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*Original Article*

# Dasatinib and interferon alpha synergistically induce pyroptosis-like cell death in philadelphia chromosome positive acute lymphoblastic leukemia

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**Abstract:** Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ALL) is a high-risk disease subtype with a dismal prognosis. Inhibiting BCR-ABL kinase alone is insufficient to eradicate Ph+ALL clones, and alternative BCR-ABL-dependent and -independent pathways need to be targeted as an effective strategy. Our study revealed that the combination of dasatinib and interferon-α showed synergistic activity against Ph+ALL, inducing mitochondrial dysfunction and causing necrosis-like cell lysis. Mechanistic studies showed that the induced cell death was caspase-3-independent. Canonical necroptosis signals, such as RIP1 and MLKL, were not activated; instead, the pyroptosis executor Gasdermin D was upregulated expression and activated. The expression levels of extracellular ATP and IL-1β were also upregulated, both of which are markers of pyroptotic cell death. In a murine Ph+ALL model, the dual drug treatment prolonged the survival of tumor-bearing mice. More importantly, we incorporated the dual drugs to maintenance therapy in 39 patients who were unfit for allogeneic stem cell transplantation (allo-HSCT). The median follow-up was 28.5 months, the 4-year disease-free survival and overall survival rates were 52.2% and 65.2%, respectively. Our data suggest that the combination of dasatinib and interferon-α has potential synergistic activity against Ph+ALL and shows promise as a maintenance therapy for Ph+ALL patients who are unfit for allo-HSCT.

**Keywords:** Philadelphia chromosome-positive acute lymphoblastic leukemia, dasatinib, interferon-α, pyroptosis, maintenance therapy

## Introduction

Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ALL) is a high-risk disease subtype with a dismal prognosis [1, 2]. The integration of BCR-ABL tyrosine kinase inhibitors (TKIs) with chemotherapy has markedly improved Ph+ALL outcomes, and TKIs have become the cornerstone of Ph+ALL treatment [3]. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a standard strategy with the highest curative potential. However, there is a substantial proportion of patients unfit for allo-HSCT due to a lack of suitable donors or existing transplant contraindications. Thus, new therapeutic strategies should be explored to target to both BCR-ABL-dependent and -independent mechanisms.

Dasatinib is a dual Src/ABL TKI that additionally targets the Lyn tyrosine kinase. Unlike the other TKIs, such as imatinib and nilotinib, dasatinib can inhibit ABL and Src kinases. The Src family kinases Lyn, Hck and Fgr are activated by Bcr-Abl in pre-B cells, both of which are required for Ph+ALL leukemogenesis [4]. Therefore, dasatinib may cause various alterations in cell signalling of Ph+ALL by inhibiting Src and ABL kinases [5]. Interferon-α (IFN-α) had shown facilitated effects on Ph+ALL in some clinical case reports and small cohort studies, even in the patients with ABL kinase mutations related to BCR-ABL-dependent resistance [6-9]. However, the mechanisms by which TKIs with IFN-α elicits a response are still largely unknown. In this study, for the first time, we reported that dasatinib and IFN-α

exerted synergistic cytotoxic effects on Ph+ALL cells by inducing pyroptosis-like cell death. More importantly, the combination of dual drugs might be used as a maintenance strategy for Ph+ALL patients who are unfit for allo-HSCT.

## Methods

### *Reagents and antibodies*

Imatinib, nilotinib, and dasatinib were purchased from LC Laboratories (Woburn, MA). The pan-caspase inhibitor Z-VAD-FMK (Z-VAD) was purchased from ENZO Life Sciences (Farmingdale, NY). The necroptosis inhibitor necrostatin-1 was purchased from Cayman Chemical (Ann Arbor, MI). Recombinant human IFN-α and mouse IFN-α were purchased from R&D Systems (Minneapolis, MN). The antibodies used for western blot and flowcytometry were listed in Table S1.

### *Cell lines, primary leukemic cells, and control cells*

The Ph+ALL cell line SUP-B15 and the B cell precursor leukemia cell line Nalm-6 were purchased from American Type Culture Collection (ATCC). These two cell lines were authenticated by short tandem repeat DNA (STR) profiling and tested for mycoplasma contamination before use. Primary leukemic cells were isolated from newly diagnosed patients with either Ph+ALL or Ph-ALL. The patients' bone marrow cells were treated with Ficoll-Paque PLUS and centrifuged to collect mononuclear cells (MNCs). Leukemic cells were further isolated from MNCs by CD34+ magnetic bead purification (Miltenyi Biotec, Gladbach). Normal peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors. Human mesenchymal stem cells (MSCs), or umbilical endothelial cells (ECs) were kindly provided by Sichuan Province Cord Blood Bank. The research protocol was reviewed and approved by the Ethics Committees of West China Hospital, Sichuan University.

### *Cell viability assay*

Cell viability following drug treatment was measured by MTT assay. SUP-15 cells were cultured in IMDM medium at a density of $2\times10^5$/ml and seeded in a 96-well plate. The cells were treated by different concentrations of dasatinib, or imatinib, or nilotinib combined with IFN-α or alone, and incubated at 37°C and 5% $CO_2$ for 4 days. Nalm-6 cells were cultured in RMPI-1640 medium at the same cell density and treated as above. The primary leukemic cells from the patients either Ph+ALL or Ph-ALL were cultured in IMDM medium at the same cell density, and treated as above. 20 μl MTT (0.5 mg/ml) was used to assess cell proliferation. The treated cells were collected and stained with trypan blue. Live and dead cells were counted, and drug interaction index was calculated by King's formula, $q=(E_{a+b})/(E_a+E_b-E_a\times E_b)$, as previously described [10].

### *Cell death assessments*

SUP-B15 cells were seeded in 24-well plate at a density of $2\times10^5$/ml cells, and treated with dasatinib (0.02 nM) or IFN-α (1000 U/ml) alone or combination for 4 days. Annexin V and propidium iodide (PI) were used to detect apoptotic cells by flow cytometry [11]. Annexin $V^+/PI^+$ cells were considered late apoptotic and necrotic cells, while necrotic cells exhibited relatively low annexin V staining.

DNA fragmentation analysis of dying cells was performed using a Quick Apoptotic DNA Ladder Detection Kit (Biovision, Milpitas, CA) as previously described [12]. TUNEL assay was performed using the YF®488 TUNEL Assay Apoptosis Detection Kit (Jiangsu, China). The results were visualized under a Nikon A1R MP+ confocal fluorescence microscope. The percentage of TUNEL positive cells was analysed by flowcytometry.

### *Mitochondrial assessments*

SUP-B15 cells were treated with dasatinib or IFN-α alone or combined for 4 days, Mito-Tracker (MIT) green (Beyotime Biotechnology, Shanghai, China) was used to stain mitochondria in live cells. The cells were also stained with PI to identify dead cells. Under fluorescence microscopy, the green fluorescence positively related to normal mitochondria, while red fluorescence referred to dead cells. In each group, about 120 cells in four fluorescence microscope field were evaluated. Image-J software was used to quantify green and red fluorescence.

The mitochondrial membrane potential (MMP) was assessed by the MMP assay kit with JC-1

(Beyotime Biotechnology, Shanghai, China). Briefly, SUP-B15 cells were treated with dasatinib and IFN-α alone or combined for 1, 2 and 3 days, respectively. Cells were harvested and suspended in 0.5 ml cultural medium and added with 0.5 ml JC-1 working solution, incubated at 37°C for 10 minutes. Then the cells were collected and analysed by flow cytometry. The red fluorescence stands for aggregated JC-1 dye located in mitochondrial and green fluorescence for monomer JC-1 dye diffused from mitochondrial. Red to green ratio was used as an indicator of MMP.

*Reactive oxygen species (ROS) measurement*

The ROS level in SUP-B15 cells treated with dasatinib and IFN-α was evaluated by the Cellular Reactive Oxygen Species Detection Assay Kit (Beyotime Biotechnology, Shanghai, China). After drugs treatment for 2 or 3 days, the cells were labelled with 10 μM fluorescent probe DCFH-DA (2', 7'-dichlorodihydrofluorescein diacetate). Cells treated with the Rosup reagent in the kit for 30 min were considered as positive control. The cells were then washed three times with serum free medium and tested by flowcytometry.

