Preclinical pharmacokinetic and pharmacodynamic evaluation of dasatinib and ponatinib for the treatment of T-cell acute lymphoblastic leukemia

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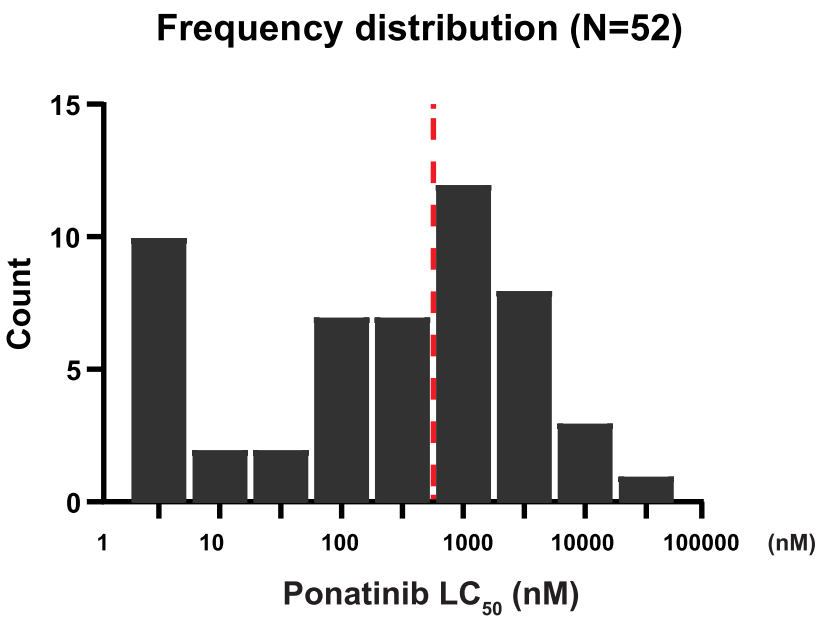
Supplemental materials included in this file:

Other Supplemental Material for this manuscript includes the following:

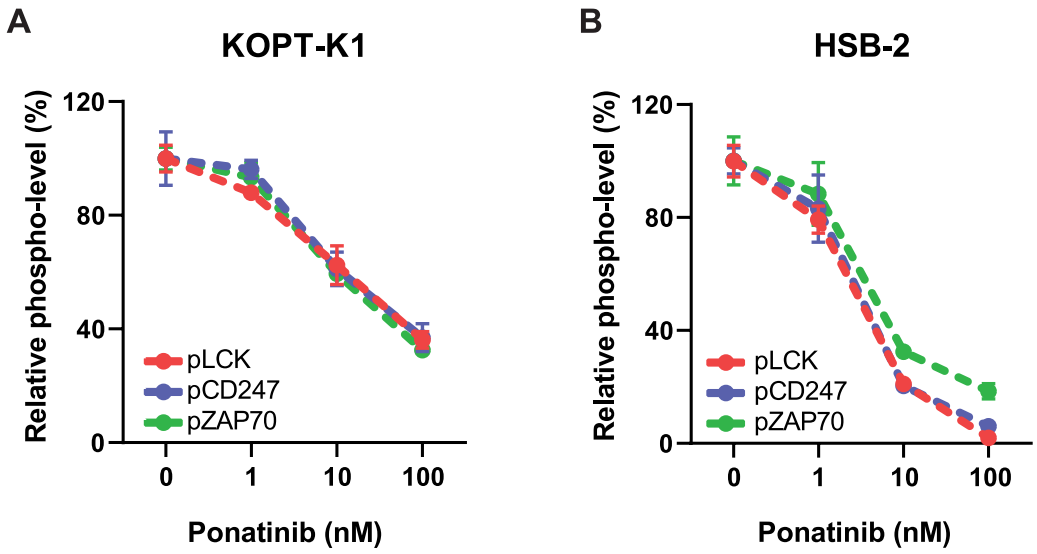
Supplemental Table 1

Supplemental Figures



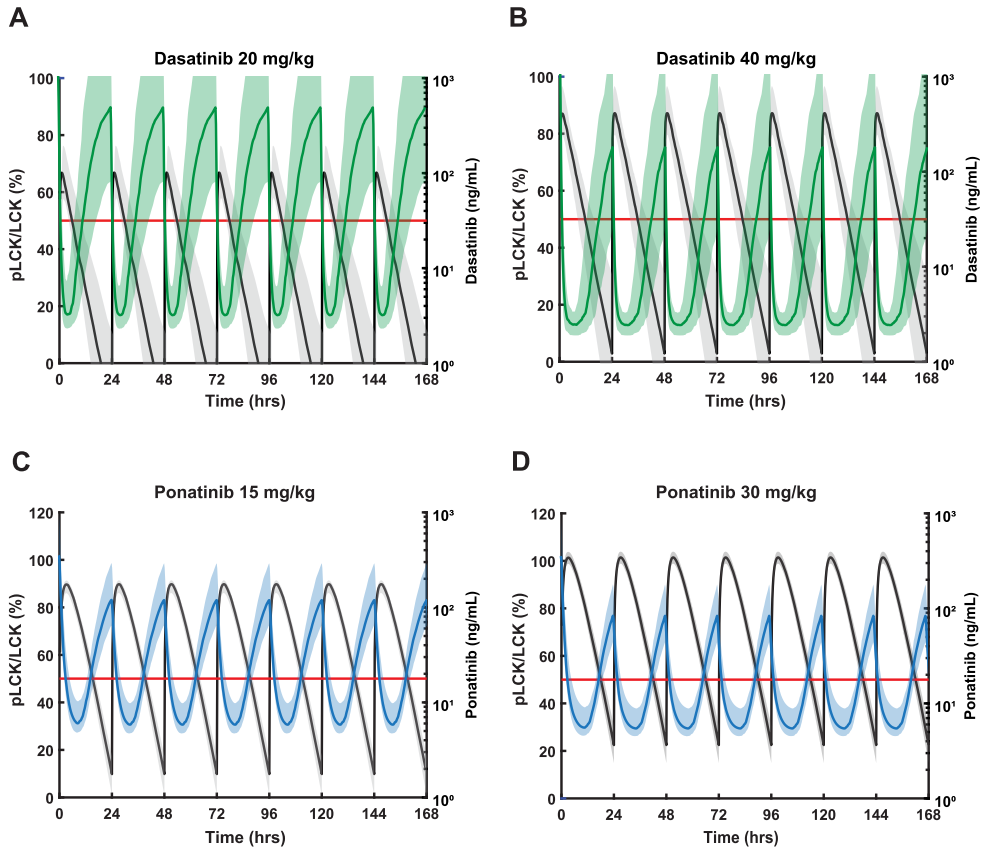
Supplemental Figure 1. Ponatinib LC_{50} distribution in T-ALL samples.

LC_{50} of all T-ALL samples tested for ponatinib (N=52) are summarized by histogram. The red dashed line indicates the median LC_{50} (432.6nM) for all samples.



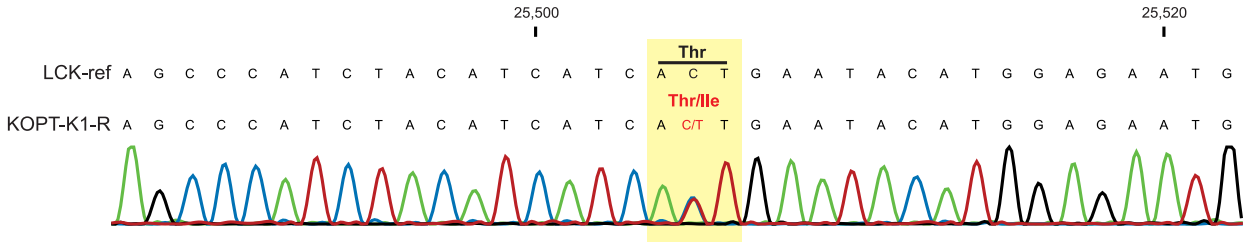
Supplemental Figure 2. Dephosphorylation of preTCR-LCK signaling molecules with ponatinib treatment in LCK-activated T-ALL cell lines.

KOPT-K1 (with *TCR-LMO2* fusion, **A**) and HSB-2 (with *TCR-LCK* fusion, **B**) were treated with ponatinib at various concentrations for 1 hr and phosphorylation of LCK, CD247 and ZAP70 were quantified by flow cytometry after intracellular staining with antibodies specific to each phospho-protein. The y axes indicate relative phosphorylation of each normalized to the level in untreated cells. Each plot is a mean from independent experiments (n=3) shown with S.D. as an error bar.



