SUPPLEMENT

TITLE: Tyrosine kinase inhibitor response of ABL-class acute lymphoblastic leukemia: The role of kinase type and SH3 domain

RUNNING HEAD: TKI sensitivity of ABL-class ALL

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MATERIAL & METHODS

Cell culture

Ex vivo experiments using primary cryopreserved or freshly harvested PDX-origin ALL cells were conducted using primary culturing medium composed of RPMI 1640 medium (Dutch modification) (ThermoFisher Scientific, #22409015). The medium was supplemented with 20% fetal calf serum (FCS), 4.2 µg/mL insulin, 3.8 µg/mL transferrin, and 5 ng/mL sodium selenite (Merck, #I1884), 0.4 mM glutamine (ThermoFisher Scientific, #25030-024), 0.25 µg/mL gentamicin (ThermoFisher Scientific, #15710-049), 100 µg/mL penicillin/streptomycin (ThermoFisher Scientific, #15140-122), and 0.125 µg/mL fungizone (ThermoFisher Scientific, #15290-026).

Ba/F3 cells (0.3x10^6 cells/mL, DSMZ, #ACC300) were routinely cultured in cell line medium containing RPMI 1640 medium with Glutamax (ThermoFisher) supplemented with 10% FCS (Bodinco), 100 µg/ml streptomycin (ThermoFisher Scientific), 0.125 µg/ml fungizone (ThermoFisher Scientific), 100 IU/ml penicillin (ThermoFisher Scientific), and Mouse IL-3 (Miltenyi Biotec, #130-096-688). The cell line was tested for *Mycoplasma* every six weeks and remained negative throughout experimental studies.

Patient-derived xenograft (PDX) model cells

Primary xenografts samples were obtained from ABL-class ALL PDX models established by intrafemoral injection of thawed primary leukemia cells into female NGS mice aged 7 to 12 weeks according to the guidelines and approval of the Central Committee for Animal Experiments of the Dutch Cancer Institute. For each primary sample, three mice were injected with 1 million viable cells per mice. The leukemic burden of PDX mice was assessed every 2-4 weeks by determining the percentage of human ALL cells in tail bleeding samples after red blood cell lysis. Lysed blood cells were stained with anti-human CD45-PE (Biolegend, #304039), anti-human CD19-BV421 (Biolegend, #302234), and/or anti-human CD5-BV421 (Biolegend, #300626 for T-ALL) antibodies, along with anti-mouse CD45-FITC (Biolegend, #103108). The percentage of human ALL cells was determined relative to the total number of stained cells. A leukemic burden ≥1% was considered positive for engraftment. Upon overt leukemia (≥30% blasts) detection, the mice were sacrificed and blasts were harvested from the bone marrow and spleen. The blast percentage was confirmed to be ≥80% using cytospins stained with May-Grünwald-Giemsa and flow cytometry with the aforementioned antibodies. Establishment of serial transplanted PDX models was previously described and approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia as described.[1–3] Serial transplanted PDX ALL cells were also subjected to DNA-based next-generation sequencing at the Children's Hospital of Philadelphia Division of Genomic Diagnostics clinical laboratory as described.[4,5]

Ex vivo experiments were conducted using the same primary culture medium as described for primary ALL cells.

Generation of ABL-class constructs

Plasmid DNA in a retroviral backbone, MSCV-IRES-GFP, on filter paper was a kind gift of CG Mullighan (*ZMIZ1::ABL1*, *RCSD1::ABL1*, *EBF1::PDGFRB*, *RCSD1::ABL2*, *SSBP2::CSF1R*).[6,7] For *ZMIZ1::ABL1*, *RCSD1::ABL1*, *EBF1-PDGFRB*, *SSBP2::CSF1R*, and *RCSD1::ABL2* fusion constructs, standard cloning was performed by addition of *PmeI* and *MluI* restriction sites flanking the fusions gene using PCR, followed by purification, digestion, and ligation (New England Biolabs, M0202L) into the lentiviral SFFV-PGK-puro backbone. For the *RCSD1::ABL1*wSH3_1 and *RCSD1*::*ABL1*wSH3_2, and *BCR::ABL1* constructs, HiFi assembly was used by primers with overlapping 5' and 3' regions to enable assembly of multiple

fragments into one construct. Subsequent assembly was performed according to manufacturers' conditions (New England BioLabs, #5520S). All PCR reactions were performed using SuperFi II DNA polymerase (ThermoFisher, #12361010). Stbl3 bacteria (ThermoFisher, C737303) were used for transduction, except for *BCR::ABL1* for which NEB® 5-alpha F'I^q Competent *E. coli* (NEB, #C2992H) was used. Before lentiviral transduction, constructs were validated using Sanger sequencing (Macrogen Europe).

