Published in final edited form as:

Pediatr Blood Cancer. 2009 December ; 53(6): 1132–1135. doi:10.1002/pbc.22083.

Flow Cytometric Determination of Src Phosphorylation in Pediatric Patients Treated with Dasatinib

Bella S. Guerrouahen^{1,2,*}, Eric Wieder, Ph. D.^{3,4,*}, Elisabeth G. Blanchard¹, Francis Y. Lee, Ph. D.⁵, Richard Aplenc, M.D.⁶, and Seth J. Corey, M.D.^{1,7,8}

¹ Division of Pediatrics, MD Anderson Cancer Center, Houston, TX

² Universite Paris-Sud 11, Paris, France

³ Department of Stem Cell Transplantation and Cellular Therapy, MD Anderson Cancer, Houston, TX

⁴ Division of Hematology/Oncology, Miller School of Medicine, University of Miami, Miami, FL

⁵ Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey

⁶ Division of Pediatric Oncology, Children's Hospital of Philadelphia, Philadelphia, PA

⁷ Department of Leukemia, MD Anderson Cancer Center, Houston, TX

⁸ Division of Hematology/Oncology and Transplantation, Department of Pediatrics and the Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, IL

Abstract

Tyrosine kinase inhibitors, such as imatinib, have dramatically improved the outcomes for patients with selected cancers. For imatinib, western blotting of phospho-CrkL was an insensitive, indirect, and descriptive method to determine drug efficacy. Greater use of targeted therapies should involve more quantitative evaluation of the target's dose-inhibition. The Src/Abl kinase inhibitor dasatinib has recently been approved for use in Ph+ leukemias after failure with imatinib. Src family kinases (SFK) also play a critical role in nonhematologic cancers. We have developed a flow cytometric assay to measure SFK autophosphorylation levels in blood mononuclear cells and observed a direct correlation between its inhibition and patient dosage. This method provides a sensitive, quick, and quantitative tool to assess drug efficacy.

Keywords

Phase I; tyrosine kinase inhibitor; Src kinases; dasatinib

Address correspondence to: Seth Corey, MD, MPH, Division of Hematology/Oncology and Transplantation, Children's Memorial Hospital, Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, 303 East Superior Street, Lurie 5-107, Chicago, IL 60611, s-corey@northwestern.edu, 312-503-6694 (office), 312-503-1181 (fax).

*BSG and EW contributed equally

BSG, EW, and EGB performed the experiments

BSG, EW, and SJC wrote the manuscript

FYL provided the drug

RA and SJC designed the phase I protocol

INTRODUCTION

With the pharmacologic inhibition of Bcr-Abl in chronic myeloid leukemia and epidermal growth factor receptor in epithelial cancers, targeted therapies have ushered in a new age of cancer therapeutics[1,2]. Two essential properties for molecularly targeted therapies are: relevance of the molecule to carcinogenesis and drug efficacy in inhibiting that molecule's function. Among the most attractive targets in cancer are tyrosine and serine/threonine kinases. Src family kinases, Akt, mTor, and vascular endothelial growth factor receptor serve as candidate targets in a wide range of hematologic and non-hematologic cancers.

Evaluation of phase I/II new agent trials requires demonstration of drug efficacy in inhibiting the target and tumor responsiveness[3]. Because of technical difficulties, few trials have however incorporated direct assessment of the target. Major limitations include the availability of primary or surrogate tissues, availability of sensitive and quantitative assays, and costs. Flow cytometry provides a sensitive and quantitative tool to measure biomarkers. Its use in immunophenotyping hematologic malignancies is now commonplace[4]. Use of phospho-specific antibodies can assess by flow cytometry *in vivo* effects of kinase inhibitors[5].

The Food and Drug Administration approved in 2006 dasatinib, a dual inhibitor of the Src and Abl tyrosine kinases for the treatment of Ph+ leukemias with imatinib resistance or intolerance to previous therapy[6]. Dasatinib is also being studied in solid tumors because of the role of Src family kinases in invasion and metastases[7,8]. To determine the efficacy of dasatinib in inhibiting Src tyrosine kinase activity, we used a commercially available phospho-specific antibody directed against Src Tyr416[9]. Conserved among the Src kinases, this residue constitutes the positive autophosphorylation site. We previously used this antibody on lysates from patients samples and found a single band at correct molecular size of phospho-SrcY416 (60 KDa). First, we demonstrated that dasatinib inhibited *ex vivo* in a dose-responsive manner phospho-Src Tyr416 in mononuclear cells. We then demonstrated that dasatinib inhibited *in vivo* in a dose-responsive manner phospho-Src Tyr416 among children and adolescents enrolled on a phase I Children's Oncology Group dasatinib study. Clinical monitoring of patients treated with Src kinase inhibitors can be performed by simple flow cytometric analysis of blood mononuclear cells, and its use can be broadened to other targets and new agents.

