Threshold Levels of ABL Tyrosine Kinase Inhibitors Retained in Chronic Myeloid Leukemia Cells Determine Their Commitment to Apoptosis

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Abstract

The imatinib paradigm in chronic myelogenous leukemia (CML) established continuous BCR-ABL inhibition as a design principle for ABL tyrosine kinase inhibitors (TKI). However, clinical responses seen in patients treated with the ABL TKI dasatinib despite its much shorter plasma half-life and the apparent rapid restoration of BCR-ABL signaling activity following once-daily dosing suggested acute, potent inhibition of kinase activity may be sufficient to irrevocably commit CML cells to apoptosis. To determine the specific requirements for ABL TKI-induced CML cell death for a panel of clinically important ABL TKIs (imatinib, nilotinib, dasatinib, ponatinib, and DCC-2036), we interrogated response of CML cell lines and primary CML cells following acute drug exposure using intracellular fluorescence-activated cell sorting and immunoblot analyses of BCR-ABL signaling, apoptosis measurements, liquid chromatography/tandem mass spectrometry of intracellular drug levels, and biochemical TKI dissociation studies. Importantly, significant intracellular TKI stores were detected following drug washout, levels of which tracked with onset of apoptosis and incomplete return of BCR-ABL signaling, particularly pSTAT5, consistent with high-affinity binding and slow dissociation from ABL kinase. Together, our findings suggest commitment of CML cells to apoptosis requires protracted incomplete restoration of BCR-ABL signaling mediated by intracellular retention of TKIs above a quantifiable threshold. These studies refine our understanding of apoptotic commitment in CML cells and highlight parameters important to design of therapeutic kinase inhibitors for CML and other malignancies.

Introduction

The clinical success of imatinib in chronic myelogenous leukemia (CML) represents a hallmark in tyrosine kinase inhibitor (TKI) therapy for the treatment of cancer. Design...
rates, with the benefit of reduced incidence of toxicity with the once-daily schedule (12). The finding that clinical efficacy can be maintained despite only transiently inhibiting BCR-ABL signaling opens an opportunity to study the mechanistic requirements for ABL TKI-induced CML cell death.

We and others have previously shown commitment of CML cells to apoptosis following potent, transient target inhibition with ABL TKIs in vitro (13–15), although differences between concentrations required to produce this effect and their relative activity against BCR-ABL kinase suggest potential involvement of previously unrecognized factors. One hypothesis, referred to as the oncogenic shock premise, holds that intense, temporary disruption of BCR-ABL activity sets up a kinetic imbalance between prosurvival and proapoptotic signaling favoring the latter, the consequence of which is irreversible commitment to apoptosis (16, 17).

We report a mechanistic evaluation encompassing transient exposure of CML cells to a panel of U.S. Food and Drug Administration-approved ABL TKIs [imatinib, nilotinib, dasatinib, ponatinib (AP24534; refs. 2, 18), as well as DCC-2036 (rebastinib)], which is entering phase II trials (3, 19)]. After transient exposure of the cells to each of these agents, we interrogate response using multiparameter intracellular fluorescence-activated cell sorting (FACS) and immunoblot analyses, apoptosis measurements, liquid chromatography/tandem mass spectrometry (LC/MS-MS), and biochemical dissociation studies of ABL from ABL TKIs. In aggregate, our findings reveal that attenuated restoration of BCR-ABL signaling correlates with apoptosis commitment and that intracellular retention of ABL TKIs above a quantifiable threshold is a critical, previously unrecognized parameter mediating this effect.

Materials and Methods

Inhibitors

All inhibitors were prepared as 10 mmol/L stock solutions in dimethyl sulfoxide (DMSO) and stored at −20°C. Serial dilutions of stock solutions were carried out just before use in each experiment.

Cell lines

Certified BCR-ABL–positive human CML blast crisis–derived K562 (American Type Culture Collection) and LAMA-84 cells (DSMZ) were maintained in RPMI-1640 supplemented with 10% FBS, 1 U/mL penicillin G, and 1 mg/mL streptomycin (complete media) at 37°C and 5% CO2. Neither of the cell lines used in this study was cultured for longer than 6 months from initial purchase or characterization. No further authentication of cell lines characteristics was done.

Collection of patient samples

Clinical samples were obtained with informed consent and under the approval of the Oregon Health & Science University (OHSU) Institutional Review Board. Bone marrow from patients was separated on a Ficoll gradient (GE Healthcare) for isolation of mononuclear cells.

Inhibitor washout protocol for CML cell lines

K562 and LAMA-84 cells (5 × 105/mL) were incubated in complete media alone or with dasatinib (10 or 100 nmol/L), ponatinib (10 or 100 nmol/L), nilotinib (500 or 5,000 nmol/L), imatinib (500 or 5,000 nmol/L), or DCC-2036 (500 or 5,000 nmol/L) for 2 hours at 37°C. Following drug exposure, cells were subjected to a series of 1 of 2 types of wash steps: resuspension in fresh inhibitor-free complete media only (quick wash) or resuspension in fresh media followed by a 5-minute incubation at 37°C (long wash). The standard washout protocol [defined to match our previously reported studies (13)] consisted of 2 quick washes followed by 1 long wash step. The expanded washout protocol involved an additional 5 wash steps (alternating long and quick washes) beyond the standard washout. Samples for apoptosis and inhibitor LC/MS-MS analyses were collected from each treatment condition at the end of the 2-hour exposure, immediately following standard washes, and immediately following extra washes. Samples for immunoblot and FACS analyses were collected before and at multiple time points following standard and expanded washout.

