Analysis of kinase inhibitors using a large array of signaling antibodies and high content screening.

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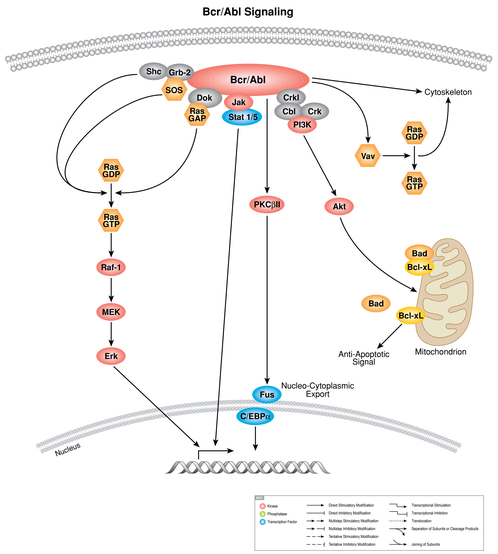
Abstract

Signaling antibodies can be used in high content screening assays to examine the efficacy of kinase inhibitors in a cellular environment. We have generated a large array of 96 signaling antibodies that can be used in cell-based immunofluorescence assays to examine signaling in many different pathways and to monitor biological processes such as proliferation, apoptosis, stress, inflammation, and cytotoxicity. In this study, we used the antibody array to compare the signaling profiles of two Bcr/Abl inhibitors, Gleevec® and Dasatinib. K562 (Bcr/Abl-positive) and SEM cells (Bcr/Abl-negative) were treated with these two inhibitors and then immunofluorescently labeled with the antibody array in 96-well plates. The plates were analyzed on a TTP LabTech Acumen eX3 high content screening platform to quantify the fluorescence intensity per cell of each antibody. The greatest drug-induced changes in Bcr/Abl-positive cells were with antibodies directed against phosphorylated targets known to be downstream of Bcr/Abl such as c-Cbl, CrkL, Gab1, and Stat. These targets were not significantly affected by either inhibitor in the Bcr/Abl-negative cells. Interestingly, there were subtle differences in the signaling profiles of the two inhibitors in the Bcr/Abl-positive cells. These results demonstrate that large arrays of signaling antibodies can be used with automated plate-based cellular assays to analyze the mutation-specific signaling profile of cells, to evaluate the efficacy of kinase inhibitors, or to examine their on- and off-target effects.



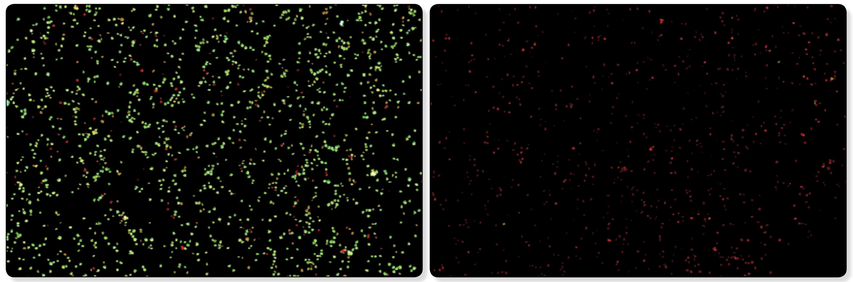
Introduction

Chronic myelogenous leukemia (CML) involves the translocation of genes for Bcr and Abl kinases (Philadelphia chromosome), resulting in the expression of the Bcr/Abl fusion protein. This abnormal protein is constitutively active and regulates proliferative and anti-apoptotic signaling pathways such as Ras/MAPK, Jak/Stat, and PI3K/Akt. Patients with CML are treated with the Bcr/Abl kinase inhibitor Gleevec® (imatinib mesylate, STI-571). Acquired resistance to Gleevec® associated with genetic amplification and point mutations in the kinase domain has led to the development of more potent Bcr/Abl inhibitors such as Dasatinib (Sprycel®, BMS-354825). Although both inhibitors target Bcr/Abl, their mechanisms of action are somewhat different. Gleevec® binds to the inactive conformation of Abl and also inhibits PDGFR and c-Kit. Dasatinib is an ATP-competitor that binds to the active and inactive conformations of Bcr/Abl and also inhibits Src family kinases, PDGFR, c-Kit, and EphA2. The goal of this study was to use a large antibody array on a high content screening platform to examine the signaling profile of Bcr/Abl-positive cells (K562) inhibited with either Gleevec® or Dasatinib. The array contains 96 different signaling antibodies and can be used to monitor most signaling pathways and diverse biological functions such as proliferation/cell cycle, apoptosis, inflammation, cytoxicity, senescence, metabolism, stress, and DNA damage.