*ATP measurement*

Intracellular and extracellular ATP levels were determined by using the ATP Assay Kit (Beyotime Biotechnology, Shanghai, China). For intracellular ATP measurement, the treated SUP-B15 cells were lysed and centrifuged at 12000 g. Supernatants (20 μl) were mixed with 100 μl ATP detection working solution in a white 96-well plate, and the luminescence was measured. The results of ATP concentration were normalized by live cells of each group. For measurement of extracellular ATP levels, 100 μl of the culture supernatant was collected and mixed with 100 μl ATP detection working solution, and the luminescence was measured as the same method.

*Transmission electron microscopy (TEM)*

The ultrastructure of drugs treated cells was examined by TEM, and the cells were processed as described previously [13]. Sections were examined with JEM-1400 Flash TEM. Morphologically normal cells and necrosis-like cells were counted on four lower power fields (3000×) in each group, then necrosis-like cells rate (ratio of necrosis-like cells to total cells) were calculated.

*Immunofluorescence analyses*

SUP-B15 cells treated with or without dasatinib and IFN-α for 48 hr were fixed with 4% paraformaldehyde and then permeabilized with 0.25% Triton X-100. The cells were then blocked with 1% BSA in PBST (PBS+0.1% Tween 20) and incubated with an anti-ENDOG antibody (Santa Cruz Biotechnology, Shanghai, China) diluted 1:50 overnight at 4°C. The cells were then incubated with Alexa Fluor 555-conjugated donkey anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA) and stained with DAPI (4', 6-diamidino-2-phenylindole).

*Western blot analysis*

The treated SUP-B15 cells were collected. The total cell lysates were obtained by cell lysate buffer containing a cocktail of protease inhibitors (Roche, Mannheim, Germany). Cytoplasmic and nuclear protein were separated and collected by the MinuteTM cytoplasmic and nuclear extraction kit (Invent Biotechnologies, Plymouth, MN). The protein concentration in the cell lysates was determined by the BCA method (CWBIO, Jiangsu, China). Equal amounts of protein were boiled in 2× Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA), resolved by SDS-PAGE, and subjected to western blot analysis with specific primary antibodies and horseradish peroxidase-labelled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA), and the signal was developed using enhanced chemiluminescence (Millipore, MA).

*ELISA*

The level of IL-1β in the culture supernatant of SUP-B15 cells was measured with a human IL-1β ELISA kit (NeoBioscience, Guangdong, China) according to manufacturer's protocols.

*Animal model*

An adoptive murine Ph+ALL model was established as previously described [4, 14]. In brief, we transfected 293T cells with a MSCV-IRES-eGFP retroviral vector carrying p210 BCR-ABL1 cDNA to prepare a viral stock, then, infected

bone marrow cells isolated from 6-week-old female C57BL/6 mice (Chengdu Dossy Experimental Animal Co., China) to overexpress p210 BCR-ABL. The infected BM cells were transplanted (1 million cells per mouse) by tail vein injection into 6-week-old female C57BL/6 recipient mice (n=9) pre-treated with 8.5 Gy irradiation. Twelve days after transplantation, the mice were subjected to dasatinib and IFN-α treatment. Dasatinib was given orally once a day at a dose of 15 mg/kg. Mouse IFN-α was given subcutaneously twice a week at a dose of 30 g/kg. There were 5 mice in the dasatinib and IFN-α combination group (n=5), the control group (n=4) were treated with PBS. The peripheral blood of mice was collected from the angular vein. The tumour burden of mice was evaluated by the percentage of $GFP^+/B220^+$ cells in peripheral blood via flow-cytometry. Treatment continued until the first leukemic mice death in the control group. All mice were euthanized at the end of study. This animal study was approved by the Animal Care and Use Committees of West China Hospital, Sichuan University.

*Immunohistochemistry*

The femurs of Ph+ALL mice were fixed, decalcified and embedded in paraffin. The paraffin sections were deparaffinized, subjected to antigen retrieval and incubated with an anti-CD45R (B220) antibody (Sungene Biotech, Tianjin, China) diluted 1:100 in TBST with 1% BSA. Antigen retrieval was performed using sodium citrate buffer (10 mM, PH 6.0) at 97°C for 10 min. The sections were cooled to room temperature for 30 min, and then incubated with 3% $H_2O_2$ for 10 min to block endogenous peroxidase activity, blocked with 5% normal goat serum in TBST for 1 h at room temperature, incubated with an anti-CD45R antibody at 4°C overnight. The sections were incubated with biotinylated rabbit anti-rat IgG and treated with streptavidin-biotin complex (Boster Biological Technology, China). The signal was then developed using a DAB detection kit (ZSGB-Bio, China).

*Patients*

To verify the clinical efficacy of the combination of dasatinib and IFN-α in Ph+ALL patients, we incorporated the two drugs to maintenance therapy in the patients hospitalized in West China Hospital from February 2016 to December 2018. We collected and analysed the patients' data in a registered clinical trial (ChiCTR1800015763). A total of ninety-eight newly diagnosed Ph+ALL patients were evaluated according the following criteria: 1) the patients received dasatinib and chemotherapy as induction therapy; 2) the patients achieved and kept the first complete remission (CR) after induction and consolidation; 3) the patients were unfit for allo-HSCT due to a lack of donors or existing transplant contraindications; and 4) the patients received maintenance therapy including IFN-α and dasatinib for over 1 month. Patients who received autologous hematopoietic stem cell transplantation were excluded. Based on the above criteria, a total of 39 Ph+ALL patients were included in the maintenance therapy. Patient relapse and loss were recorded to calculate overall survival (OS) and disease-free survival (DFS). Written informed consent was obtained from all patients. The study was reviewed and approved by the Ethics Committees of West China Hospital, Sichuan University.

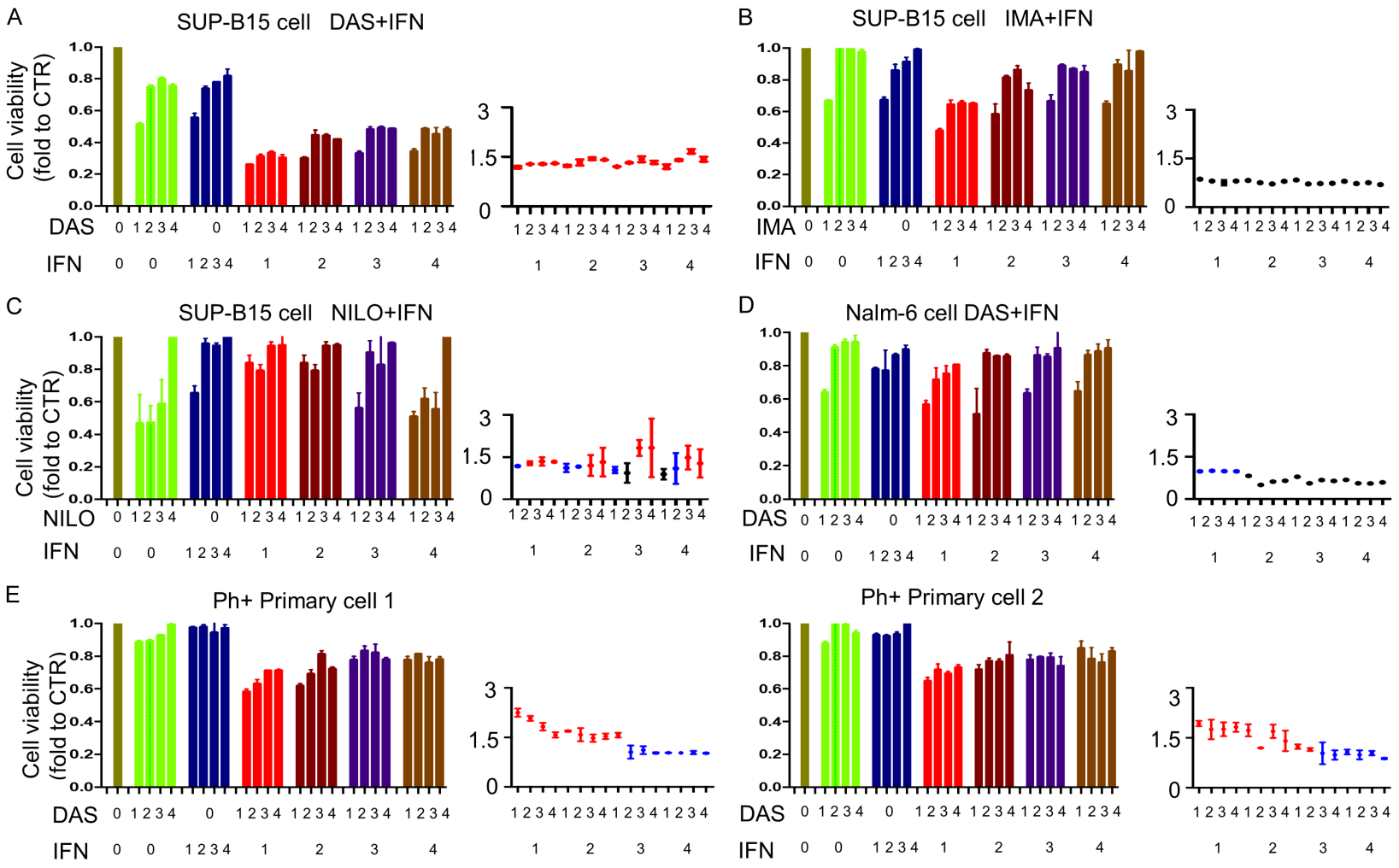
*Statistical analysis*

All data are shown as the mean ± standard deviation. Two-tailed Student's *t*-test was used to determine statistical significance, and $P \leq 0.05$ was considered statistically significant. The duration of CR was compared between different treatment groups by the log-rank test. The DFS and OS rates of patients were estimated using the Kaplan-Meier method.