Virus production and transduction of Ba/F3 cell lines

Human embryonic kidney 293T (HEK293T) cells were cultured in DMEM, high glucose, Glutamax medium (Gibco) supplemented with 2% PSF (Gibco, #31966-047) and 10% FCS (Bodinco). Transfection of HEK293T cells was carried out when they reached 80% confluence, following manufacturer instructions. Specifically, the transfection process involved using 1.6 µg pVSV-G (Addgene, Cambridge, Massachusetts, USA, plasmid #12259), 3.7 µg pPAX2 (Addgene plasmid 12260), and 10.7 µg of plasmid DNA, along with X-tremeGENE HP DNA Transfection Reagent (Sigma Aldrich, #6366244001). After 24 hours, fresh medium was added to the cells. The virus supernatant was collected on the second and third day after transfection, and cell debris was removed by filtration through a 0.45µm filter. The virus particles were then obtained by centrifugation at 32,000rpm for 2 hours at 4°C using the Optima XE-90 ultracentrifuge (Beckman Coulter). Subsequently, the virus was aliquoted and stored at -80°C until further use. To determine the optimal virus concentration, virus titration was performed on Ba/F3 cells. Ba/F3 cells were transduced and subsequently selected by puromycin for 3 weeks, followed by murine IL-3 (mIL3) selection until stable cell growth was observed. Presence and functioning of the fusion genes were confirmed by PCR. After selection, cells were cultured in medium with mIL3 until use in downstream experiments.

Co-culture assays

To determine the survival of leukemic cells after TKI exposure, leukemic cells of ABL-class ALL patients were seeded at a density of 0.9-1.1×10^6 cells/well in co-culture with primary pediatric mesenchymal stromal cells (MSCs, 2.5×10^4 cells) in a 48-well plate at 37°C with 5% CO2. This was followed by a three-day exposure of four concentrations of imatinib (1.95-125 µM, Sigma, #SML1027), dasatinib (1.95-125 nM, Abcam, #ab142050), or bosutinib (0.016-2 µM, Sigma, #PZ0192). We selected the drug concentrations to create a dose-response curve representing our cultures' *ex vivo* response. Encompassing a range from levels below therapeutic efficacy to those potentially saturating ensures a thorough understanding of the drug's impact. In cases where there were limited cell numbers, priority for exposure was given to imatinib, followed by dasatinib, and then bosutinib. After three days, leukemic cells and MSCs were harvested, stained with Brilliant Violet 421 anti-human CD19 antibody (Biolegend, #302234), Alexa Fluor 750 CD73, CD146, and CD166 antibodies (R&D Systems, #FAB5795S, #FAB932S, #FAB6561S), Sytox Red/Green dye (ThermoFisher Scientific, #S34859/#S34860) and subsequently cell viability was measured using the CytoFLEX flow cytometer (Beckman Coulter). Within the MSC-negative fraction, the percentage of Sytox-negative and CD19-positive cells was determined using Kaluza software (version 2.1), and graphs were generated using GraphPad Prism software (version 9.5.1). Data were included in this study if at least 5% of the initially seeded ALL cells of the untreated samples survived. Cell survival relative was calculated relative to the untreated control.

MTT assays

To determine the metabolic activity after TKI exposure, cells were exposed to six or seven concentrations of imatinib (0.008-7.8 µM), dasatinib (0.122-125 nM), or bosutinib (0.08-5 µM) in a 96-well plate at 37°C with 5% CO2 in duplicate for three days at a cell density of 0.3 × 10^6 cells/mL for (transduced) Ba/F3 cells. We adjusted the drug concentration ranges for Ba/F3 cells compared with the

primary material to create a dose-response curve representing our cultures' *in vitro* response. Unexposed Ba/F3 cells were included as control samples. After three days, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL in PBS) was added to the cells, the cells were incubated at 37°C with 5% CO2 for 2-6 hours and subsequently the dissolved with acidified isopropanol (0.04 N HCl). A spectrophotometer (Molecular Devices) at wavelengths of 562 nm and 720 nm was used to measure the absorbance using Softmax Pro 7.0.3 software. Data was included in this study if the optical density of the controls was ≥50, the variation between the control wells was ≤20%, and the variation between duplicates was ≤20%. The metabolic activity of the treated samples was calculated relative to the metabolic activity of the untreated samples.