MATERIALS AND METHODS

Chemical reagents and antibodies. Bristol-Myers Squibb Pharmaceuticals (Princeton, NJ) provided dasatinib. A 10 mM stock solution was prepared by dissolving the compound in DMSO and stored at -20°C . Rabbit phospho-Src Tyr416 antibody (Cell Signaling, Beverly, MA) was used in an indirect staining process in combination with a FITC-conjugated secondary antibody (FITC-Goat anti-rabbit antibody). As a staining control, a mouse IgG was used in combination with a FITC-goat-anti-mouse secondary antibody (Santa Cruz Antibodies, Santa Cruz, CA). CD3 conjugated with PE (Becton Dickinson, Mountain View, CA) and CD14 FITC (Invitrogen, Carlsbad, CA) validated scatter gating of T lymphocyte and monocyte subpopulations.

Patients samples and mononuclear cells isolation. Samples were obtained from patients enrolled on the Children's Oncology Group phase I dasatinib study following institutional-approved informed consent and normal donors following informed consent at MD Anderson Cancer Center (Houston, TX). Patients received dasatinib twice daily for 28 days. One course of therapy will be 28 days. Patient samples were collected on day 1, 8 and 28 of dasatinib treatment. Received samples were < 5 ml of whole blood. Mononuclear cells from

were isolated from fresh whole blood samples. Briefly, the whole blood was transferred into a new 15 mL tube and 1 mL of citric acid-sodium acetate-dextrose (ACD) was added. 6 mL of PBS was added and the tube was gently mixed. The diluted blood was layered onto histopaque 1083 (Sigma) and centrifuged at 2000 rpm for 30 minutes. The mononuclear cell layer was recovered and spun at 1300 rpm for 10 minutes. To lyse red blood cells, 2 mL of 0.3% NaCl was added for 30 seconds followed by addition of 10 mL of PBS. Patient samples were frozen in 90% FBS/10% DMSO. Mononuclear cells from healthy donors were isolated and treated 1 hour at 37°C by DMSO or dasatinib diluted in RPMI/20% FBS then frozen in 90% FBS/10% DMSO.

Phospho-epitope staining for flow cytometry. Samples were thawed in 37°C water bath, transferred to 15 mL tube with RPMI plus 10% FBS and spun at 1200 rpm for 5 minutes. Resuspended in RPMI plus 10% FBS, the cells were fixed with 100 μ L of 16% formaldehyde room temperature (RT). The samples were then spin at 500 g for 5 minutes. Resuspending with vigorous vortexing in 1 mL of ice-cold methanol permeabilized cells. Samples were kept at -20°C overnight. Cells were then washed in staining buffer and resuspended in 300 μ L of staining buffer. 100 μ L were aliquoted for the following studies: unstained, stained with phospho-Src Tyr416, or staining control. Samples were washed and resuspended in 50 μ L PBS plus BSA 1% for the incubation with the secondary goat anti-rabbit FITC antibody. After a 20 minute incubation in dark, the samples were spun at 500 g for 5 minutes and fixed in 1% paraformaldehyde. An aliquot of cells from a healthy donor was not subjected to permeabilization, and the cells were stained simultaneously with CD3-PE and CD14-FITC.

Flow cytometry analysis. Samples were analyzed with a FACSCalibur (Becton Dickinson, San Jose, CA) in channel FL1 for FITC or FL2 for PE. Histograms of cell numbers vs. logarithmic integrated fluorescence intensity were recorded and analysed by Flow Jo Software (Ashland, OR). Data were plotted in Prism software (GraphPad Software, LaJolla, CA). To address staining variability from day to day and between samples, a ratio was calculated between the geometric mean fluorescence of the specific phospho-Src staining and the control staining, and we then compared the difference in this ratio at day 8, or 28 versus day 1 (patients samples) or at different doses of dasatinib in a normal donor.

RESULTS

To evaluate the technique for its ability to efficiently and reproducibly measure fluctuations in phosphorylation of Src Tyr416 inside the cells, an experiment was conducted with PBMC's from a healthy donor. Figure 1A shows the result of flow cytometric analysis with treated mononuclear cells by dasatinib (100 ng/mL) or its control (DMSO). By gating on scatter properties and costaining with CD3 or CD14 antibody, we identified and gated the monocyte population and then displayed the data on a histogram. We observed clear separation between the dasatinib-treated sample versus the DMSO control sample. To confirm specificity and sensitivity of the assay, we determined a dose-inhibition in phospho-Src Tyr416 in normal monocytes. To reproduce the processing of the clinical specimens, the samples were frozen for batch analysis. After thawing, samples were stained and analyzed for phospho-Src Tyr416 content. Figure 1B showed that with increasing doses of dasatinib, phospho-Src Tyr416 decreases, corresponding to the dasatinib IC₅₀ of ~ 1 nM for SFK.