## Results

*Dasatinib and interferon-α exhibit synergistic activity against Ph+ALL*

Our results showed that dasatinib and IFN-α had synergistic activity against Ph+ALL cells. At all tested concentrations, dasatinib synergized with IFN-α significantly inhibited the growth of SUP-B15 cells (**Figure 1A**). Interestingly, under the similar treatment conditions with IFN-α, nilotinib showed partial synergized effect, while imatinib did not synergize (**Figure 1B**, **1C**). Furthermore, dasatinib and IFN-α had no synergistic effect on Ph-ALL cell line Nalm-6 (**Figure 1D**). The dual drugs combination exerted partially synergistic cytotoxic effects on primary Ph+ALL cells but not primary Ph-ALL cells (**Figure 1E**, **1F**).


A
SUP-B15 cell DAS+IFN
Cell viability (fold to CTR)
DAS
IFN
B
SUP-B15 cell IMA+IFN
IMA
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C
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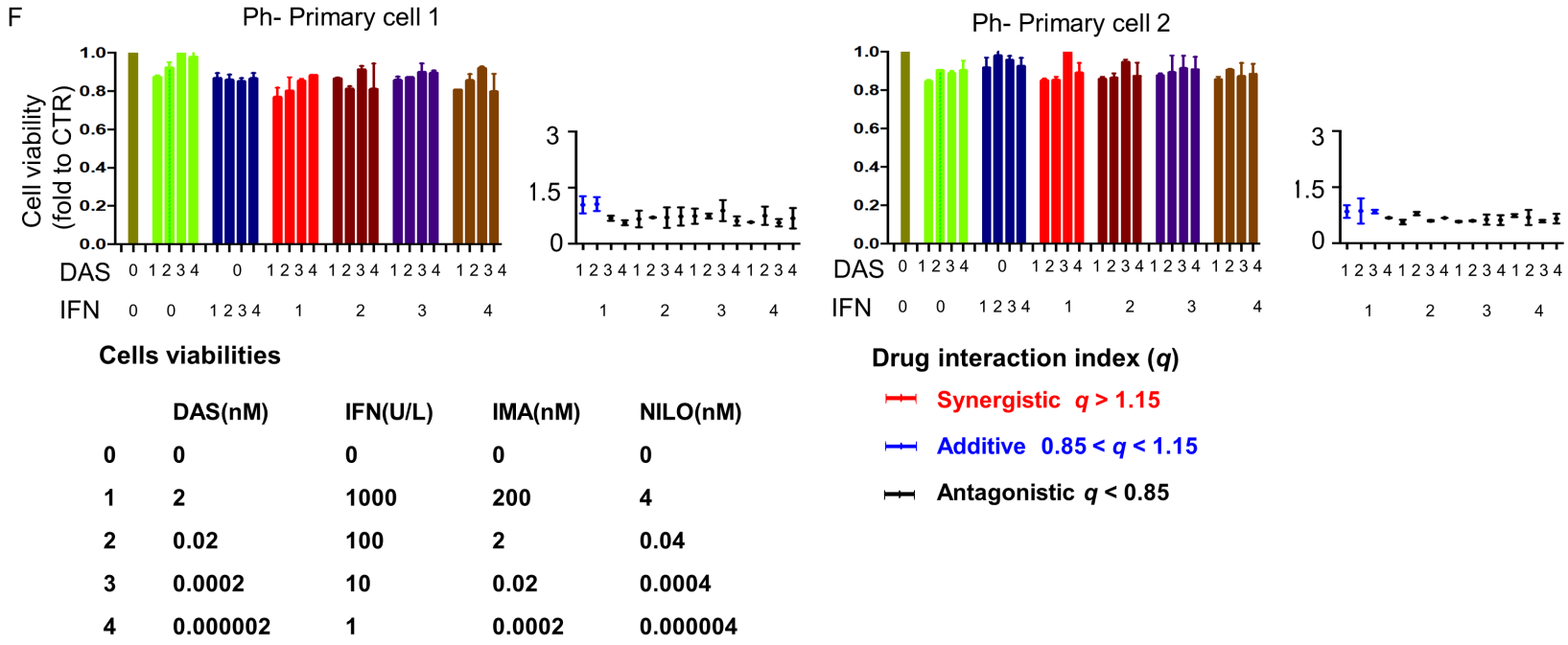


| | DAS(nM) | IFN(U/L) | IMA(nM) | NILO(nM) |
|---|---|---|---|---|
| 0 | 0 | 0 | 0 | 0 |
| 1 | 2 | 1000 | 200 | 4 |
| 2 | 0.02 | 100 | 2 | 0.04 |
| 3 | 0.0002 | 10 | 0.02 | 0.0004 |
| 4 | 0.000002 | 1 | 0.0002 | 0.000004 |

**Figure 1.** Combinational effects of tyrosine kinase inhibitors and IFN-α on acute lymphoblastic leukaemia cells. A. SUP-B15 cells were treated by titration doses of dasatinib (0, 0.000002, 0.0002, 0.02, or 2 nM) and IFN-α (0, 1, 10, 100, or 1000 IU/ml) for 4 days. The cells viabilities were assessed (left) and interaction index of drug combinations were calculated (right). B. SUP-B15 cells were treated with imatinib (0, 0.0002, 0.02, 2, or 200 nM) and IFN-α (same as above); C. SUP-B15 cells were treated with nilotinib (0, 0.004, 0.4, 40, or 4000 pM) and IFN-α (same as above); D. Nalm-6 cells were treated with dasatinib and IFN-α at the above same doses for 4 days. E. Primary leukemic cells from two Ph+ALL patients were treated with dasatinib and IFN-α at the above same doses for same duration. F. Primary leukemic cells from two Ph-ALL patients were treated with dasatinib and IFN-α at the above same doses for same duration.

