome imatinib resistance in accelerated CML has been the focus of many recent investigations. In the literature, at least three 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\textsuperscript{31}, BMS-354825\textsuperscript{38}, and AP23464\textsuperscript{39}. In addition, the Bcr-Abl chaperone heat shock protein 90 (Hsp90) inhibitors geldanamycin and 17-allyaminogelanamycin (17-AAG) have also been shown to inhibit the growth of imatinib-resistant hematopoietic cells found in patients with T315I and E255K mutation\textsuperscript{40}. Second, co-treatment of imatinib-resistant cells with antileukemic agents such as As\textsubscript{2}O\textsubscript{3}, decitabine, the farnesyl transferase inhibitor SCH66336, and the histone deacetylase inhibitors suberoylanilide hydroxamic acid (SAHA) and butyrate could enhance the antiproliferative activity of imatinib\textsuperscript{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\textsuperscript{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 resistance.

Despite reduced binding affinity, imatinib 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. To
examine this premise, we evaluate the effect of OSU-03012, a celecoxib-derived PDK-1 inhibitor but lacking CO-2 inhibitory activity, on imatinib resistance in two clinically relevant cell lines, Ba/F3p210^{E255K} and Ba/F3p210^{T315I} as compared to 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 PTEN function\textsuperscript{29}. 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 p70^{S6K}, which 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/F3p210^{Bcr-Abl} irrespective of the Bcr-Abl mutations. The lack of cross-resistance of OSU-03012 and imatinib underscores the functional relevance of targeting PDK-1/Akt signaling in imatinib-resistant mutant cells. It is especially noteworthy that OSU-03012 showed augmenting effects with imatinib on apoptosis in mutant imatinib-resistant cells at therapeutically attainable concentrations (≤ 5 µM), shifting the dose response curves by more than one log unit. For example, the IC\textsubscript{50} and IC\textsubscript{80} values for imatinib in the presence of 5 µM OSU-03012 in Ba/F3p210^{T315I} cells were 1 and 2.5 µM, respectively, vis-à-vis 30 and 65 µM for imatinib alone. This synergy is in sharp contrast to earlier reports that many antileukemic agents such as As\textsubscript{2}O\textsubscript{3}, decitabine, and SCH66336 could not synergize with imatinib in inhibiting the growth of Ba/F3p210^{T315I} cells\textsuperscript{30,32}. 

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Our data suggest that the ability of OSU-03012 to facilitate imatinib-mediated antiproliferative effects in these imatinib-resistant cells could be rationalized by the mechanistic synergy at the phospho-Akt level, i.e., 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-resistant cells sensitive to the residual antiproliferative activity of imatinib. 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 NCI. Our data indicate that oral administration of this agent in tumor-bearing nude mice even at 200 mg/kg for one month gave rise to no weight loss or apparent toxicity at necropsy (unpublished). Thus, this combination represents a viable strategy for clinical testing in imatinib-resistant CML.
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