Calculation of area under the dose-response curve

The half maximal inhibitory concentration (IC_{50}) did not accurately capture the *ex vivo* sensitivity of the primary material. This was evident in cases where samples exhibited a response to treatment, yet their survival exceeded 50%, leading to an IC50 value that erroneously implied a complete lack of response. Therefore, we used the Z-score of the area under curve (AUC) derived from the dose-response curve of co-culture assays is used as a measure for TKI sensitivity. The average survival between each pair of consecutive concentrations was summed and multiplied by the log10 difference between two concentrations. Subsequently, the Z-score was calculated based on the *BCR*::*ABL1* reference cohort and the ABL-class samples together. Measured AUC values that exceeded the maximum value indicative for no response were adjusted to the maximum value, which was 150 arbitrary units (AU) for imatinib, 180 AU for dasatinib, and 210 AU for bosutinib. Subsequently, the mean AUC of the total cohort was subtracted from the AUC of sample of interest and divided by the standard deviation of the AUC of the total cohort to obtain the sample's Z-score.

Calculation of half-maximal inhibitory concentration

In this study, the half maximal inhibitory concentration (IC_{50}) derived from the dose-response curve of the MTT assays is used as a measure for TKI sensitivity. The IC_{50} value per sample was calculated by the following formula: $\frac{(X\%-50)}{X\%-Y\%} * (Ycon - Ycon) + Ycon$ with X_{conc} being the lowest concentration with more than 50% survival, $X_{\%}$ being the percentage survival at that concentration, Y_{conc} being the lowest concentration with less than 50% survival, and $Y_{\%}$ the percentage survival at that concentration.

Comparison of tyrosine kinase sensitivity

Sensitivity to imatinib, dasatinib, and bosutinib of the patients' samples was compared with the sensitivity of *BCR*::*ABL1*-positive samples by performing a Kruskal-Wallis test with a Dunn's *post hoc* test for multiple comparisons. The correlation between the Z-score of the AUC of imatinib, dasatinib, and bosutinib for the *BCR*::*ABL1* reference samples, *ABL1*-fused ALL samples, and *PDGFRB*-fused ALL samples was calculated using the Spearman correlation coefficient.

Whole exome sequencing

DNA extraction was done using the Qiagen DNeasy Blood & Tissue kit (Qiagen, cat#69504) or TRIzol reagent. Concentrations of extracted DNA were measured using the DeNovix instrument (Wilmington) and the Qubit dsDNA broad range kit from ThermoFisher Scientific. Subsequent steps, including library preparation and DNA sequencing, were performed by Novogene (Hong Kong, China). The Agilent SureSelect Human All ExonV6 kit (Agilent Technologies) was used for library preparation, and sequencing was conducted on an Illumina NovaSeq6000 platform using paired-end sequencing with 150 bp fragment reads, aiming for a read depth of 150X. For alignment to the reference genome (GENCODE v29 GRCh38), Minimap2 (version 2.12) was used. Variant calling was done using Mutect

(version 2.2). Somatic variants were identified by filtering out variants found in the germline control. Variants present in the general population at a frequency of over 1%, as indicated by dbSNP release 155, were excluded. Further filtering excluded variants with fewer than 10 variant reads, non-synonymous variants, and variants situated in non-coding regions. The analysis specifically focused on major variants (with a variant allele frequency ≥25%) in leukemia-associated genes described by Brady et al..[8] Additionally, only variants annotated as (likely) pathogenic according to the ClinVar database were considered.