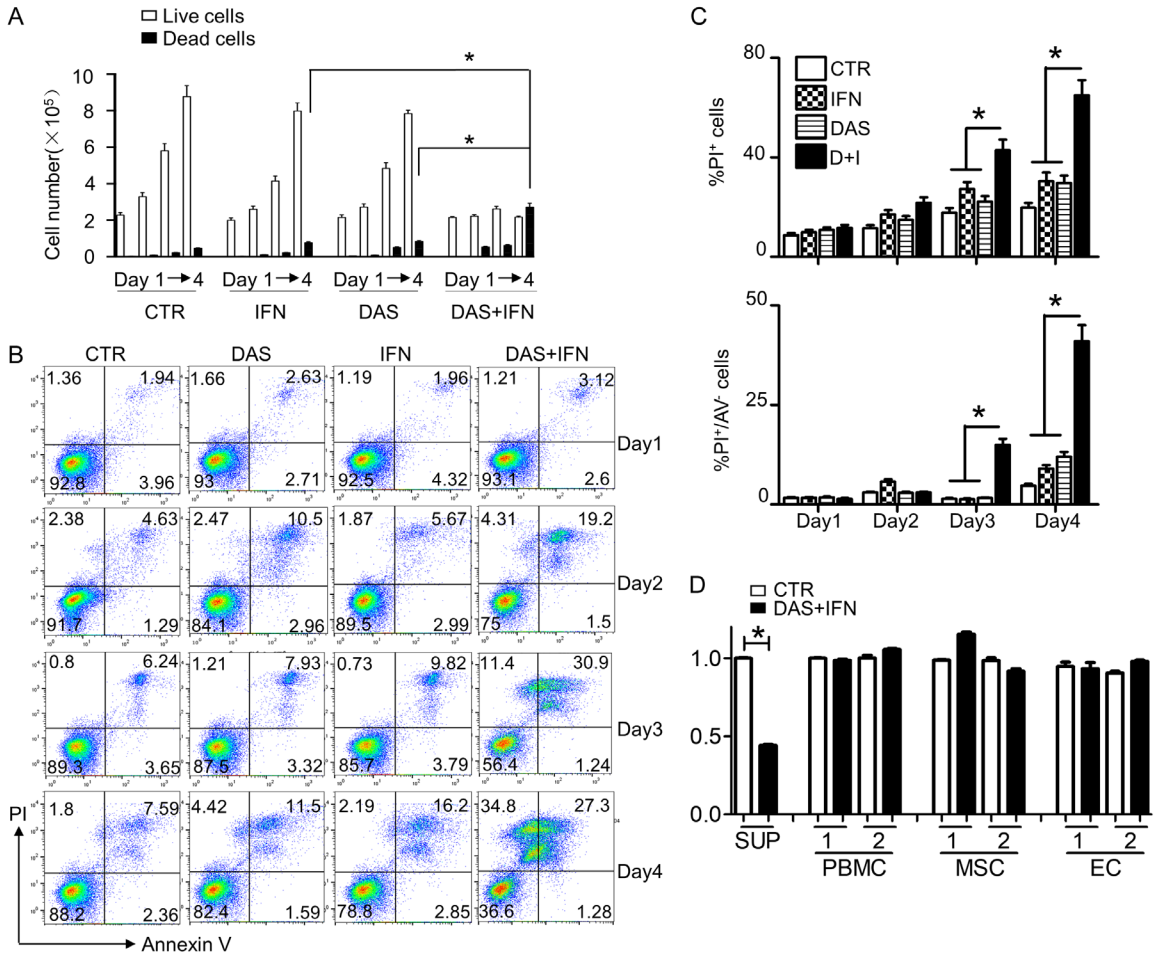


**Figure 2.** Dasatinib and interferon-α induce acute lymphoblastic leukaemia SUP-B15 cells death. A. SUP-B15 cells were seeded in 24-well plate at a density of $2\times10^5$ cells per well. The cells were treated with dasatinib (0.02 nM), IFN-α (1000 IU/ml) or their combination for up to 4 days. The cells were harvested each day, stained with Trypan blue, and then visualized under a microscope to count live and dead cells. B. Following the same procedure, the cells were harvested and subjected to flow cytometry analysis of annexin V and PI staining. C. Quantification of flow cytometry analysis. The upper panel shows the percentage of $PI^+$ and annexin $V^+$ cells, and the lower panel shows the percentage of $PI^+$ and annexin $V^-$ cells. D. Non-tumorous PBMCs, MSCs, and ECs, all of which were isolated from healthy donors, were treated with dasatinib (0.02 nM) and IFN-α (1000 IU/ml) for 4 days, and cell viability was assessed. SUP-B15 cells were subjected to the same treatment and used as positive controls. The standard deviation in PBMC corresponded to triplicate of healthy cells in the test. The #1 and #2 indicated results from two different donor cells (*P<0.05).

To further investigate dasatinib- and IFN-α-induced cell death, we treated SUP-B15 cells with either drug alone or combination for different lengths of time. The cells were stained with trypan blue, and then live cells and dead cells were visualized by contrast phase microscopy. As shown in **Figure 2A**, the dual drug-induced cell death occurred on days 3 and 4 after drug exposure. Apoptotic analysis using Annexin V and PI also showed delayed cell death on day 3 and day 4 (**Figure 2B**, **Figure 2C** upper panel). We also observed that there were many cells presented Annexin $V^{Low}/PI^+$, which is characteristic of late stage apoptotic or necroptotic cells (**Figure 2C** lower panel). We detected few early-stage apoptotic cells, which were Annexin $V^+/PI^-$. This result might indicate that the death of Ph+ALL cells induced by the drug combination was not canonical apoptosis. Finally, we examined the cytotoxic effects of the drug combination on normal cells. As shown in **Figure 2D**, at the concentrations we used, the drugs had no cytotoxic effects on healthy donor-derived PBMCs, MSCs, or ECs. Our results suggested that dasatinib and IFN-α had synergistic activity against Ph+ALL cells in vitro.

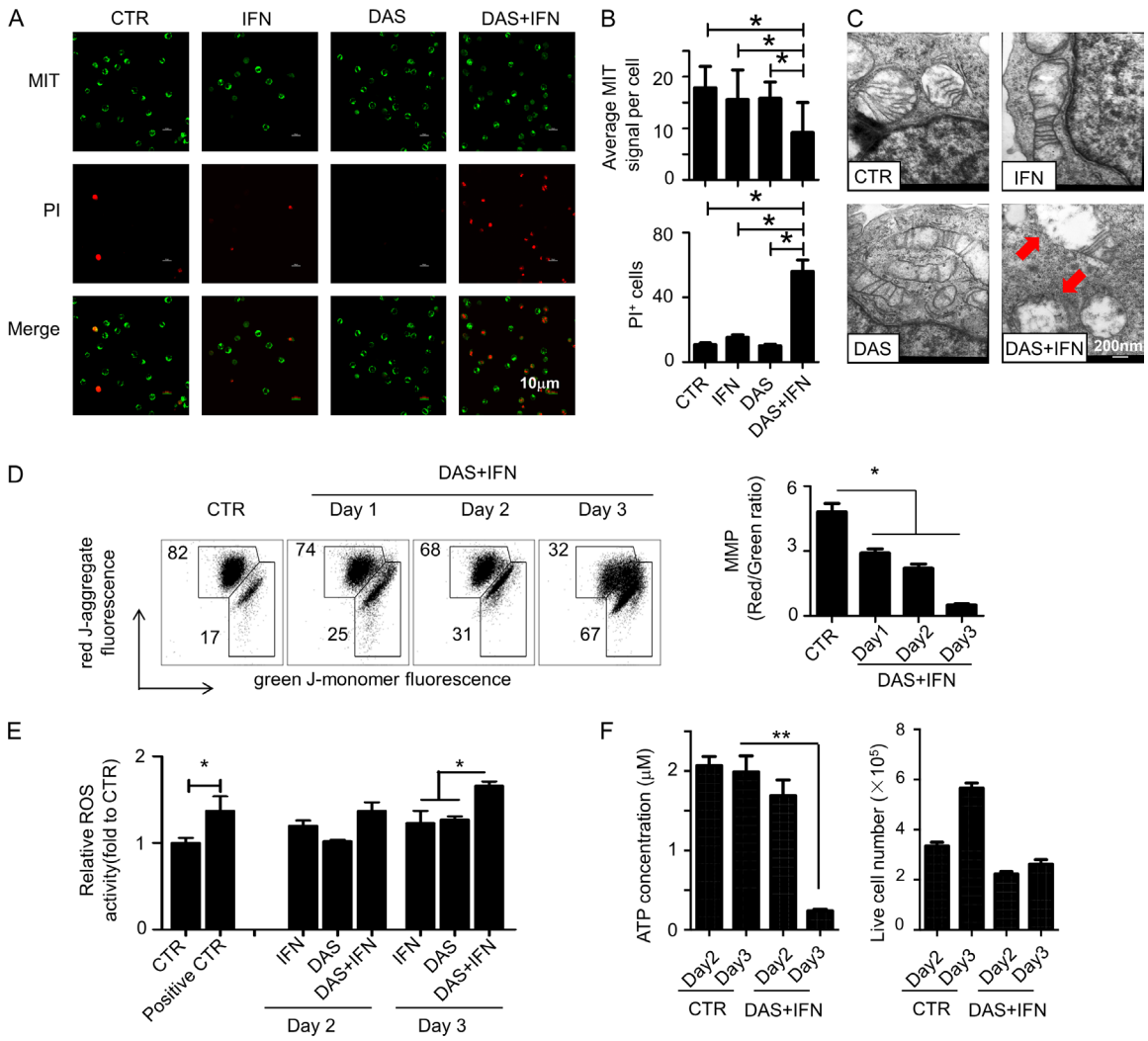


**Figure 3.** Dasatinib and interferon-α induce mitochondrial dysfunction. SUP-B15 cells were treated with dasatinib (0.02 nM), IFN-α (1000 IU/ml) or their combination for 3 days. A. The cells were stained with MIT and PI, and visualized under a fluorescence microscope. B. Quantification of MIT staining. The upper panel shows the average fluorescence intensity of MIT. The lower panel shows the number of $PI^+$ cells in each sample. C. Transmission electron microscopy (TEM) showed significantly abnormal mitochondrial morphology in the dual drugs group. D. The mitochondrial membrane potential (MMP) was assessed by JC-1 dye (upper panel), and Quantification of MMP assessment was showed in lower panel. E. The intracellular ROS level was evaluated by flow cytometry. The ROS level in the cells treated with dual drugs was remarkably increased. F. The intracellular ATP concentration was evaluated (corrected by live cell number, left panel), corresponding live cell number were showed in right panel (*P<0.05).