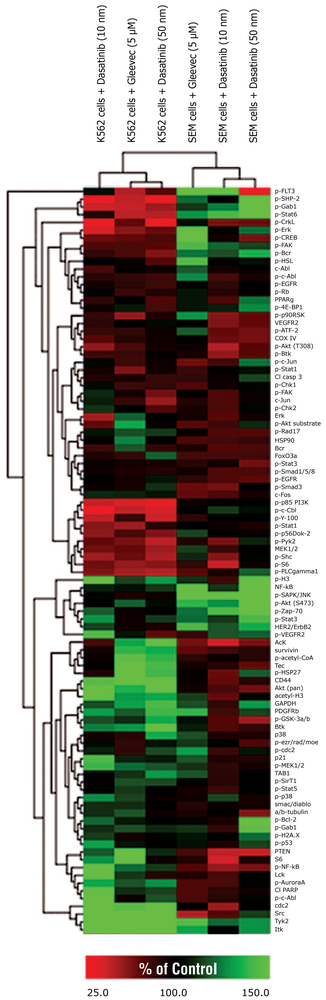
Materials and Methods

K562 (Bcr/Abl) and SEM (FLT3 amplification) cells were treated with vehicle (dimethyl sulfoxide) or the Bcr/Abl inhibitors Gleevec® (5μM) or Dasatinib (10nM or 50nM) for 3 hours at 37˚C. Cells were fixed with 4% formaldehyde for 15 minutes and permeabilized with -20˚C 100% methanol. Cells were then stained with a panel of 96 signaling antibodies from Cell Signaling Technology overnight at 4˚C. After primary antibody incubation, the cells were labeled with anti-rabbit or anti-mouse Alexa Fluor® 488-conjugated secondary antibodies for 1 hour at 22˚C. Nuclei were labeled with propidium iodide for at least 30 minutes at 22˚C. Imaging and fluorescence quantification were performed on a TTP Labtech Acumen eX3 high content screening platform. The mean fluorescence intensities per cell were normalized to 100% of the vehicle control. The heat map was generated and unsupervised clustering was performed using The Institute for Genomic Research MultiExperiment Viewer (TIGR MeV) software.

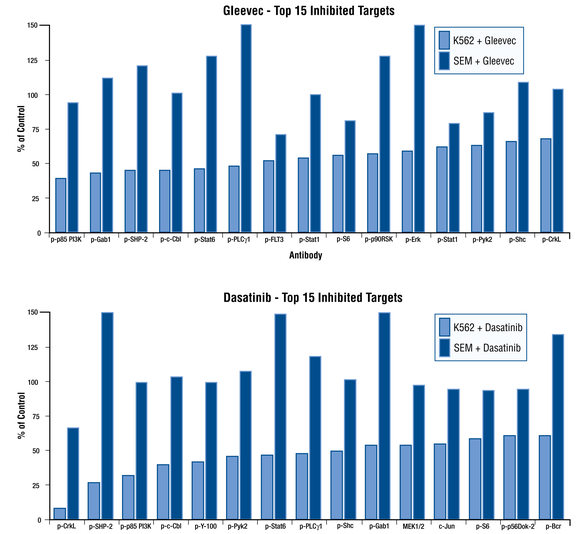
Results



Representative images of an inhibited target. K562 cells were treated with vehicle only (left) or 50nM Dasatinib (right) and labeled with phospho-CrkL antibody.



Unsupervised clustering of antibody array data. Responses were reported as either a percent decrease (red) or increase (green) in fluorescence intensity compared to vehicle. Both drugs inhibited Bcr/Abl-specific signaling pathways in K562 but not SEM cells



Graphs representing the inhibition of K562 versus SEM cells. Responses of the 96 endpoints were sorted according to % inhibition as compared to vehicle. The top 15 inhibited targets are represented in these graphs (Gleevec-top, Dasatinib-bottom). The magnitude of the response and the targets affected were slightly different between the two compounds.

Conclusions

- Both Gleevec® and Dasatinib similarly inhibited proliferation and anti-apoptotic markers, and other signaling proteins known to be downstream of Bcr/Abl in K562 cells but not in SEM cells (FLT3 amplification).
- There were subtle differences in the magnitude of inhibition of signaling proteins downstream of Bcr/Abl between the two drugs in K562 cells, likely due to the differences in their mechanism of action.
- Dasatinib inhibited some signaling proteins in SEM cells that were not affected by Gleevec®, indicating that Dasatinib may be inhibiting other kinases in addition to Bcr/Abl in these cells.
- The Acumen eX3 high content screening platform can effectively quantify fluorescence intensity from cells in the same plate labeled with antibodies that have very different fluorescence intensities.
- Large arrays of activation state-specific antibodies can be used to profile cellular signaling, to identify a disease-specific signaling fingerprint, or to examine on-and off-target effects of compounds in high throughput cell-based assays.