

Dasatinib targets chronic myeloid leukemia-CD34⁺ progenitors as effectively as it targets mature cells

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Online Supplementary Design and Methods

Cell lines

K562 (*BCR-ABL1*⁺) cells were obtained from the American Type Culture Collection (ATCC, Manassas, USA). K562-Dox (ABCB1 over-expressing cells), VBL100 (ABCB1 over-expressing cells) and CCRF-CEM (parental cells for VBL100) were obtained from Professor Leonie Ashman (University of Newcastle, Newcastle, Australia). An ABCG2-overexpressing cell line was generated by stably transfecting an ABCG2 plasmid in K562 cells (K562-ABCG2).¹

Patient samples

Blood was collected from newly diagnosed CML-CP patients. All samples were collected after obtaining informed consent; importantly all samples were collected before starting IM therapy. Mononuclear cells (MNCs) were isolated from blood using Lymphoprep (Axis-Shield PoC As, Oslo, Norway) density gradient centrifugation. CD34⁺ progenitor cells were isolated from the MNC by using Miltenyi AutoMACS Pro Separator and CD34 Progenitor Cell Isolation Kit human (MACS; Miltenyi Biotech, Germany). CD34⁺ cell yield varied from patient to patient, depending on their white blood cell count at presentation and percentage of CD34⁺ cells in blood. In patients who had leukopenia, we could isolate up to 2 × 10⁵ CD34⁺ cells, while in other newly diagnosed patients we could isolate between 2 × 10⁶ to 7 × 10⁷ CD34⁺ cells (*Online Supplementary Figure S1A*). CD34⁺ cell purity was checked by staining cells with anti-CD34⁺ PE (BD Biosciences, San Jose, CA, USA). In the majority of patients, CD34⁺ purity ranged from 81% to 97% (*Online Supplementary Figure S1B*; representative CD34⁺ purity).

IUR assays were performed on thawed cryopreserved cells. Cryopreservation was achieved by placing cells in media containing 10% dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany), followed by controlled rate freezing and storage in liquid nitrogen vapor.

TKI and other inhibitors

Dasatinib and ¹⁴C-dasatinib (¹⁴C-Das) were kindly provided by Bristol-Myers Squibb (New Brunswick, USA). ¹⁴C-Das was dissolved in ethanol at 1 mg/mL. Non-radiolabeled dasatinib was prepared at a concentration of 10 mM in DMSO. Nilotinib, IM, and ¹⁴C-imatinib (¹⁴C-IM) were kindly provided by Novartis Pharmaceuticals (Basel, Switzerland). Stock solutions of IM and ¹⁴C-imatinib were prepared at 10 mM and 1 mg/mL in distilled

water, respectively. The ABCB1 inhibitor PSC833 (provided by Novartis Pharmaceuticals, Basel, Switzerland), and the Fumitremorgin C analog Ko143 (ABCG2 inhibitor, supplied by Dr John Allen, Centenary Institute of Cancer Medicine and Cell Biology, NSW, Australia) were used at 10 μM and 0.5 μM, respectively. Verapamil was purchased from Abbott (Australasia).

Intracellular drug uptake and retention assay (IUR)

The ¹⁴C-Das intracellular drug uptake and retention assay (IUR) was based on our previously published methodology.¹ In brief, 2 × 10⁵ cells were incubated at 37°C with ¹⁴C-Das (100 nM, 1 μM and 2 μM) or ¹⁴C-IM (0 to 2 μM). Isotopes were resuspended to 1 mg/mL and specific activities were 3.3 MBq/mg for ¹⁴C-IM and 1.18 MBq/mg for ¹⁴C-Das. After incubation, the cellular and aqueous phases were separated, and incorporation determined using a Top Count Microplate Beta Scintillation counter (Perkin Elmer, Boston, USA) following the addition of Microscint 20 (Perkin Elmer) scintillation fluid. IUR assays were performed in triplicate and repeated if the assay results were not concordant.

Western blot analysis

Western blot analysis for phosphorylated Crkl (p-Crkl) was performed as previously described.¹ Briefly, 2 × 10⁶ patient MNC and CD34⁺ cells of newly diagnosed CML-CP patients were incubated with dasatinib (0, 2, 5, 10 and 20 nM) with or without ABCB1 (PSC-833) or ABCG2 (Ko143) inhibitors for 2 h. Cells were then washed with cold phosphate buffered saline (PBS) and lysed in 20 μL of Laemmli's buffer by boiling for 12 min. Protein lysates were resolved on an SDS/10% (w/v) polyacrylamide gel, then electrophoretically transferred to a PVDF membrane (GE Healthcare, Buckinghamshire, UK). Following blocking, the membrane was probed with anti-Crkl antibody (Santa Cruz, CA, USA), detected with ECF substrate (GE Healthcare) and analyzed by Fluor Imager analysis (Molecular Dynamics, Sunnyvale, USA). Signals were quantified using Image Quant software (Molecular Dynamics) and the ratio of p-Crkl to total Crkl was determined.