Inhibitor washout protocol for primary CML cells

Mononuclear cells (5 × 105 cells/mL) from patients with CML were incubated in complete media (supplemented with 100 μmol/L β-mercaptoethanol) alone or with ABL TKIs (same concentrations as for cell line protocol above) for 4 hours at 37°C. Following drug exposure, cells were subjected to the standard or expanded washout protocol (identical to those conducted in cell lines). Samples for apoptosis and inhibitor LC/MS-MS analyses were collected from each treatment condition at the end of the 4-hour exposure and immediately following standard and expanded washout. Samples for immunoblot and FACS analyses were collected at the end of the 4-hour exposure and at 24 hours after standard and expanded washout.

Apoptosis assay

At each relevant time point within the washout protocols described earlier, cells from each treatment condition were plated in triplicate in a 96-well plate (5 × 104/well), incubated at 37°C, and apoptosis was measured at 72 hours after the start of the experiment using the Guava Nexin assay (Millipore). Results are reported as the mean percentage Annexin V-positivity; error bars represent SEM.

Immunoblot analysis for Crkl phosphorylation

For CML cell lines, cells were collected for each treatment condition at 0, 0.5, 2, 4, 8, 12, 24, 30, and 36 hours after either standard or expanded washout (as described earlier) and lysed (5 × 105 cells/30 μL) in boiling SDS-PAGE loading buffer supplemented with protease and phosphatase inhibitors for 10 minutes. All lysates were subjected to SDS-PAGE and immunoblotted with anti-CrkL antibody C20 (Santa Cruz Biotechnology). Phosphorylated and nonphosphorylated CrkL forms were distinguished on the basis of differential band migration, and band signal intensities were quantified by densitometry on a Lumi Imager (Roche) and expressed as...
percentage phosphorylated CrkL. [calculated as (pCrkL)/(pCrkL + non-pCrkL) × 100%].

Phosflow analysis
For CML cell lines, cells were collected for each treatment condition at the end of the 2-hour drug exposure and at 0, 0.5, 2, 4, and 8 hours after either standard or expanded washout (as described earlier). For experiments involving primary CML mononuclear cells, where sufficient cell numbers were available, samples were collected at the end of the 4-hour drug exposure and at 24 hours after standard and expanded washout (see above protocol). Samples were prepared by incubating cells (1 × 10^6) in Cytofix buffer (BD) at 37°C for 15 minutes and resuspending in PBS (Gibco) supplemented with 10% DMSO for storage at −80°C before analysis. Upon thawing of samples, fixed cells were permeabilized with Perm Buffer III (BD) for 30 minutes on ice. Samples were stained for pCrkL (Alexa647) and pSTAT5 (Alexa488) and analyzed using a Canto II instrument (BD) and FlowJo or FCS Express (De Novo Software).

Detection of inhibitors by LC/MS-MS
For CML cell lines, cellular and media fractions were separated by centrifugation for each treatment condition at the end of the 2-hour drug exposure, immediately following standard and expanded washout (as described earlier), and at 2 hours after the expanded washout. For experiments involving primary CML mononuclear cells, where sufficient cell numbers were available, cellular and media fraction samples were collected at the end of the 4-hour drug exposure and immediately following standard and expanded washout. Cellular fraction lysate samples were prepared by washing cells (1 × 10^6) once with PBS, resuspending in hypotonic lysis buffer [5 mmol/L Tris, 5 mmol/L EDTA, 5 mmol/L EGTA in double-distilled water (ddH2O); pH 7.0], shearing with a 27-gauge needle, and incubating on ice for 15 minutes. Samples were spun down and clarified lysates isolated. All samples (both cellular and media fractions) were stored at −80°C before analysis. Amounts of dasatinib, ponatinib, nilotinib, imatinib, and DCC-2036 in cellular and media fractions were determined by LC/MS-MS using an adaptation of the method of Haouala and colleagues (20); for full details, see Supplementary Materials and Methods. Briefly, samples were thawed and extracted in acetonitrile containing internal standard (sorafenib) and analyzed using an Applied Biosystems/MDS SCIEX 5500QTRAP triple-quadrupole hybrid linear ion trap mass spectrometer. Compounds were quantified using multiple reaction monitoring (MRM). Results for intracellular inhibitor levels are reported in ng per 1 × 10^6 cells and concentrations of inhibitor in media are reported in nmol/L.

ABL kinase inhibitor dissociation studies
The rate of dissociation of dasatinib, ponatinib, nilotinib, imatinib, and DCC-2036 from ABL kinase was determined using the LanthaScreen Eu Kinase Binding Assay (Life Technologies); staurosporine was also included as a control. For full details, see Supplementary Materials and Methods. Briefly, full-length purified, recombinant His-tagged ABL kinase protein (either unaltered or tyrosine dephosphorylated) was preincubated with a biotin-labeled anti-His-tag antibody and Europium chelate-labeled streptavidin in saturating concentrations of dasatinib, ponatinib, nilotinib, imatinib, DCC-2036, or staurosporine for 2 hours at room temperature. This mix was subsequently diluted into solution containing excess of Alexa647-labeled kinase ligand (Kinase Tracer 178), where dissociation of the inhibitor is followed by rapid binding of tracer. Upon tracer binding, productive time-resolved fluorescence resonance energy transfer (TR-FRET) signal was measured over time using a PHERAStar Plus instrument (BMG Labtech) to establish dissociation curves. Off-rate data are reported as t1/2 values in hours.