*Dasatinib and interferon-α induce Ph+ALL cell mitochondrial dysfunction*

Since mitochondria may initiate diverse cell death signals [15], we examined the mitochondrial function of Ph+ALL cells after dasatinib and IFN-α exposure. First, we stained SUP-B15 cells with MIT fluorescent dye on day 3 after drug treatment, before visible cell death occurs. As shown in **Figure 3A** and the quantitative results in **Figure 3B**, the dual drug-treated SUP-B15 cells exhibited a decreased MIT signal, which indicated loss of mitochondrial membrane potential. TEM also showed morphological changes in mitochondria on day 3 after dual drugs exposure: the edges of mitochondria were indistinct, and cristae structure vanished (**Figure 3C**). While control or single agent-treated cells exhibited normal mitochondrial morphology.

The MMP assay showed a continuous decrease in mitochondrial membrane potential after cells were exposed to the drug combination,

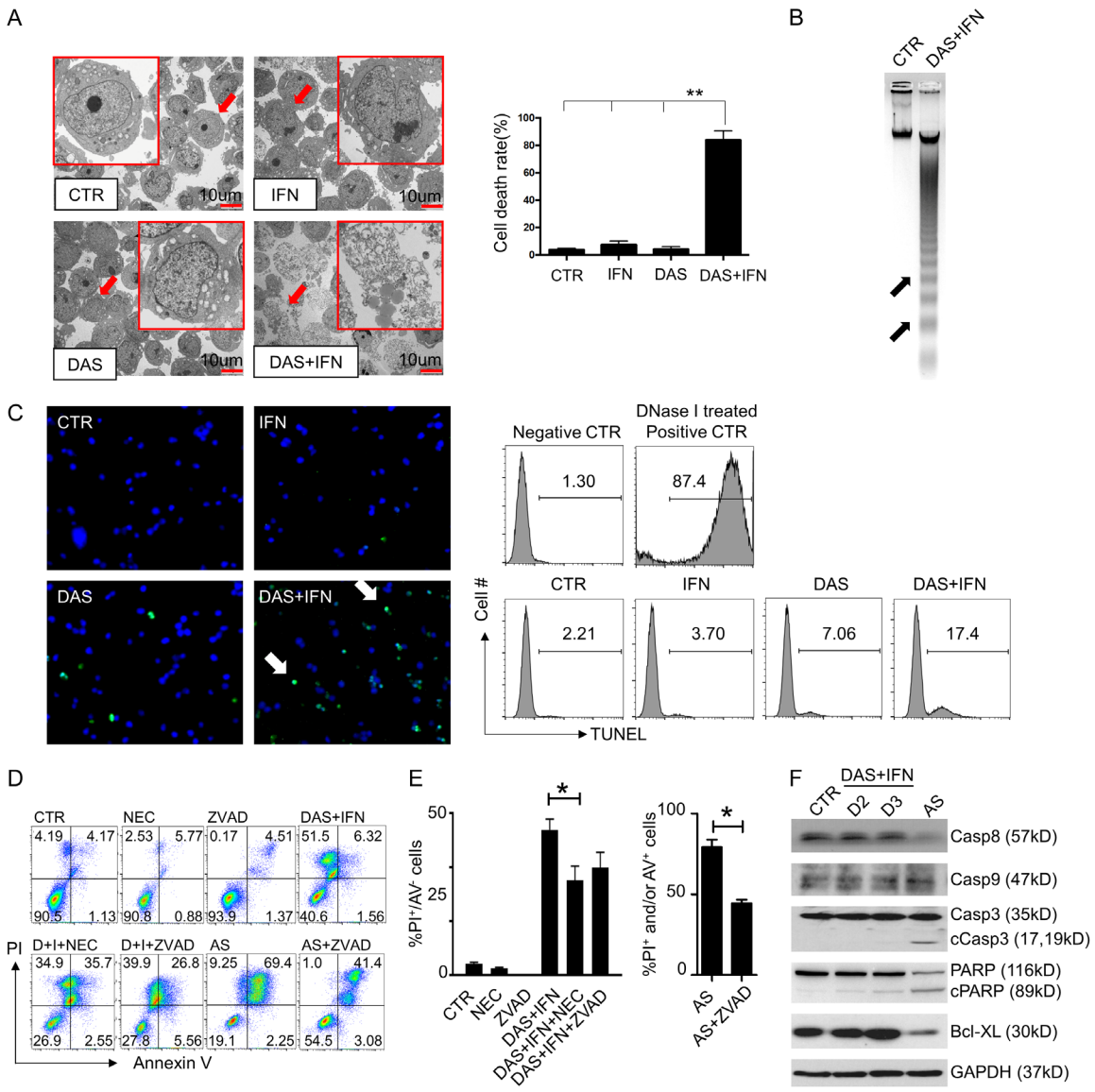


**Figure 4.** Dasatinib and interferon-α induce necroptosis-like, caspase-3-independent cell death. SUP-B15 cells were treated with dasatinib (0.02 nM), IFN-α (1000 IU/ml) or their combination for 4 days. A. Transmission electron microscopy displayed the cells necrosis-like decomposition (left figures), and right panel shows the quantification of necrosis-like cell rate (ratio of necrosis-like cells number to total cells number). B. DNA ladder assay shows that the dual drug-treated cells exhibited apoptotic DNA fragmentation. C. DNA fragments of cells were marked with TUNEL staining, then visualized under microscope (left figures) or analysed with flowcytometry (right panel). D. SUP-B15 cells were treated with dasatinib and IFN-α as described above, in the presence of Z-VAD (ZVAD, 20 μM) or necrostatin-1 (NEC, 500 nM). The cells treated with arsenic trioxide (AS, 50 μM) were used as positive controls for cell apoptosis. The cells were subjected to flow cytometry analysis of annexin V and PI staining. E. Quantification of the data from the above experiments. F. The levels of apoptosis-related proteins were measured by western blotting (*P<0.05).

but before the visible cell death occurs (**Figure 3D**). We also assessed cellular ROS activity and the intracellular ATP concentration, both of which reflect mitochondrial function. As shown in **Figure 3E** and **3F**, the dual drugs exposure resulted in increased ROS activity and decreased intracellular ATP concentration.

*Dasatinib and interferon-α induce necrosis-like, caspase-3-independent cell death*

TEM showed that on day 4, after dasatinib and IFN-α exposure, the cells were lysed and exhibited a ruptured cell membrane (**Figure 4A**). This is necrosis-like morphological change [16].

However, the DNA ladder assay showed that the dual drug-treated cells exhibited apoptotic DNA fragmentation, which is a marker of apoptotic activation of endonucleases (**Figure 4B**). Additionally, we performed the TUNEL assay to evaluate apoptotic DNA fragmentation. The result revealed that many but not all dual drug-treated cells had fragmented DNA (**Figure 4C**, left figures). We also used flow cytometry to measure the percentage of TUNEL positive cells under above conditions, the dual drugs induced about 17% of TUNEL positive cells (**Figure 4C**, right panel). Next, we treated SUP-B15 cells with either the apoptosis inhibitor Z-VAD-FMK (Z-VAD) or the necroptosis inhibitor necrostatin-1 (NEC) to inhibit the dual drug-induced cell death. To our surprise, Z-VAD had only a minimal effect, while NEC partially reduced the dual drug-induced $PI^+/AV^-$ cell population. Z-VAD effectively inhibited cell apoptosis of SUP-B15 cells induced by arsenic trioxide, which was used as a positive control (**Figure 4D**, **4E**, right panel). Western blotting showed that the dual drugs treatment could not induce caspase 3 activation or PARP cleavage (**Figure 4F**). Only a small amount of cleaved PARP was detected in the dual drug-treated cells. Cleavage of caspase 8, 9 and Bcl-xL was not observed in the dual drug-treated cells. Our findings suggested that dasatinib and IFN-α induced necroptosis-like caspase-3-independent SUP-B15 cell death.