To test whether detection of patient treatment could be evaluated through this technique, samples were obtained from patients enrolled on the Children's Oncology Group dasatinib phase I trial. Mononuclear cells from patients enrolled in a time course of dasatinib treatment were isolated and then frozen. Three patients had samples from days 1 and 8, one patient had samples from days 1 and 28, and four patients had samples from days 1, 8, and 28. The samples were then stained for phospho-Src Tyr416 or staining control. There was

insufficient number of cells from patient samples to make lysates for validation by western blot. Others and we have used this antibody to demonstrate SFK activation [10]. Figure 2A shows the staining distribution for phospho-Src Tyr416 and staining control in a representative patient pre-chemotherapy. We observed a dasatinib dose-dependent decrease in phospho-Src Tyr416 in gated monocytes (Figure 2B). The dose response is illustrated by the difference in geometric means at day 8 or 28 compared to the day before chemotherapy. Six of seven patients showed a reduction in phospho-Src Tyr416 at day 8. The three samples from patients treated at the highest dose of dasatinib (110 mg/m²/day) demonstrated the greatest reduction in phospho-Src levels.

DISCUSSION

We developed a flow cytometric assay to measure the activated form of Src family kinases, phospho-SrcTyr416, in blood mononuclear cells with commercially available antibody. We demonstrated its ability to detect decreased levels of phospho-Src Tyr416 in blood mononuclear cells from patients who received the highest dose of the Src/Abl kinase inhibitor dasatinib in a COG phase I trial. This novel assay should prove useful in directly monitoring drug efficacy in future clinical trials. The strengths of this assay are widespread availability of flow cytometers, low-cost reagents (~\$13.00/sample), and small blood volume.

Acknowledgments

This work was supported by grants RO1-CA108922, Leukemia SPORE P50, and JP McCarthy Development Fund, to S.J.C. The authors thank Drs. Yvon Cayre and Sean Hartig and the technicians at flow cytometry facility in the stem cell transplantation and cellular therapy department at MD Anderson Cancer Center for their advice.

References

1. Baselga J. Targeting tyrosine kinases in cancer: the second wave. *Science*. 2006; 312(5777):1175–1178. [PubMed: 16728632]
2. Krause DS, Van Etten RA. Tyrosine kinases as targets for cancer therapy. *N Engl J Med*. 2005; 353(2):172–187. [PubMed: 16014887]
3. Sausville EA, Elsayed Y, Monga M, et al. Signal transduction--directed cancer treatments. *Annu Rev Pharmacol Toxicol*. 2003; 43:199–231. [PubMed: 12195027]
4. Craig FE, Foon KA. Flow cytometric immunophenotyping for hematologic neoplasms. *Blood*. 2008; 111(8):3941–3967. [PubMed: 18198345]
5. Irish JM, Kotecha N, Nolan GP. Mapping normal and cancer cell signalling networks: towards single-cell proteomics. *Nat Rev Cancer*. 2006; 6(2):146–155. [PubMed: 16491074]
6. Kantarjian H, Jabbour E, Grimley J, et al. Dasatinib. *Nat Rev Drug Discov*. 2006; 5(9):717–718. [PubMed: 17001803]
7. Le Tourneau C, Faivre S, Raymond E. New developments in multitargeted therapy for patients with solid tumours. *Cancer Treat Rev*. 2008; 34(1):37–48. [PubMed: 17983706]
8. Kolb EA, Gorlick R, Houghton PJ, et al. Initial testing of dasatinib by the pediatric preclinical testing program. *Pediatr Blood Cancer*. 2008; 50(6):1198–1206. [PubMed: 17914733]
9. Luo FR, Barrett YC, Yang Z, et al. Identification and validation of phospho-SRC, a novel and potential pharmacodynamic biomarker for dasatinib (SPRYCELtrade mark), a multi-targeted kinase inhibitor. *Cancer Chemother Pharmacol*. 2008
10. Dos Santos C, Demur C, Bardet V, et al. A critical role for Lyn in Acute Myeloid Leukemia. *Blood*. 2007; 111:2269–2279. [PubMed: 18056483]

Figure 1.

Effect of dasatinib evaluated in mononuclear cells from healthy donors. A) Mononuclear cells freshly isolated were treated ex vivo for 1 hour at 37°C with dasatinib (100 ng/ml) or DMSO diluted in RPMI/20% FBS, frozen and stained for phospho-Src Tyr416, CD3 and CD14. B) Dasatinib dose-inhibition of phosphorylation of Src Tyr 416 was performed with 1, 5, 10, 50, 100 ng/ml or DMSO. Samples were then analyzed by flow cytometry. Percentage of equivalent monocytes between the different conditions was gated, and geometric mean was calculated using FlowJo software. The ratio of the geometric mean (phospho-Src Tyr416 vs. staining control) was calculated to provide a phospho-Src index and the data were plotted in Prism software (1B).

Figure 2.

Inhibition of Src Tyr416 phosphorylation in primary mononuclear cells exposed to dasatinib at day 8 and/or day 28. A) An example of phospho-Src Tyr416 staining pre-chemotherapy in a patient sample. Mononuclear cells isolated from COG patients were stained for phospho-Src Tyr416 or with a staining control. B) Phospho-protein flow analysis was performed in 20 samples from 8 patients. Geometric means were calculated by FlowJo, and the difference of the geometric mean noted as the phospho-Src index (phospho-SrcY416 vs. staining control) was calculated for day 8, 28 or before treatment. The difference between day 8 or 28 to day 1 was plotted in Prism (n=12). Each circle represents a change in the phospho-Src-Index from the pre-treatment phospho-Src-Index value for a single patient sample.