Results
Commitment of BCR-ABL-positive cells to apoptosis following acute exposure to ABL TKIs is not fully explained by relative potency for BCR-ABL inhibition
We established a 2-tier washout system (Fig. 1A) to evaluate whether acute exposure to ABL kinase inhibitors is necessary and sufficient to irrevocably commit BCR-ABL-positive cells to apoptosis. In line with previous reports from our laboratory and others, continuous exposure of K562 cells to dasatinib at 10 or 100 nmol/L resulted in substantial onset of apoptosis as measured by percentage of Annexin V–positive cells (Fig. 1B; refs. 13, 14). Implementation of our previously established in vitro standard washout procedure after 2 hours of exposure to dasatinib, designed to emulate the clinical situation of acute dasatinib exposure (serum half-life: 3–5 hours; ref. 13), abolished onset of apoptosis for cells treated with 10 nmol/L dasatinib but only slightly diminished the extent of apoptosis induced in 100 nmol/L dasatinib-treated cells compared with continuous exposure (Fig. 1B).

Although dasatinib’s short 3- to 5-hour plasma half-life is in sharp contrast to that of other clinically available ABL TKIs (t1/2 > 15 hours; refs. 9, 21), the variation in potency, target selectivity, and drug-binding properties among these different inhibitors provides a valuable means for further interrogating the mechanisms responsible for apoptotic commitment following acute ABL TKI exposure in CML cells. Dasatinib and ponatinib represent highly potent, multikinase TKIs, whereas nilotinib, imatinib, and DCC-2036 are less potent TKIs with reduced off-target kinase profiles (Supplementary Fig. S1; refs. 2, 3, 22). Thus, we carried out parallel washout experiments at physiologically relevant concentrations to examine the properties of each of these 5 clinically relevant ABL TKIs with respect to triggering of apoptosis. Among these, ponatinib is approximately equipotent with dasatinib as an ABL TKI (Supplementary Fig. S1; ref. 2), suggesting that if absolute potency against BCR-ABL were the sole determinant of irrevocable commitment to apoptosis, the 2 TKIs would be expected to elicit similar concentration-dependent levels of apoptosis. However, we found acute exposure to 10 or 100 nmol/L ponatinib followed by standard washout resulted in minimal or no reduction in apoptosis, respectively, compared with continuous exposure at these same concentrations (Fig. 1B).
These differences between dasatinib and ponatinib were also observed for a second BCR-ABL-positive cell line (LAMA-84; Supplementary Fig. S2A).

Consistent with previous studies (13, 15), experiments involving the less potent, more restricted kinase off-target profile ABL TKI nilotinib confirmed a pattern of concentration-dependent commitment to apoptosis similar to dasatinib following standard washout protocol (Fig. 1B). In contrast, imatinib- and DCC-2036–induced apoptosis returned to approximately baseline levels observed in untreated cells following standard washout conditions even at high concentration (5,000 nmol/L); similar results and trends were obtained using LAMA-84 cells (Supplementary Fig. S3A). Notably, DCC-2036 is roughly comparable with nilotinib in terms of relative potency against BCR-ABL (Supplementary Fig. S1; refs. 19, 23).

The intriguing differences in apoptosis-triggering capacity between TKIs with nearly identical equimolar potency against BCR-ABL led us to hypothesize that these distinct properties could potentially be attributable to fortuitous off-target effects that reinforce ABL TKI effectiveness in a multifactorial way or to differences in the extent of TKI removal from the cells following implementation of the same standard washout protocol. To investigate whether differences not addressed by the standard washout protocol could be discerned, an expanded washout protocol consisting of 5 additional wash steps beyond the standard 3 washouts were implemented (Fig. 1A). Strikingly, when the additional expanded washout protocol was applied to K562 cells initially treated with 100 nmol/L dasatinib, a moderate reduction in the extent of apoptosis was achieved (Fig. 1B). This was also true for 10 nmol/L ponatinib and 5,000 nmol/L nilotinib, where apoptosis levels were reduced to background levels matching the untreated control. In contrast, levels of apoptosis in cells treated with 100 nmol/L ponatinib were unaffected by inclusion of the expanded washout steps (Fig. 1B). Similar results and trends were obtained for LAMA-84 cells (Supplementary Figs. S2A and S3A). These results suggest that a pool of drug remains sequestered within CML cells following acute exposure, and in the case of exposure to 100 nmol/L ponatinib, these levels remain above a threshold required to induce maximal apoptosis even after implementing the 2-tier washout protocol. Taken together, these data suggest that commitment of CML cells to apoptosis following acute

Figure 1. Commitment of BCR-ABL–positive cells to apoptosis following acute exposure to ABL TKIs varies by inhibitor, concentration, and extent of washout. A, schematic outlining general experimental washout protocol. Briefly, BCR-ABL positive cells were incubated in complete media alone or in the presence of each of 5 clinically relevant ABL TKIs either continuously (72 hours) or for a short exposure (2 hours for cell lines, 4 hours for primary cells). For acute exposure conditions, cells were subjected to our previously published 3 wash steps protocol (standard washout) followed by an additional 5 wash steps (expanded washout) and cultured in fresh complete media for the remainder of the 72-hour experiment. B, levels of apoptosis in K562 cells following continuous or acute exposure to ABL kinase inhibitors. Cells were incubated in the presence of the indicated inhibitor concentrations for 2 hours, and samples were collected just before washout and immediately following standard and expanded washout. Annexin V-positivity was measured at 72 hours after the start of the experiment, and bars represent the mean of at least 3 independent experiments carried out in triplicate ± SEM.
exposure to ABL TKIs is: (i) dependent upon the extent of drug washout, and (ii) not fully explained by absolute potency against BCR-ABL.

**BCR-ABL signaling is not fully restored under conditions that commit CML cells to apoptosis following ABL TKI exposure**

To explore this situation further in the context of BCR-ABL signaling, we conducted an assessment of each of these 5 ABL TKIs using a sensitive immunoblot assay that allows direct, same-lane comparison of tyrosine-phosphorylated and non-phosphorylated levels of the direct BCR-ABL substrate CrkL. (24) and 2 intracellular FACS-based readouts of BCR-ABL tyrosine phosphorylation substrates (pSTAT5 and pCrkL).