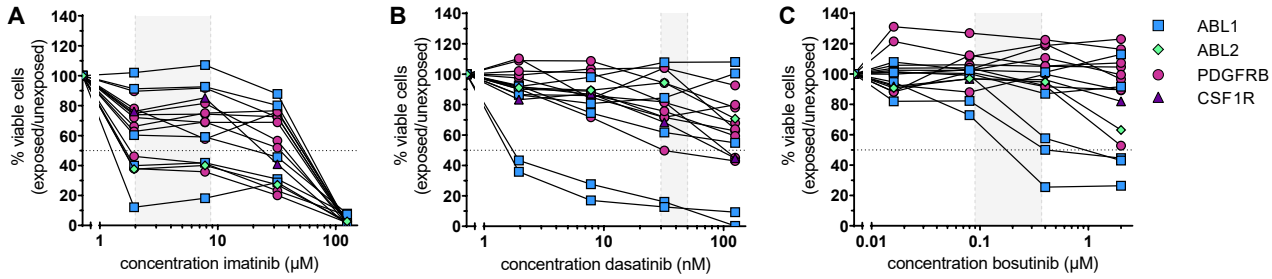
SUPPLEMENTAL TABLES

Supplemental Table 1: Patient characteristics

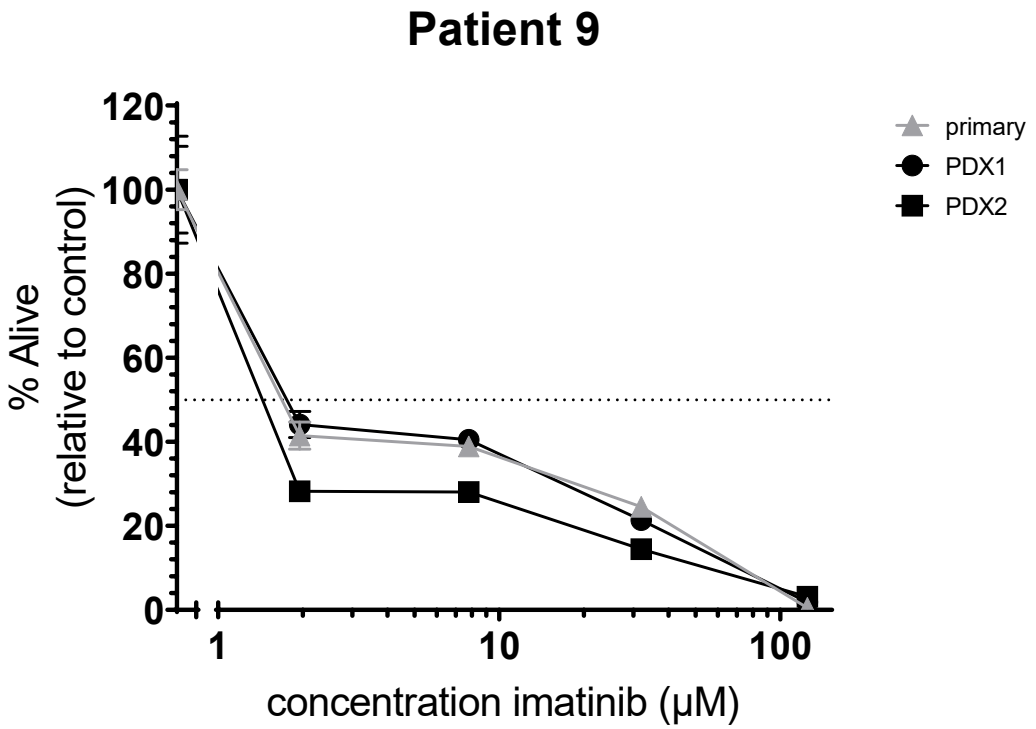
Patient nr	Sample type	Sample origin	Involved Lineage	Fusion	Sex	Age
P1	diagnosis	Primary xenograft	B	*RCSD1*::*ABL2*	M	4
P2	diagnosis[i]	Primary xenograft	B	*NUP214*::*ABL1*	F	16
P3	diagnosis	Primary	T	*ZMIZ1*::*ABL1*	F	6
P4	diagnosis	Primary	B	*RCSD1*::*ABL1*	M	11
P5	diagnosis	Primary xenograft	B	*ZMIZ1*::*ABL1*	M	1
P6	diagnosis	Primary	T	*NUP214*::*ABL1*	M	11
P7	diagnosis	Serial transplant	B	*NUP214*::*ABL1*	M	14
P8	diagnosis	Serial transplant	B	*ETV6*::*ABL1*	M	5
P9	relapse	Primary; primary xenograft	T	*JAKMIP2*::*PDGFRB*	F	3
P10	diagnosis	Primary	B	*EBF1*::*PDGFRB*	M	8
P11	relapse	Primary xenograft	B	*EBF1*::*PDGFRB*	M	19
P12	diagnosis	Primary xenograft	B	*EBF1*::*PDGFRB*	M	14
P13	relapse	Primary xenograft	B	*CCDC88C*::*PDGFRB*	M	2
P14	diagnosis	Primary	B	*EBF1*::*PDGFRB*	M	5
P15	diagnosis	Primary xenograft	B	*EBF1*::*PDGFRB*	M	14
P16	diagnosis	Primary xenograft	B	*EBF1*::*PDGFRB*	M	13
P17	diagnosis	Primary	B	*EBF1*::*PDGFRB*	M	10
P18	diagnosis	Serial transplant	B	*EBF1*::*PDGFRB*	M	11
P19	diagnosis	Primary	B	*SSBP2*::*CSF1R*	M	10
P20	diagnosis	Serial transplant	B	*SSBP2*::*CSF1R*	F	10