*Dasatinib and interferon-α induce pyroptosis-like cell death*

Since dasatinib targets both Src kinase and BCR-ABL, we assessed Src cell signalling. As shown in **Figure 5A**, the dual drugs treatment had an additive inhibitory effect on pAbl, pSrc and pJAK kinase activity. We also found that the addition of the Src kinase inhibitor PP2 increased imatinib and IFN-α induced cell death (**Figure 5B**). These results indicated that dasatinib-mediated Src kinase inhibition might be required for the synergistic effect of the drug combination against Ph+ALL. We did not detect upregulation of the expression of RIP1 and pMLKL, which are hallmarks of necroptosis signalling [17]. Consistently, the LC3B expression level indicated that autophagy cell signalling was not activated (**Figure 5C**) [18]. Alternatively, we found that the protein endonuclease G (EndoG) translocated from cytoplasm to nucleus (**Figure 5D**). EndoG is a mitochondrial nuclease activated during caspase-independent apoptosis [19]. Immunofluorescence staining showed that the dual drugs treatment stimulated EndoG nuclear translocation (**Figure 5E**). Therefore, EndoG might contribute to DNA fragmentation after the dual drugs treatment (**Figure 4B**, **4C**). Furthermore, we examined caspase 1 and Gasdermin D (GSDMD), both of which are main executors of cell pyroptosis, an inflammatory form of necroptotic cell death [20]. The dual drugs treatment induced downregulation of pro-caspase 1 (**Figure 5F**). Interestingly, the expression of both GSDMD and its cleaved form, cGSDMD, were upregulated after treatment, suggesting that pyroptosis signalling was activated. In addition, we found that extracellular ATP and IL-1β concentrations were increased after drug treatment (**Figure 5G**, **5H**). Previous studies suggested that pyroptotic cells release proinflammatory substances, such as ATP and IL-1β [21]. Our findings suggested that the dual drugs treatment induced a pyroptosis-like cell death in Ph+ALL cell.

*Dasatinib and interferon-α show anti-leukemia activity in a Ph+ALL mouse model*

To examine the anti-leukemia activity of dasatinib and IFN-α in vivo, we established a murine Ph+ALL model. Murine lymphoblast cells were oncogenically transformed by overexpression of BCR-ABL (p210) (**Figure 6A**). As shown in **Figure 6B**, $GFP^+/B220^+$ leukemic cells were present in the peripheral blood cells of a tumour-bearing mouse. The tumour-bearing mice were treated with either solvent control or the combination of dasatinib and IFN-α. The dual drug-treated mice had significantly fewer $B220^+$ tumour cells in the BM than control-treated mice (**Figure 6C**). Flow cytometry analyses also showed that the treatment group exhibited consistently low number of circulating tumour cells (**Figure 6D**). The survival of the dual drug-treated mice was prolonged compared with PBS-treated group (**Figure 6E**).

*Dasatinib and interferon-α show promise as a maintenance therapy for Ph+ALL patients*

To verify the effect of dasatinib and IFN-α, we incorporated the dual drugs to maintenance therapy in a registered clinical trial. A total of 98 Ph+ALL patients with a median age of 40 years old (range, 15-73 years) were enrolled in

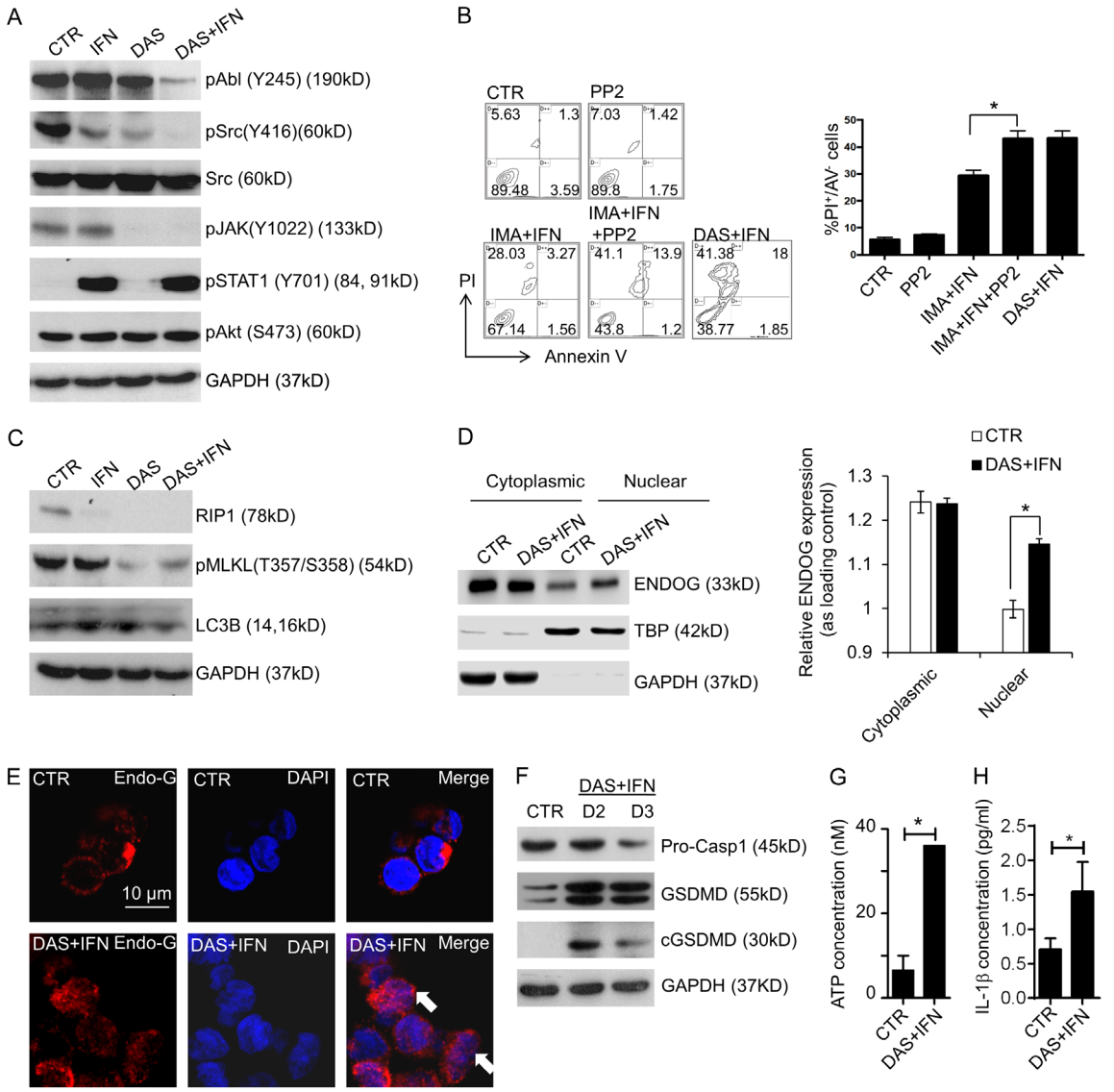


**Figure 5.** Dasatinib and interferon-α induce pyroptosis-like cell death. (A) SUP-B15 cells were treated with dasatinib (0.02 nM), IFN-α (1000 IU/ml) or their combination for 3 days. The levels of target to Src signalling proteins in the cells were measured by western blotting. (B) SUP-B15 cells were treated with imatinib (2 nM) and IFN-α (1000 IU/ml), the Src inhibitor PP2 (20 μM), or their combination for 3 days. The cells were subjected to flow cytometry analysis of annexin V and PI staining (left panel), and the results were quantified (right panel). (C) SUP-B15 cells were treated with dasatinib (0.02 nM) and IFN-α (1000 IU/ml) for 3 days, the levels of target to autophagy and necroptosis signalling proteins were measured by western blotting. (D) SUP-B15 cells were treated as described above, the level of endonuclease G (EndoG) was measured by western blotting. (E) SUP-B15 cells were treated as described above, subjected to immunofluorescence staining for EndoG, and visualized by confocal microscopy. (F) SUP-B15 cells were treated as described above, and the levels of target to pyroptosis signalling proteins were measured by western blotting. In addition, under the same treatment conditions, the extracellular ATP level and IL1-β level were measured, and the results are shown in (G and H), respectively (*P<0.05).