Comparison of signaling events following acute exposure to dasatinib and ponatinib revealed fundamentally different situations (Fig. 2). In K562 cells exposed to dasatinib, treatment for 2 hours with concentrations of 10 and 100 nmol/L reduced pCrkL to less than 10% and nearly undetectable levels compared with untreated cells, respectively, as determined by immunoblot analysis (Fig. 2A). However, while both the standard and expanded washout procedures restored pCrkL signaling in cells treated with 10 nmol/L dasatinib to levels comparable with pretreatment, the 100 nmol/L dasatinib...
treatment followed by standard washout resulted in less rapid return of pCrkL signaling to levels approaching those of pretreatment. In all cases, the 2-tier washout gave more consistent, slightly higher levels of pCrkL restoration compared with the standard washout protocol (though differences were not dramatic), with the substantial recovery of pCrkL signal occurring as early as the 2 hours after washout time point. Although pCrkL signaling differences were slight for the standard versus expanded washout for 100 nmol/L dasatinib (Fig. 2A), given the apparent difference in the extent of apoptosis (Fig. 1B), we more quantitatively explored the relationship between even slightly attenuated signal restoration and extent of apoptosis using intracellular FACS analysis of pCrkL and pSTAT5. The tyrosine-phosphorylation status of STAT5 provides a more sensitive surrogate measure of extent of BCR-ABL signaling restoration (25), and by extension, is a better indicator than pCrkL for low levels of residual TKI. Importantly, we found pSTAT5 signal in the 100 nmol/L dasatinib-treated cells showed a greater degree of restoration at every time point after expanded washout than the corresponding standard washout time point (Fig. 2B and Supplementary Table S1), and yet signal was never fully restored, consistent with results in Fig. 1B.

In the case of ponatinib, pCrkL signal was only partially reduced (~25%–50% of pretreatment levels) following 2 hours treatment at a concentration of 10 nmol/L, whereas 100 nmol/L treatment inhibited pCrkL signal to essentially undetectable levels by immunoblot (Fig. 2A). Upon washout under the standard or expanded protocol, limited restoration of pCrkL levels was achieved in cells treated with 10 nmol/L ponatinib despite substantial reduction in the extent of apoptosis in the case of the expanded washout compared with the standard washout protocol. Once again, however, this subtle difference was most discernible in the levels of pSTAT5 (Fig. 2B and Supplementary Table S1). In marked contrast to findings for dasatinib, upon washout of 100 nmol/L ponatinib under the standard or expanded protocol, minimal restoration was observed as monitored by pCrkL immunoblot or FACS measurements of intracellular pCrkL and pSTAT5 (Fig. 2 and Supplementary Table S1), suggesting that TKI cannot be washed out readily and that BCR-ABL remains inhibited.

Exposure of K562 cells to nilotinib for 2 hours resulted in near complete pCrkL signal blockade with 500 nmol/L and complete interruption with 5,000 nmol/L, although substantial restoration of pCrkL was only apparent in cells treated with 500 nmol/L nilotinib following standard washout protocol as gauged by immunoblot analysis (Fig. 3A). Consistent with apoptosis data, the expanded washout protocol resulted in pCrkL levels approximating pretreatment levels as judged by FACS for both concentrations tested (Fig. 3B). In contrast to nilotinib but consistent with our earlier apoptosis findings, acute exposure to either 500 or 5,000 nmol/L imatinib showed minimal or partial interruption of pCrkL signal, respectively, which was subsequently restored to levels at or near pretreatment following standard and expanded washout protocols (Fig. 3A); complete restoration was apparent for both pCrkL and pSTAT5 by FACS (Fig. 3B and Supplementary Table S1). Finally, despite showing significant signal blockade at the end of drug exposure, cells treated with 500 nmol/L DCC-2036 showed rapid and complete restoration following both wash regimens. However, cells treated with 5,000 nmol/L DCC-2036 showed similar trends in signaling inhibition as nilotinib. The extent of signaling recovery after standard washout conditions was considerably greater for DCC-2036 than for nilotinib by immunoblot and FACS (Fig. 3), consistent with differences in apoptotic commitment (Fig. 1B). Similar results and trends were seen using LAMA-84 cells (Supplementary Figs. S2A and S2C and S3A and S3C). Together, these findings suggest that apoptotic commitment in CML cells tracks with incomplete restoration of BCR-ABL signaling and, for certain TKIs, can be rescued with a more thorough washout protocol, implicating drug retention properties of ABL TKIs as a mediator of this effect.

ABL TKIs are retained intracellularly following drug washout and intracellular TKI levels track with apoptosis and signaling inhibition

The potential existence of a functionally important, difficult-to-remove residual intracellular drug pool has major consequences for inhibitor design. To detect and quantitatively determine the amount of residual TKI present in CML cells following acute drug exposure, we developed a highly sensitive LC/MS-MS-based assay for each of the 5 ABL TKIs under investigation and collected intracellular and media fractions for evaluation. This analysis revealed dramatic variance in levels of retained TKI in K562 cells among the 5 inhibitors following both standard and expanded washout after acute drug exposure (Fig. 4A, left). For 10 nmol/L dasatinib treatment, intracellular levels were 0.06 and 0.04 ng/10⁶ cells after standard and expanded washout protocols, respectively (Fig. 4A, left), and these both corresponded with background levels of apoptosis (Fig. 1B). In contrast, cells exposed for 2 hours to 100 nmol/L dasatinib showed intracellular levels of 0.56 and 0.35 ng/10⁶ cells after standard and expanded washout protocols, respectively, with both conditions associated with concentration-dependent increased levels of apoptosis (Fig. 1B). Further regression analysis of the association between intracellular TKI levels after washout and levels of apoptosis revealed a nonlinear, concentration-dependent relationship consistent with intracellular TKI levels above a critical threshold being necessary for commitment to apoptosis (Fig. 4B). Thus, the LC/MS-MS technique is capable both of establishing a threshold for onset of apoptosis and of quantitating and distinguishing differences in amounts of functionally important residual TKI.