[i]sample obtained from day 33 after two weeks of TKI treatment; M = male; F = female; Age in years

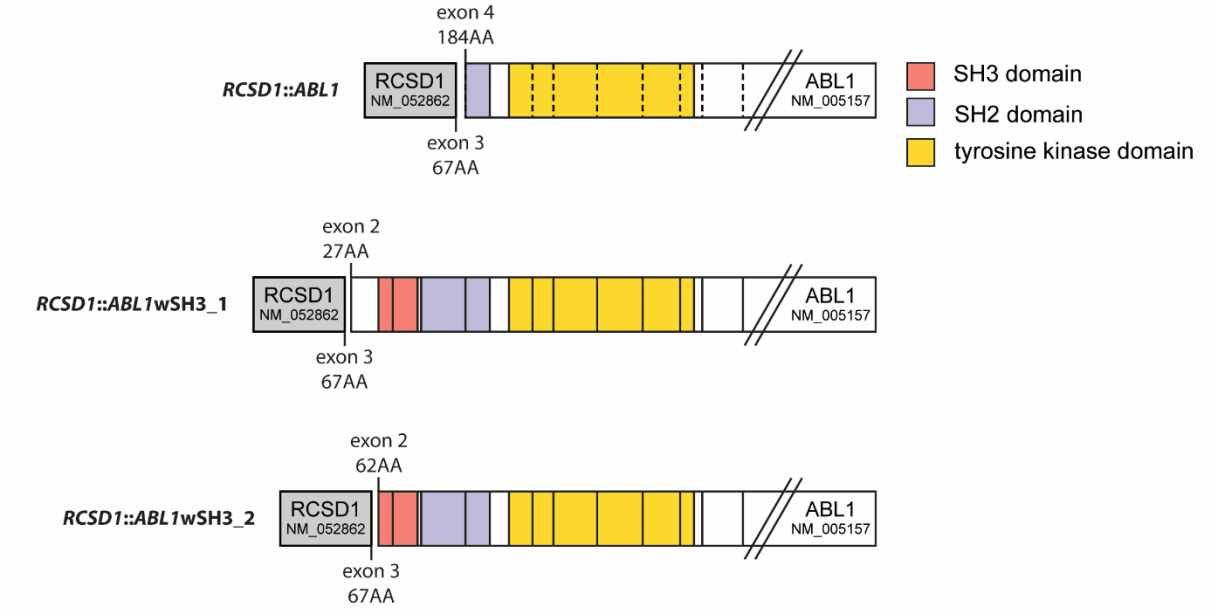
SUPPLEMENTAL FIGURES



Supplemental Figure S1: Dose-response curves of ABL-class patients with clinical achievable plasma concentrations highlighted. A) Dose-response curves of imatinib colored per tyrosine kinase gene. The highlighted area in grey represents the range of clinical achievable plasma concentrations of pediatric patients treated with treated with 440 mg/m2 imatinib daily.[9] B) Dose-response curves of dasatinib colored per tyrosine kinase gene. The highlighted area in grey represents the steady-state plasma average concentrations for pediatric patients treated with 60 mg/m2 dasatinib daily.[10] C) Dose-response curves of bosutinib colored per tyrosine kinase gene. The highlighted area in grey represents the steady state to maximum plasma concentration of pediatric patients treated with 300 to 400 mg/m2 bosutinib daily.[11]



Supplemental Figure S2: Dose-response curve of patient 9 performed on primary material and primary xenografted material of the same sample. Overlapping dose-response curves for imatinib for patient 9, *ex vivo* imatinib sensitivity was measured using primary material and primary xenografted material (PDX 1/2). Every point represents the mean of 3 technical replicates +/- the standard deviation.



Supplemental Figure S3: Schematic representation of original and modified *RCSD1::ABL1* constructs. Schematic representation of the original SH3-lacking *RCSD1::ABL1* construct, and the two modified SH3 domain-containing *RCSD1::ABL1* constructs, either containing the ABL1 starting at amino acid (AA) 27 (*RCSD1*::*ABL1*wSH3_1) or starting at amino acid 62 (*RCSD1*::*ABL1*wSH3_2). Amino acid numbers refer to amino acid in wild-type protein of RCSD1 NM_052862 and ABL1 NM_005157.

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