SUPPLEMENTARY MATERIALS AND METHODS

Cellular proliferation assays for CML cell lines

K562 and LAMA-84 cells (4 x 10^3 cells/well) were plated in quadruplicate and incubated for 72 h in complete media alone or with graded concentrations of dasatinib (0.1-100 nM), ponatinib (0.1- 100 nM), nilotinib (5-5000 nM), imatinib (5-5000 nM), or DCC-2036 (5-5000 nM) at 37 °C. Cellular proliferation was measured by methanethiosulfonate (MTS)-based assay (CellTiter 96® Aq_{ueous} One; Promega), and IC_{50} values were calculated using Graphpad Prism software.

BCR-ABL phosphotyrosine immunoblot analysis

K562 and LAMA-84 cells (1 x 10^6 per condition) were cultured overnight in complete media alone or in the presence of graded concentrations of dasatinib (0.1, 1, 10, 100 nM), ponatinib (0.1, 1, 10, 100 nM), nilotinib (5, 50, 500, 5000 nM), imatinib (5, 50, 500, 5000 nM), or DCC-2036 (5, 50, 500, 5000 nM). Cells were harvested by centrifugation and clarified lysates were subjected to SDS-PAGE, transferred to Immobilon-FL® PVDF membranes, blocked in AquaBlock™ reagent (EastCoast Bio), and probed using anti-phosphotyrosine (4G10) anti-actin (Clone C4; Millipore) antibodies. Immunoblots were visualized using an Odyssey Imager (LI-COR). Bands corresponding to phospho-BCR-ABL (pBCR-ABL) and actin were quantitated densitometrically and normalized to each untreated control. To account for subtle loading differences, pBCR-ABL signal was normalized to actin levels in the same sample; IC_{50} values for BCR-ABL tyrosine phosphorylation were calculated for each inhibitor using Graphpad Prism software.

Continuous drug exposure assay

K562 cells (5 x 10^5 cells/mL) were incubated in complete media alone or with graded concentrations of dasatinib (0.1, 0.25, 0.5, 1, 10, 100 nM), ponatinib (0.1, 1, 2.5, 5, 10, 100 nM), nilotinib (5, 10, 25, 500, 500, 5000 nM), imatinib (5, 50, 500, 1000, 2500, 5000 nM), or DCC-2036 (5, 10, 25, 50, 500, 5000 nM) at 37 °C. For consistency with washout protocols, samples were collected for signaling, LC/MS/MS, and apoptosis analyses at 2 h and 72 h after the start of the experiment, respectively.

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 h drug exposure, immediately following standard and expanded washout (as described above), and at 2 h 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 h 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 mM Tris, 5 mM EDTA, 5 mM EGTA in ddH_2O; pH 7.0), shearing with a 27-gauge needle, and incubating on ice for 15 min. Samples were spun down and clarified lysates isolated. All samples (both cellular and media fractions) were stored at -80 °C prior to analysis. Amounts of dasatinib, ponatinib, nilotinib, imatinib, and DCC-2036 in cellular and media fractions were determined by LC/MS/MS using an adaption of the method of Haouala and colleagues (46). 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 used in triple quadrupole mode. The mass spectrometer was equipped with a TurboIonSpray® ESI source operated in the positive mode. The LC consisted of a Shimadzu (Columbia, MD) SIL-20AC XR auto-sampler, a CBM-20A system controller, two LC-20AD XR LC pumps, a DGU-20 A5 in-line solvent degasser, and a CTO-20A column oven. Compounds were quantified using multiple reaction monitoring (MRM). Optimal instrument parameters for the MRM transitions for each compound were determined by direct infusion of purified compounds. Linear least-square regression of spiked naïve cell lysate and media dilution series was used for quantification, and data acquisition and quantitative processing were accomplished with Analyst software (version 1.5). Results for intracellular inhibitor levels are reported in ng per 1×10^6 cells; concentrations of inhibitor in media are reported in nM. The lower limit of quantitation (LLOQ) for each compound was: dasatinib 0.25 ng/mL, ponatinib 0.025 ng/mL, nilotinib 0.1 ng/mL, imatinib 0.25 ng/mL, and DCC-2036 0.1 ng/mL. For all compounds, the

relative standard deviation at the LLOQ was <5% and the signal-to-noise ratio was >10:1, except for dasatinib (6:1). All analytical work was conducted in the OHSU Bioanalytical Shared Resource core facility.

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. Full-length purified, recombinant ABL kinase protein containing a His-tag was used in its active (untreated) and dephosphorylated (YOP Protein Tyrosine Phosphatase-treated) forms (7). ABL kinase (10 nM) was pre-incubated with a biotin-labeled anti-His-tag antibody (200 nM), Europium chelate-labeled streptavidin (200 nM), and saturating concentrations of dasatinib, ponatinib, nilotinib, imatinib, DCC-2036, or staurosporine for 2 h at room temperature. The concentrations of inhibitor used were chosen based on prior competitive binding assay results (data not shown) in order to achieve saturation of the kinase. In a white, 96-well assay plate, the pre-incubation mix was diluted 1:100 with a solution containing excess Kinase Tracer 178 (20.2 nM), an Alexa-647-labeled kinase ligand. Following dilution, samples were read over time to monitor change in TR-FRET signal, as the inhibitor dissociates and the tracer rapidly binds free kinase. TR-FRET values were measured using a PHERAStar Plus instrument (BMG Labtech, Germany) with excitation at 340 nm followed by detection of emission at 615 nm (donor signal) and 665 nm ($_{\text{acceptor}}$ signal). Emission intensities were collected over 200 µs following a post-excitation delay of 100 µs. TR-FRET values were calculated as the ration of acceptor fluorescence intensity to donor fluorescence intensity and normalized as a percentage of control wells containing no inhibitor (100%) or 10 µM dasatinib (0%). Data were then fit to an equation for exponential association:

$$Y = Y_{max} \times \left[1 - e^{(-k_{\text{obs}} \times X)}\right]$$

where X is time and the curve starts at zero and ascends to Y_{max} with the observed rate constant k_{obs}. Off-rate data were reported as the observed dissociative half-life ($t_{1/2,obs}$) where $t_{1/2,obs} = 0.693/k_{obs}$.