this trial. They received induction therapy with dasatinib (100 mg/day) and a VP regimen (4 mg vindesine weekly and 1 mg/kg/day prednisone for 4 weeks). After CR, young patients aged <55 years received consolidation treatment consisting of 3 courses of dasatinib plus the DVP regimen (45 mg/m$^2$ daunorubicin weekly, 4 mg vindesine weekly, and 1 mg/kg/day prednisone for 4 weeks); in patients aged ≥55 years, daunorubicin was omitted from the consolidation regimen. Forty-three patients were intent of maintenance therapy; 4 of them

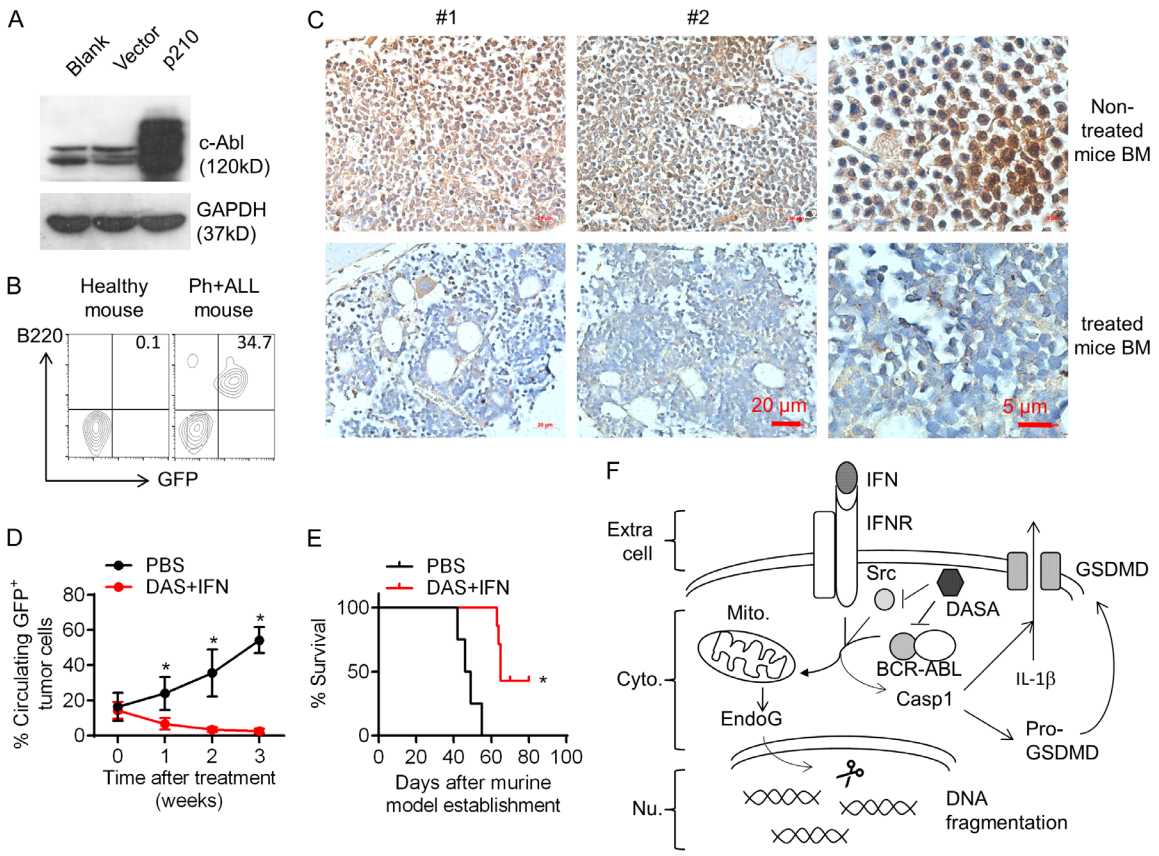


**Figure 6.** Dasatinib and interferon-α treatment efficacy in vivo in a Ph+ leukaemia mouse model. A. Virus-infected murine NIH/3T3 cells were subjected to western blotting. B. After the Ph+ALL mouse model was established, mouse peripheral blood cells were analysed by flow cytometry (one representative result out of four). Percentage of $GFP^+/B220^+$ positive cells indicated the tumour burden of mouse. C. Representative IHC staining images of mouse bone marrow B220 positive cells in control and dual drug-treated group. D. Tumour burden during treatment was analysed by measuring the percentage of circulating $GFP^+/B220^+$ cells in the peripheral blood. E. Survival of the control-treated and dual drug-treated mice. F. Cell signal diagram related to the dual drug-induced Ph+ALL cell death.

discontinued IFN-α within one month due to intolerance. A total of 39 Ph+ALL patients received maintenance therapy consisting of dasatinib and IFN-α plus VP chemotherapy for over 1 month (100 mg dasatinib daily; 3 million units IFN-α 2-3 times per week; 4 mg vindesine on day 1 and day 11, and 1 mg/kg/day prednisone on day 1 to 7 and day 11 to 17 monthly in the first year; once every two months in the second year; and once every three months in the third year). A total of 16 cycles of prophylactic intrathecal chemotherapy (methotrexate, cytarabine with dexamethasone) were administered for all the patients. The patients' characteristics are summarized in **Table 1**, and the patient recruitment flowchart is shown in **Figure 7A**. The median follow-up was 28.5 months (range, 6.5 to 58.8 months), 21 of 39 patients survive in CR1 now. Eighteen patients experienced relapse, 12 of whom died and 6 of whom are still alive. The 4-year DFS and OS rates were 52.2±8.3% and 65.2±8.3%, respectively, the median DFS and OS rates are not reached. Interestingly, the Kaplan-Meier curves of DFS and OS in this cohort reached a plateau from the third year (**Figure 7B**).

## Discussion

In this study, we showed that a combination of dasatinib and IFN-α synergistically induced Ph+ALL cell death but had limited effects on Ph-ALL cells and non-tumorous cells. Dasatinib and IFN-α induced Ph+ALL cell lysis occurred several days after continuous the dual drugs exposure, and cell death could be reduced by an insufficient the dual drugs exposure time or

**Table 1.** Patient characteristics

| | |
|---|---|
| Number of patients | 98 |
| Gender (%) | |
| male | 53 (54.1) |
| female | 45 (45.9) |
| Age | |
| range | 15~73 |
| mean ± SD | 39.9±13.7 |
| median | 40 |
| Age group (%) | |
| ≥55 yrs | 16 (16.3) |
| <55 yrs | 82 (83.7) |
| WBC count at diagnosis $\times 10^9$/L | |
| range | 0.36~479.73 |
| mean ± SD | 81.6±109.2 |
| median | 34.3 |
| Hyperleukocytosis (%) | |
| yes (≥30) | 47 (48.0) |
| no (<30) | 51 (52.0) |
| BCR/ABL transcript subtype | |
| p190 | 53 (54.1) |
| p210 | 38 (38.8) |
| unknown | 7 (7.1) |
| Immunophenotype (%) | |
| pro-B | 2 (2.0) |
| common-B | 86 (87.8) |
| pre-B | 5 (5.1) |
| unknown | 5 (5.1) |
| MRD (%)* | |
| positive | 63 (64.3) |
| negative | 35 (35.7) |

*MRD positivity was defined as a BCR/ABL level greater than 0.01%.

sequential treatment with each drug. This finding might indicate that death stimuli accumulate within the cells when multiple signalling pathways are altered simultaneously by dasatinib and IFN-α. Mitochondrial dysfunction occurred immediately after the dual drugs exposure and continued until cell lysis. Therefore, the dual drug-induced alterations in cell signalling might integrate to affect mitochondria and lead to cell death. Furthermore, we found that the dual drug-induced Ph+ALL cell death was not a canonical form of cell death. First, caspase-3 was not activated, and the apoptosis inhibitor Z-VAD did not affect cell death. These results indicated that the cell death was not apoptosis. Second, although the dual drug-induced cell death caused membrane rupture and cellular organelle swelling, both of which are characteristic features of cell necrosis, the dead cells exhibited DNA fragmentation, which is usually mediated by active endonucleases in apoptotic cells [22]. Our results suggested that endonuclease function was carried out by EndoG in the dual drug-treated cells. Third, although GSDMD activation and pyroptotic release of IL-1β and ATP were observed after dual drugs treatment, we only found the activation of caspase 1, which are upstream of GSDMD, the activation of upstream caspase 4/5 was not detected. These findings suggested that the dual drug-induced Ph+ALL cell death was pyroptosis-like but also exhibited features of caspase-independent apoptosis [23]. It has long been recognized that the features of different types of cell death, such as apoptosis, necroptosis and pyroptosis, and their regulatory cell signals are not completely distinct [24]. Therefore, the dual drug-induced cell death might also be a mixture of a variety of forms of cell death.