Given ponatinib’s pronounced capacity for inducing apoptosis and for sustaining marked inhibition of BCR-ABL signaling despite extensive drug washout, ponatinib intracellular drug retention was compared with dasatinib (which features comparable molecular weight and potency for BCR-ABL to that of ponatinib), revealing multiple differences. First, despite a 10-fold difference in initial exposure concentration, results with 10 nmol/L ponatinib treatment were similar to those achieved with 100 nmol/L dasatinib: ponatinib intracellular levels were 0.24 and 0.14 ng/10⁶ cells following standard and expanded washout, respectively (Fig. 4A, left), with the latter...
Extent of restoration of BCR-ABL signaling activity following washout of less potent, more selective ABL TKIs tracks with conditions committing cells to apoptosis. K562 cells were incubated alone or in the presence of 500 and 5,000 nmol/L nilotinib, imatinib, or DCC-2036 for 2 hours, subjected to standard and expanded washout, and collected at the indicated time points after washout for analysis by immunoblot (A) and Phosflow FACS analyses (B). For immunoblot analyses, the phosphorylated and nonphosphorylated forms of CrkL were resolved by SDS-PAGE, blotted using a total CrkL antibody, and results are expressed as percentage pCrkL, with the red dashed line indicating the level of percentage pCrkL in untreated K562 cells. For Phosflow FACS analyses, cells were fixed, permeabilized, and stained using Alexa647-pCrkL- and Alexa488-pSTAT5-conjugated antibodies. Results are displayed, for comparison purposes, as the overlaid signal peak traces of isotype control, untreated cells, and each indicated time point after washout. Vertical, black dashed lines highlight the peak signal in untreated K562 cells for reference.
ABL TKIs are retained intracellularly following washout of drug from culture media and define thresholds of apoptotic commitment. A, residual levels of ABL TKIs detected after washout in isolated intracellular and media fractions. Following acute (2 hours) exposure to ABL TKIs, K562 cells were collected just before washout, immediately following standard and expanded washout, and 2 hours after completion of expanded washout. Cellular and media ABL TKIs detected after washout in isolated intracellular and media fractions. Following acute drug exposure at least 3 replicate experiments before washout, immediately following standard and expanded washout, and 2 hours after completion of expanded washout. Cellular and media fractions were isolated by centrifugation, and cells were washed in PBS and subjected to hypotonic lysis on ice. Clarified intracellular lysate and culture media samples were analyzed for levels of ABL TKIs by LC/MS-MS and results are reported as the mean ng/10^6 cells and nmol/L in media, respectively, of to the indicated intracellular TKI threshold values and compared using a two-tailed Student test. Statistically significant **, P < 0.01; ***, P < 0.001, respectively.

inducing apoptosis only slightly above background (Fig. 1B). Second, cells treated with 100 nmol/L ponatinib retained substantially more drug following the acute exposure period compared with 100 nmol/L dasatinib treatment such that reduced intracellular ponatinib levels achieved with the standard and expanded wash protocols (2.11 and 0.87 ng/10^6 cells, respectively) did not produce a significant reduction in the level of apoptosis compared with continuous treatment.
and only minimally restored BCR-ABL signaling (Figs. 1B and 2).

Among the 3 tested less potent, more selective ABL TKIs, K562 cells most readily retained imatinib following standard and expanded washout of 5,000 nmol/L treatment (Fig. 4A, left), although given the approximately 5- to 20-fold reduced potency of imatinib compared with either nilotinib or DCC-2036 (Supplementary Fig. S1; refs. 19, 23), these intracellular levels of imatinib were not sufficient to induce significant apoptosis above background (Figs. 1B and 4B). Comparison of nilotinib and DCC-2036 also highlighted fundamental differences in the properties of these 2 inhibitors in that treatment with 5,000 nmol/L of either TKI resulted in equal amounts of drug retained after standard washout (Fig. 4A, left), yet only nilotinib under these conditions resulted in substantial apoptosis (Fig. 1B). Furthermore, intracellular nilotinib levels were markedly reduced after expanded versus standard washout (0.03 vs. 0.59 ng/10^6 cells, respectively), which tracked with abrogation of apoptosis induction. Similar intracellular TKI retention findings were obtained with all tested inhibitors using LAMA-84 cells (Supplementary Figs. S2B and S3B). These findings suggest that either the majority of the residual TKI pool retained within a CML cell following washout is not bound to BCR-ABL or that auxiliary, off-target kinase pathways may also help mediate commitment to apoptosis.

To rule out the possibility that, either by equilibration with intracellular stores and/or despite extensive washout and several cycles of media and tissue culture plastic ware exchange, the media still contained appreciable concentrations of TKI sufficient to produce an unrecognized continuous exposure situation, we determined the minimum media concentration of each TKI sufficient to trigger apoptosis under continuous exposure conditions (Supplementary Fig. S4A). Thresholds for continuous media concentrations triggering substantial apoptosis (>50% that for the highest concentration tested) were: 1 nmol/L dasatinib, 2.5 nmol/L ponatinib, 25 nmol/L nilotinib, 500 nmol/L imatinib, and 50 nmol/L DCC-2036. For dasatinib, imatinib, and DCC-2036, TKI in the media fraction was either not detected or below the lower limit of quantitation (LLOQ) of the LC/MS-MS-based assay subsequent to either wash protocol (Fig. 4A, right). In conditions following standard or expanded washout where media TKI was detected, concentrations were well below the aforementioned thresholds for triggering substantial apoptosis (Supplementary Fig. S4A), consistent with residual intracellular stores serving as the source of functionally important TKI. In contrast, while media TKI concentrations detected following washout of lower treatment concentrations of ponatinib and nilotinib were below thresholds inducing substantial apoptosis, media levels of TKI for 100 nmol/L ponatinib and 5,000 nmol/L nilotinib following standard washout protocol were 6.68 and 25.5 nmol/L, respectively, likely reflecting slow equilibration of high-level intracellular stores (Fig. 4A, right). Media TKI levels for both drugs were reduced to below apoptosis-inducing thresholds after implementing the expanded washout.