Supplemental Figure 3. PK and PD simulations of dasatinib and ponatinib in mice.

A,B, The steady-state after seven doses of dasatinib 20 mg/kg (**A**) or 40 mg/kg (**B**) given daily are simulated using PD data from two T-ALL PDX models (PDX #2 and #3). The median, and 10^{th} and 90^{th} percentile model estimated phosphorylated LCK (pLCK) levels are shown by the green curve and shaded regions, respectively. **C,D,** The steady-state after seven doses of ponatinib 15 mg/kg (**C**) or 30 mg/kg (**D**) given daily are simulated. The blue curve and shaded regions indicate the median, 10^{th}-90^{th} percentile model estimated pLCK levels. In all simulations, the black solid curve and shaded regions indicate the median, 10^{th}-90^{th} percentile model estimated drug concentrations. The right y axes, plasma drug concentrations; the left y axes, pLCK levels normalized to the untreated mice. The red lines indicate model estimated pLCK levels equal to 50%. 100 ng/ml is equivalent to 204.9nM for dasatinib, and 187.8nM for ponatinib.



Supplemental Figure 4. Sanger sequencing confirmed *LCK* mutation in dasatinib-resistant KOPT-K1 cells.

Sanger sequencing results confirmed T316I mutation occurred in LCK kinase domain in KOPT-K1 cells that had acquired resistance to dasatinib. The top sequence is representative of the wildtype LCK kinase domain.

Supplemental Methods

Human leukemia cell lines and chemicals

Human T-ALL cell lines KOPT-K1 (gifted by Dr. Takaomi Sanda at National University of Singapore) and HSB-2 (purchased from DSMZ) used in this study were authenticated by STR analysis and cultured in RPMI-1640 (ThermoFisher, #11875093) containing 10% fetal bovine serum at 37 °C with 5% CO_2. Drugs used for drug sensitivity assays were purchased from MedChemExpress: dasatinib (#HY10181), ponatinib (#HY12047) and saracatinib (#HY10234).

Ex vivo leukemia drug-sensitivity assay

Drug response of human ALL blasts was evaluated using a mesenchymal stem cells (MSC) co-culture system and determined by either 1) flow cytometry assays or 2) imaging-based assay, as described previously (1, 2). Briefly, hTERT-immortalized MSCs were first seeded one day prior to the assay, followed by plating leukemia cells with serial drug solution (9.766-10 000nM) diluted in the AIM V^{TM} serum free medium (Gibco, #12055083). Duplicates were included for each of the drug concentrations. After 96 hrs of incubation at 37°C with 5% CO_2, leukemia cells were subject to either cell viability assay, where drug-induced death was estimated by comparing leukemia cells treated with medium alone. Cell viability data were analyzed by either FlowJo (version 10.7.2) or Harmony high-content imaging and analysis software (version 4.9). Quality control was performed to remove cases with low viability, namely <500 viable blast cells in each well and AOPI < 20% on day 4 in the control wells. The concentrations of drug lethal to 50% of the cells (LC_{50}) were calculated by a four-parameter dose-response model. For cases in which even the lowest drug concentration killed >50% of leukemia cells, LC_{50} was assigned as half of the minimum tested concentration. Conversely, for cases with >50% viability even at the highest drug concentration, LC_{50} values were assigned as twice of the highest tested concentration.