Dasatinib is a dual Src/ABL TKI that additionally targets the Lyn tyrosine kinase. Recently, Scherr et al. found that dasatinib enhances the expression of BIM and reduces the expression of Bcl-xL and MCL-1 in mitochondria. Dasatinib-associated cytotoxicity heavily depends on BIM, suggesting that inhibition of Src kinases may also converge on the induction of mitochondrial apoptosis [25]. Another study by Mariani et al. showed that BMI1 silencing induces the upregulation of the expression of many IFN-α-response genes, suggesting that the growth-promoting effects of BMI1 in Ph+ ALL cells may also depend on negative regulation of the IFN-α-response pathway [26]. These findings support the use of the combination of dasatinib and IFN-α for Ph+ALL treatment.

In addition to performing in vitro and in vivo studies using cell lines and an animal model, we incorporated the combination of dasatinib and IFN-α to maintenance therapy for Ph+ALL patients. Currently, it is recommended that all Ph+ALL patients fit for allo-HSCT should receive HSCT to achieve long-term DFS [27]. However, there is a substantial proportion of patients who are unfit for allo-HSCT because of age, comorbidities, donor availability, and/or socioeconomic issues. In various trials, the ratio of Ph+ALL patients who received allo-HSCT varied from 30% to 60% [27, 28]. In our

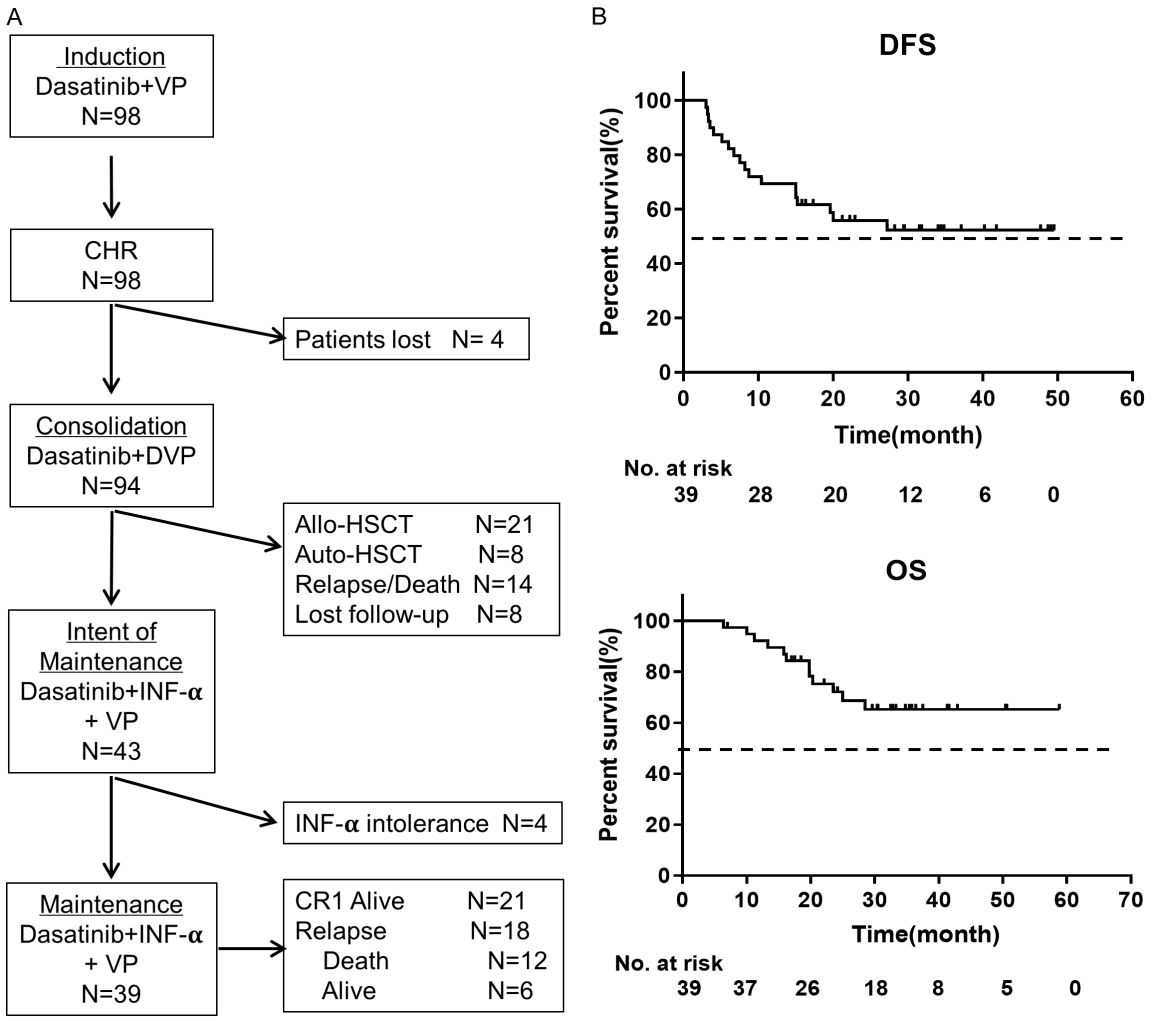


**Figure 7.** Dasatinib and interferon-α combination therapy for Ph+ALL maintenance. A. Flowchart of patient recruitment for the cohort study. B. The Kaplan-Meier curves of DFS and OS of the patients who received the dual drugs treatment for disease maintenance.

cohort, 21.4% (21 out of 98) of patients received allo-HSCT, and 8.2% (8 out of 98) received auto-HSCT. There is no standard treatment regimen for Ph+ALL patients who do not receive transplantation, and these patients in general have a poor prognosis. In our nontransplant cohort, the 4-year DFS and OS rates were 52.2% and 65.2%, respectively, and the combination of dasatinib and IFN-α was well tolerated by most Ph+ALL patients during long-term maintenance. Previous case reports suggested that the combination of dasatinib and IFN-α was effective in a few specific Ph+ALL cases [8, 9]. In those reported cases, the patients were resistant to a single TKI agent, either imatinib or dasatinib, but responsive to dual drugs therapy. Whether dual drugs therapy overcomes TKI resistance needs to be further investigated. Larger systematic clinical trials and further mechanistic studies are warranted.

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## Disclosure of conflict of interest

None.

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**Table S1.** The list of antibodies for western blot and flowcytometry

| Item | Antibody | Cat No. | Company |
|---|---|---|---|
| Western blot | Caspase 8 | Cat No. 4790 | Cell Signalling Technology (MA, USA) |
| | Caspase 3 | Cat No. 9662 | |
| | Caspase 9 | Cat No. 9508 | |
| | PARP | Cat No. 9542 | |
| | P-MLKL (T357/S358) | Cat No. 14516 | |
| | RIP1 | Cat No. 4926 | |
| | P-Src (Y416) | Cat No. 6943 | |
| | Src | Cat No. 2109 | |
| | c-ABL | Cat No. 2862 | |
| | P-c-ABL (Y425) | Cat No. 2868 | |
| | P-STAT1 (Y701) | Cat No. 9167 | |
| | Bcl-xL | Cat No. 2764 | |
| | P-Akt (S473) | Cat No. 9271 | |
| | GAPDH | Cat No. 2118 | |
| | RIP3 | Cat No. sc-374639 | Santa Cruz Biotechnology Co. (CA, USA) |
| | caspase 1 | Cat No. sc-56036 | |
| | ENDOG | Cat No. sc-365359 | |
| | GSDMD | Cat No. sc-81868 | |
| | LC3 | Cat No. L7543 | Sigma-Aldrich (MO, USA) |
| | P-JAK1 (Y1022) | Cat No. BS4108 | Bioworld Technology, Inc. (Jiangsu, China) |
| | TBP | Cat No. 22006-1-AP | Proteintech Group, Inc. (IL, USA) |
| Flowcytometry antibody | murine B220-eFluor 780 | Cat No. 47-0452-82 | Thermo Fisher Scientific (MA, USA) |
| | istotype controls | | |
| | Annexin V | Cat No. FXP018 | Beijing 4A Biotech Co. Ltd. (Beijing, China) |
| | Propidium iodide (PI) | | |