Flow cytometry analysis

ABCB1 and ABCG2 expression was assessed by using flow cytometry analysis of K562, K562-Dox, K562-ABCG2 cells and CML-CD34⁺ cells. Cells were stained with phycoerythrin conjugated (PE) anti-ABCB1 antibody (Immunotech, France) and

anti-ABCG2 (R & D Systems, Minneapolis, USA). Data were acquired on an FC500 (Beckman Coulter, USA) flow cytometer and were analyzed using CXP (Beckman Coulter, USA) and FCS Express V3 (De Novo Software, USA) analysis software.

ABCB1 and ABCG2 efflux pump activity

Cellular efflux activity was assessed using Rhodamine-123^{2,3} and Pheophorbide-A,⁴ fluorescent substrates of ABCB1 and ABCG2, respectively. Rhodamine-123 (Invitrogen, USA) is a fluorescent substrate which diffuses into the cell and is effluxed by ABCB1.^{2,3} Hence, cells with low or no ABCB1 expression will have high intracellular fluorescence. On the other hand, in cells with high ABCB1 expression the Rhodamine-123 fluorescent is low. Thus, this assay assesses ABCB1 activity and can be complementary to ABCB1 expression measured by flow cytometry. Previous studies have demonstrated good correlation between ABCB1 expression^{2,3} and intracellular Rhodamine-123 fluorescence. K562, K562-Dox cells and CD34⁺ cells were incubated with Rhodamine-123 (0.5 µg/mL) with or without PSC833 (10 µM, ABCB1 inhibitor), nilotinib (1 to 10 µM), or dasatinib (10 nM to 1000 nM) for 30 min at 37°C. After washing with cold

PBS, cells were incubated for 1 h with PSC-833, nilotinib or dasatinib, but without Rhodamine-123. Cells were washed with cold PBS and were analyzed by flow cytometry and Rhodamine-123 mean fluorescent intensity (MFI) was assessed.

Similarly, Pheophorbide-A (kindly provided by Dr. Robey, NIH, Bethesda, USA) is a fluorescent substrate effluxed by the ABCG2 efflux pump and, therefore, has been used to assess ABCG2 efflux activity.⁴ The ABCG2 activity assay was performed analogous to the Rhodamine-123 assay described above using 10 µM Pheophorbide-A and Ko143 as a specific inhibitor for ABCG2.

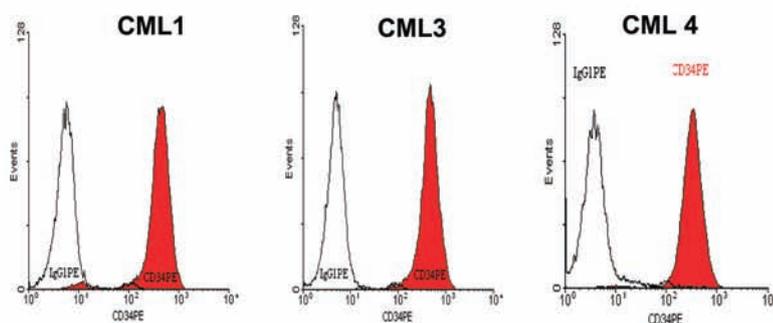
Statistical analysis

Statistical significance between two groups was assessed by Mann-Whitney test using GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego California USA; www.graphpad.com).