The substantial intracellular stores of TKI we observed following acute drug exposure and the media concentrations of ponatinib and nilotinib detected after standard washout suggested that drug retained intracellularly may also continue to leach out of the cells after washout completion. To confirm this, we also collected intracellular and media fractions from each treatment condition at 2 hours after completion of the expanded washout protocol. In every case, intracellular concentration of TKI was decreased relative to that immediately after washout completion (Fig. 4A, right) and paired with a concomitant slight increase in TKI levels in the media (Fig. 4A, left). These data suggest that detectable concentrations of ABL TKIs in the media following washout are likely attributable to variable kinetics of drug slowly escaping from intracellular stores, possibly as apoptosis takes place.

**Primary CML cells show intracellular TKI retention-dependent commitment to apoptosis and highlight prolonged efficacy of ponatinib**

To confirm that our findings from CML cell lines about intracellular TKI retention and commitment to apoptosis extend to primary CML cells, we conducted similar washout studies using mononuclear cells isolated from patients with newly diagnosed CML. Absolute levels of apoptosis achieved with continuous exposure were slightly less in primary cells compared with those observed with cell lines. Consistent with trends observed with K562 and LAMA cells, only dasatinib, ponatinib, and nilotinib triggered substantial apoptosis over untreated following standard washout, and among these, only ponatinib (100 nmol/L) induced substantial, though slightly reduced, levels of apoptosis following the expanded washout protocol (Supplementary Fig. S5). Also, in register with CML cell line findings, levels of each TKI retained intracellularly were serially reduced following the standard and expanded washout protocols, which paralleled ultimate rescue of apoptotic commitment for all inhibitors except ponatinib (Supplementary Fig. S5).

On the basis of the informative differences we observed between equimolar treatments of dasatinib and ponatinib in cell lines, we further examined this specific comparison in washout experiments using primary cells from an additional patient with newly diagnosed CML (12/209; Fig. 5). Findings for 10 nmol/L ponatinib mirrored those of 100 nmol/L dasatinib with a modest decrease in apoptosis levels following standard washout and subsequent return to baseline levels following expanded washout. Cells treated with 100 nmol/L ponatinib showed increased levels of apoptosis overall that were only reduced following expanded washout, and even then not completely back to baseline (Fig. 5A).

As our previous findings in K562 and LAMA cells suggested pSTAT5 represents a more sensitive biomarker for resolving subtle differences in BCR-ABL signal attenuation and that absolute levels of apoptosis following continuous dasatinib or ponatinib treatment in primary CML samples were slightly lower at 72 hours compared with those seen with CML cell lines, we focused our FACS-based analysis on pSTAT5 levels. As expected, absolute differences in signal shift were reduced compared with CML cell lines but trends were consistent, with pSTAT5 inhibition following acute 100 nmol/L dasatinib treatment (4 hours) partially and fully rescued to
pretreatment levels by 24 hours after standard and expanded washouts, respectively (Fig. 5B, top). In contrast, in cells treated with 100 nmol/L ponatinib, pSTAT5 remained inhibited at 24 hours after standard washout at levels comparable with those just before washout under treatment. Levels of pSTAT5 approaching but not quite reaching those of untreated cells were observed by 24 hours after expanded washout (Fig. 5B, bottom).

Consistent with the slightly reduced absolute numerical differences but preserved data trends between CML cell lines and primary CML cells, we observed slightly increased intracellular retention of ponatinib versus dasatinib, with after washout reduction in intracellular levels of both TKIs tracking with varying levels of pSTAT5 signal restoration and apoptosis (Fig. 5C). Notably, even intracellular levels of ponatinib as low as 0.05 ng/10^6 cells after washout were sufficient to induce a substantial increase in apoptosis; in contrast, this threshold for dasatinib was approximately 2- to 3-fold higher after washout (Fig. 5C). Taken together, these findings validate the relationship between apoptotic commitment, intracellular retention of ABL TKIs, and incomplete restoration of BCR-ABL signaling in primary patient cells and suggest that among the inhibitors tested, ponatinib shows the most robust prolonged efficacy extending to intracellular levels of drug below the apoptosis-triggering thresholds of all 4 other ABL TKIs. This may suggest that a higher proportion of retained ponatinib is bound to BCR-ABL target than for other inhibitors.

**Differences in kinase: inhibitor dissociation rates among ABL TKIs largely parallel acute exposure apoptosis trends**

We observed through sensitive LC/MS-MS-based assays and apoptotic quantitation in both CML cell lines and primary CML cells that (i) different ABL TKIs are retained intracellularly to different extents after washout, and (ii) comparable intracellular amounts of approximately equipotent TKIs with respect to BCR-ABL do not ensure comparable apoptotic induction. One possible factor contributing to the variability in degree of washout efficiency between different TKIs is differences in binding properties of each TKI to the BCR-ABL kinase domain, with the presumption that kinase-bound inhibitor is less readily washed out of the cells. To explore this question, we used a TR-FRET-based assay (see Materials and Methods) to evaluate relative rates of dissociation of all 5 ABL...
Intracellular Drug Retention Mediates Apoptosis Commitment in CML

TKIs from purified ABL kinase (26). Among these inhibitors, imatinib and ponatinib represented the 2 extremes of very fast and extremely slow off-rates, respectively (Fig. 6), with ponatinib’s remarkably long residence time profile resembling that of irreversible kinase inhibitors. Dasatinib, nilotinib, and DCC-2036 dissociation kinetics were intermediate between imatinib and ponatinib. Differences in dissociation rates were observed between the catalytically active and inactive conformations of the ABL kinase domain, consistent with previously characterized crystallographic-binding evidence for each compound (3, 27). These findings suggest that certain ABL TKIs, most notably ponatinib, may remain bound to BCR-ABL kinase for long periods following acute drug exposure, enabling prolonged intracellular retention, and partial signal disruption after washout.