Dasatinib and ponatinib quantification for PK evaluation

Drug concentrations were measured by liquid chromatography with tandem mass spectrometry using Waters ACQUITY separation system and TQD triple-quadrupole system (Waters Corporation, MA) in the same way as described previously(3). Separation was achieved on a Waters ACQUITY BEHC18 column (1.7 µm, 50 x 2.1 mm) with a Waters ACQUITY in-line filter at 40°C maintained by a column heater. Autosampler temperature was kept at 15 ± 5°C. The gradient mobile phase was composed of 0.1% formic acid in acetonitrile (B) and 10 mM ammonium acetate in H_2O (pH 3.8). After injection, the B was kept 20% for 30 sec and increased to 80% in the next 1 min. The column was then equilibrated at the initial condition (B at 20%) for 2 min before next injection. The flow rate was 0.9 ml/min and the separation was completed within 3.6 min. The mass spectrometer was operated in the positive mode, which was controlled by Masslynx 4.1 software (Micromass, UK). The analysis was performed in MRM mode and the following mass ions (m/z) were used for detection: m/z 488.1>401.1 for dasatinib and 496.2>405.9 for [$^2H_8$]-dasatinib as internal standard; 533.3 >260.1 for ponatinib and 541.3> 260.1 for [$^2H_8$]-ponatinib as standard. The tandem MS conditions were set as follows: capillary voltage: 0.6 kV; cone voltage: 60V; source temperature: 150°C; desolvation temperature: 450°C; cone gas flow: 10 L/h; desolvation gas flow: 900 L/h and collision energy: 32 v for dasatinib, ponatinib and internal standards. Calibration and QC samples were prepared in human plasma by dilution of the dasatinib stock solution (1 mg/ml in methanol) to final concentration from 1 to 1 000 ng/ml. Weighted linear least-squares regression ($1/X^2$) as the weighting factor was employed to define the calibration curves using the ratios of the peak area of the analytes and the internal standards. For plasma sample processing, 150µl extraction solvent (200 ng/ml [$^2H_8$]-dasatinib or [$^2H_8$]-ponatinib in the mixture of methanol and acetonitrile [1:1,v/v]) was pipette into 50µl of human plasma sample. The sample was vortex-mixed for 30 sec and centrifuged for 8 min at 10 000 rpm. 10µl of supernatant was injected into LC-MS for analysis.

***In vitro* cell viability assay**

CTG (Cell-Titer-Glo) assay was used to determine cell lines' sensitivity to dasatinib or ponatinib. Cells were resuspended at the density of 120 000 live cells/ml in RPMI1640 supplemented with 10% FBS, and 24µl of cell suspension was plated on 384-well plates (2 880 cells/well). Drug stocks at 10 mM were store at -80°C and thawed at room temperature just before the assay. Drug solutions were made at serial concentration range between 0.5-50 000nM for each drug with the medium and 6µl of the drug solution was added to the cell suspension in each well. After three days of incubation at 37°C/5% CO_2, 25µl of CTG solution (Promega, #G9241) was added to each well. Cells were then briefly incubated at room temperature, followed by luminescence measurement. Cell viability was assessed by comparing the luminescence of cells treated with drugs with those treated with medium alone.

Phospho-flow of LCK, CD247, and ZAP70

Human T-ALL leukemic cell lines (KOPT-K1 and HSB-2) were treated with dasatinib or ponatinib for 1 hour and followed by phospho-flow cytometric analysis as described previously(1). Briefly, cells were treated with either dasatinib or ponatinib for 1 hr, then collected and resuspended in cold PBS. Cells were fixed by adding pre-warmed (37°C) Lyse/Fix buffer (BD, #558049) and then mixed well. After incubated at 37°C for 10 min, cells were resuspended in pre-chilled Phosflow Perm Buffer III (BD, #558050) to be permeabilized for 30 min on ice. The cells were stained with anti CD3 antibody (BD Pharmingen, #558117) and phospho-antibodies targeting SRC-Y418/LCK-Y394 (BD Phosflow, #560095; 1:100), CD247-Y142 (BD Phosflow, #558448; 1:50), ZAP70-Y319 (BD Phosflow, #557881; 1:50) and then run on a flow cytometer. All phospho-flow data were analyzed using FlowJo.

Development of dasatinib-resistant T-ALL cell model

To establish a dasatinib-resistant cell line model, KOPT-K1 cells were cultured in the presence of dasatinib, starting at 1nM and gradually increasing to 500nM over five weeks. while their viability

was monitored. After dasatinib was washed out, CTG assay was used to confirm the acquired resistance to dasatinib and Western blotting was for the decline in pLCK inhibition, which details were described in the main text. Genomic DNA was extracted (QIAGEN, # 69504) from both parental and dasatinib-resistant cell lines and their mutational status was examined by whole genome sequencing (WGS). For WGS, libraries were prepared using the HyperPrep Library Preparation Kit (Roche PN 07962363001) and were sequenced on a NovaSeq 6000 (Illumina). The analysis was performed following procedures established previously(4). *LCK* mutational status at T316 was also confirmed by Sanger sequencing using the following PCR and sequencing primers: PCR-Fw GGCCGCTGAGGTGATGAGAG, PCR-Rv CTCCAGGGGGCAGGAAAGC, Seq-Fw CCCAGGACAGCTGCCTGGC, Seq-Rv CTCAAGAAACCCTCCTTGCT.

References

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