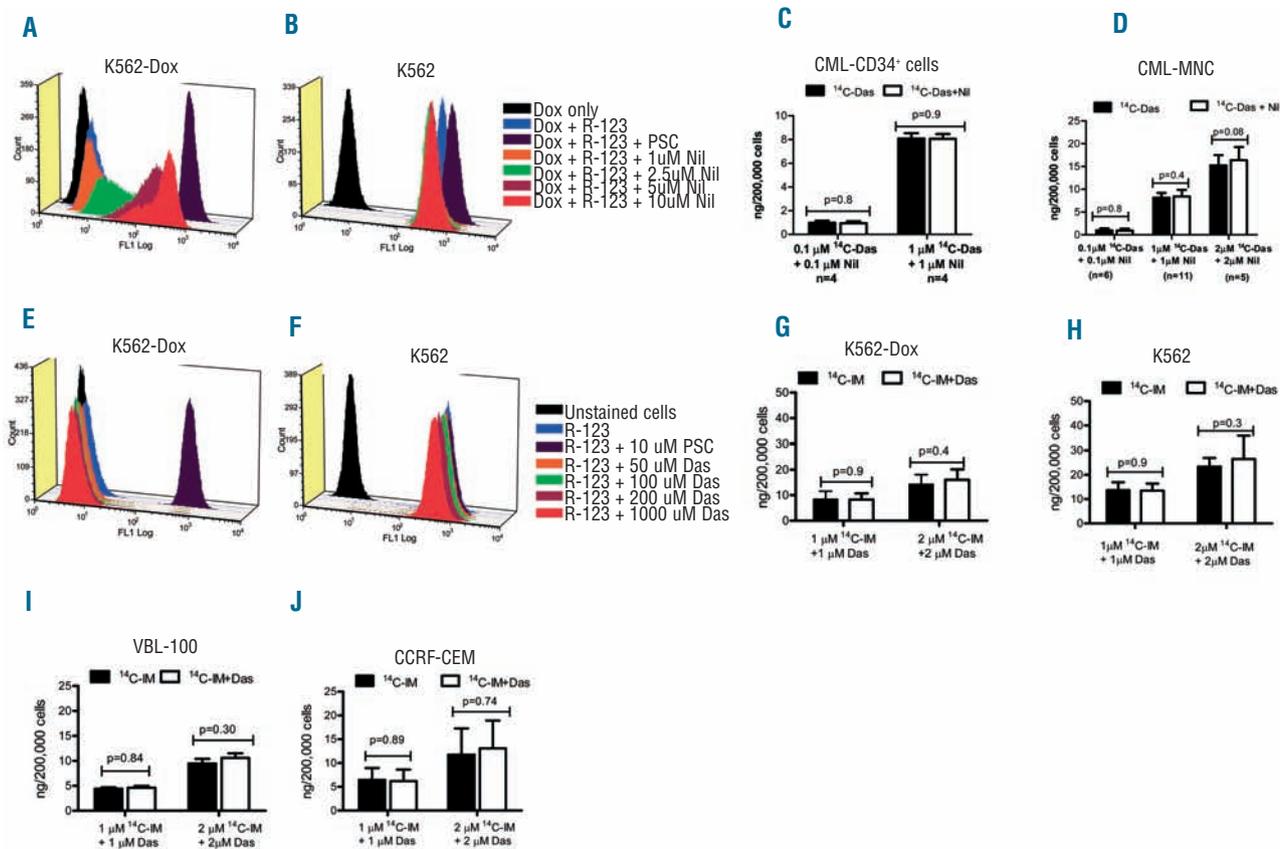
A

Patient	Total CD34 ⁺ cells isolated
CML1	2.3x10 ⁷
CML2	6.5x10 ⁷
CML3	3.3x10 ⁷
CML4	2.1x10 ⁷
CML5	11.8x10 ⁷
CML6	7.6x10 ⁷
CML7	1.2x10 ⁷
CML8	19.5x10 ⁷
CML9	6.3x10 ⁷

B



Online Supplementary Figure S1. Number of CD34⁺ cells isolated at diagnosis and CD34⁺ cell purity. (A) Number of CD34⁺ cells isolated by magnetic assisted cell sorting (MACS) at diagnosis in individual patients. (B) As shown the purity of CD34⁺ cells at the time of experiments were 81-97% in most of patients (representative plot from 3 patients).



Online Supplementary Figure S2. Nilotinib (Nil) does not increase dasatinib (das) intracellular concentration in CML CD34⁺ cells. In contrast to nilotinib, dasatinib is not an ABCB1 inhibitor. (A) In K562-Dox cells Rhodamine-123 (R-123) fluorescence was significantly lower than K562 cells. In K562-Dox cells nilotinib inhibited the ABCB1 mediated Rhodamine-123 efflux and increased Rhodamine-123 MFI (PSC833 is a positive control). (B) In K562 cells, Rhodamine-123 is not effluxed; neither nilotinib nor PSC 833 increase Rhodamine-123 MFI. (C-D) Nilotinib did not increase ¹⁴C-dasatinib (¹⁴C-Das) IUR in CML CD34⁺ cells and MNC. (E) In contrast to nilotinib, dasatinib (10 to 1000 nM) did not change Rhodamine-123 MFI (PSC-833 is a positive control for the assay) in K562 Dox cells. (F) In K562 cells, Rhodamine-123 is not effluxed actively and neither PSC 833 nor dasatinib change the Rhodamine-123 MFI. (G-J) Dasatinib did not change ¹⁴C-Imatinib (¹⁴C-IM) IUR in K562-Dox and VBL100 (ABCB1 over-expressing cells) and their parental cells K562 and CCRF-CEM, respectively.

References

- Hiwase DK, Saunders V, Hewett D, Frede A, Zrim S, Dang P, et al. Dasatinib cellular uptake and efflux in chronic myeloid leukemia cells: therapeutic implications. *Clin Cancer Res*. 2008;14(12):3881-8.
- ten Hoeve J, Arlinghaus RB, Guo JO, Heisterkamp N, Groffen J. Tyrosine phosphorylation of CRKL in Philadelphia+ leukemia. *Blood*. 1994;84(6):1731-6.
- Oda T, Heaney C, Hagopian JR, Okuda K, Griffin JD, Druker BJ. Crkl is the major tyrosine-phosphorylated protein in neutrophils from patients with chronic myelogenous leukemia. *J Biol Chem*. 1994; 269(37):22925-8.
- Hatzieremia S, Jordanides NE, Holyoake TL, Mountford JC, Jorgensen HG. Inhibition of MDR1 does not sensitize primitive chronic myeloid leukemia CD34+ cells to imatinib. *Exp Hematol*. 2009;37(6):692-700.