Discussion

The clinical success of the TKI paradigm in CML has motivated analogous strategies for many malignancies. As such, the ability of dasatinib to induce durable responses with once-daily dosing despite a much shorter plasma half-life than imatinib (3–5 vs. >18 hours) and subsequent rapid restoration of BCR-ABL activity (10, 11) has prompted investigation into the mechanistic requirements for ABL TKI-induced CML cell death.

In vitro drug washout protocols have previously shown apoptotic commitment in CML cells following acute exposure to ABL TKIs despite only transient inhibition of BCR-ABL activity (13–15). Among the potential explanations offered for this phenomenon is the oncogenic shock hypothesis, which holds that temporary disruption of prosurvival and proapoptotic signaling sets up a kinetic imbalance that guarantees irreversible commitment to apoptosis (16, 17). Much like a tightrope walker who loses contact with the high wire, the cell’s fate is sealed upon satisfaction of a single condition. Enforcement of the concept of oncogenic shock as applied to ABL TKIs requires only that transient, potent interruption of BCR-ABL signaling is both necessary and sufficient to irrevocably commit CML cells to apoptosis.

In our studies in CML cells, the condition of complete interruption of BCR-ABL signaling was met following 2-hour exposure to physiologically relevant concentrations of all TKIs except imatinib (at both 500 and 5,000 nmol/L for nilotinib and DCC-2036; at 100 nmol/L for dasatinib and ponatinib; Figs. 2 and 3), yet this did not result in irrevocable onset of apoptosis in all cases, particularly following more extensive washout protocol (Fig. 1B). This suggests that the extent of ABL TKI-induced apoptosis can indeed be modulated following acute, potent target inhibition, thus calling the oncogenic shock explanation into question. Moreover, the oncogenic shock concept implies that levels of BCR-ABL signaling restoration following acute, potent target inhibition do not alter commitment to programmed cell death. In contrast, we found that sensitive monitoring of BCR-ABL activity (particularly as measured by levels of pSTAT5; Supplementary Table S1) revealed an association for all ABL TKIs between incomplete restoration of signaling and increased levels of apoptosis (Figs. 1B, 2, and 3). Importantly, while previous studies of acute dasatinib-induced cell death reported full (14) or approaching full (13) restoration of pCrkL levels rapidly following washout, extent of pSTAT5 signal restoration following washout was not evaluated. Notably, FACS analysis of pSTAT5 and pCrkL in ponatinib-treated cells at 24 hours following washout revealed in select cases a subpopulation of cells undergoing early apoptosis that showed reduced levels of both phospho-proteins (data not shown), resulting in a reduction in mean signal for the sample (Supplementary Table S1). However, we did not observe this to be a robust difference and this was less pronounced or not observed for other inhibitors at the same time point. Given our observations that for select TKI treatment conditions (including 100 nmol/L dasatinib) pCrkL, but not pSTAT5 signal was completely restored following washout (Fig. 2B and Supplementary Table S1), this suggests that STAT5 phosphorylation may serve as a more sensitive metric for monitoring low level changes in BCR-ABL signaling activity.

Investigation into the source of incomplete BCR-ABL signaling restoration following drug washout revealed varying levels of TKI retained within CML cells as detected by sensitive
Intracellular levels of TKI were reduced for all ABL TKIs after our standard washout protocol and reduced further following expanded washout, which tracked with rescue of BCR-ABL signaling activity and reduction in apoptosis (Figs. 1B, 2, and 3; Supplementary Table S1). These findings provide evidence for a functionally significant pool of residual TKI following drug washout capable of attenuating BCR-ABL signaling, consistent with results for low-level continuous inhibition of BCR-ABL (Supplementary Fig. S4B). In addition, the nonlinearity of the relationship between specific levels of intracellular ABL TKI and levels of apoptosis after washout suggests a threshold-based requirement for triggering apoptotic commitment in CML cells (Fig. 4B). In fact, for the 3 TKIs for which we observed a capacity for apoptotic induction following acute drug exposure at the physiologically relevant concentrations tested (dasatinib, ponatinib, and nilotinib), we used receiver–operator characteristic (ROC) analysis based on induction of apoptosis comparable with that of continuous drug exposure to help guide selection of threshold values and found after washout samples with intracellular TKI levels above these thresholds to have significantly increased levels of apoptosis compared with those below the threshold (Fig. 4C). For example, standard washout of 10 nmol/L dasatinib-treated cells apparently reduced the intracellular stores of TKI below an apoptosis-triggering threshold (Figs. 1B and 4). In contrast, standard washout following acute exposure to 100 nmol/L ponatinib or 100 nmol/L dasatinib reduced intracellular TKI to levels approaching but still above such a threshold for each drug, and 100 nmol/L ponatinib-treated cells retained TKI at levels well above a threshold for apoptosis commitment, such that no reduction in apoptosis was discernible relative to continuous ponatinib treatment (Figs. 1B and 4). An inhibitory threshold that must be exceeded to commit CML cells to apoptosis is consistent with the oncogenic shock premise. However, our data suggest that the threshold is not dictated by potent, acute inhibition of BCR-ABL alone. At minimum, commitment to apoptosis also requires residual TKI levels sufficient to provide functionally relevant low-level continuous inhibition of BCR-ABL signaling.

Although dasatinib is the only 1 of 5 TKIs we tested which features a short plasma half-life clinically (10, 11), many of the key findings from this study were made based on valuable comparisons with other ABL TKIs. To mimic the plasma pharmacokinetics of dasatinib for all ABL TKIs examined, our standard washout protocol was designed, through use of serial volume exchanges, to reduce initial media TKI levels to femtomolar concentrations, which is well below detection limits of LC/MS-MS. Thus, while for several treatment conditions (including 10 nmol/L dasatinib) washout did reduce levels of TKI in the culture media to undetectable levels, it was somewhat surprising to find TKI present at very low concentrations in the media fraction following washout (Fig. 4, right). Although in almost all cases these media concentrations of TKI were alone too low to contribute significantly to increased apoptosis via continuous exposure in K562 cells (Supplementary Fig. S3A), the 2 exceptions were 100 nmol/L ponatinib and 5,000 nmol/L nilotinib (Fig. 4, right), which showed levels of 6.68 and 25.5 nmol/L, respectively. Importantly, media levels of both drugs were reduced to sub-apoptosis–contributing levels following expanded washout, suggesting they cannot alone explain the induction of apoptosis under these conditions (Fig. 4, right and Supplementary Fig. S3A). Furthermore, rather than inefficiency of the drug washout protocol, the low levels of TKI detected in the media following washout may reflect differences in dynamics of cellular drug efflux mechanisms in response to significant intracellular levels of each drug (28–34) and/or differences in subcellular drug sequestration (35). Indeed, a recent study found that overexpression of the drug transporters ABCB1 or ABCG2 rescued K562 cells from apoptosis following exposure to very high concentrations of imatinib (36). However, while the cellular drug transporter-mediated uptake and efflux of imatinib has been reasonably well-studied (29, 33), application of these findings to newer ABL TKIs including dasatinib, nilotinib, and ponatinib has been limited because of differences in transporter substrate specificity (28, 30–32, 34). Regardless of the specific transport mechanisms, our findings that a measurable decrease in intracellular TKI levels at 2 hours after the expanded washout was paralleled by a slight increase in levels of TKI in the media (Fig. 4A) are consistent with the results for the native c-ABL protein is normally tightly regulated within the cell by autoinhibitory mechanisms involving the N-terminal SH3, SH2, and cap domains, maintaining the kinase’s catalytically inactive conformation (37, 38). Furthermore, wild-type c-ABL has also been shown to exhibit greater protein stability than constitutively activated, tyrosine phosphorylated c-ABL in vitro (half-life: 18 vs. 7 hours, respectively; ref. 39). Interestingly, although imatinib showed very rapid dissociation from ABL kinase, intracellular levels...
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The drive to better understand and exploit the mechanisms of TKI uptake and retention during the conformational transition between the catalytically active and inactive states (3), may initially be slower to bind BCR-ABL such that the 2- to 4-hour drug exposure period was insufficient to resolve potential acute exposure efficacy. In contrast, continuous exposure for 72 hours to 25 to 50 nmol/L DCC-2036 induced substantial apoptosis in K562 cells over untreated (Supplementary Fig. S4A). Importantly, however, washout studies conducted using cells from a CML blast crisis patient harboring a dominant BCR-ABL T315I mutation revealed decreased retention of dasatinib, nilotinib, and imatinib (in contrast to relatively unchanged levels of the ABL D315I inhibitors ponatinib and DCC-2036) after standard washout compared with similar experiments in primary CML cells harboring unm umted BCR-ABL (Supplementary Figs. S5 and S6), further highlighting the importance of drug binding in intracellular retention.

The findings of our comprehensive investigation into the requirements of ABL TKIs to commit CML cells to apoptosis following acute drug exposure underscore the importance of intracellular drug residence time. The drive to better understand and exploit the mechanisms of TKI uptake and retention has received considerable recent attention (4, 28–34, 36, 40–42). Our findings provide novel insights into the importance of previously unrecognized intracellular drug retention threshold levels required for apoptotic induction and identify ponatinib as a TKI with a kinase residence time profile reminiscent of irreversible kinase inhibitors (26, 43). This has particular application to drug design and development, where currently many compounds that may feature highly favorable target specificity and potency may be excluded from further development clinically based on plasma pharmacokinetic profiles. The oncogenic shock model would suggest that to maximize efficacy and minimize toxicity the key design features of novel compounds should be extreme kinase target potency and rapid clearance from both the plasma and tissues. In contrast, our findings heavily challenge the oncogenic shock concept by arguing strongly that absolute potency is not sufficient to mediate efficacy and that residual intracellular TKI above a critical threshold level is required. Furthermore, exploiting the basis of the extremely slow dissociation properties of TKIs like ponatinib may aid in maximizing efficacy of a small molecule even at lower doses. Although extensive studies have been conducted confirming the therapeutic and prognostic value of monitoring drug levels in plasma of patients with CML (9, 44, 45), this approach does not provide direct information about the intracellular stores of drug, which seems to be of critical importance particularly in the case of TKIs featuring short plasma half-lives such as dasatinib. Consideration and monitoring of intracellular drug retention properties will yield improved candidate compounds for clinical development and complementary data for interpreting and defining rationale behind optimal dosing regimens for molecularly targeted therapies in CML and other malignancies.

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References


Threshold Levels of ABL Tyrosine Kinase Inhibitors Retained in Chronic Myeloid Leukemia Cells Determine Their Commitment to Apoptosis

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